Journal of Diabetes Research

# Nontraditional Therapy for Diabetes and Its Complications 2021

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# Contents

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*Review Article*

# Evidence and Potential Mechanisms of Traditional Chinese Medicine for the Adjuvant Treatment of Coronary Heart Disease in Patients with Diabetes Mellitus: A Systematic Review and Meta-Analysis with Trial Sequential Analysis

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Traditional Chinese medicine (TCM) has long been used to treat diabetes mellitus and angina. It has also gained widespread clinical applications in China as a common adjuvant treatment. Although there is high-quality evidence that TCM is effective in regulating glucose and lipid metabolism, the cardiovascular protective effect of TCM in the treatment of diabetes mellitus has not been fully elucidated, especially in patients with both diabetes mellitus and coronary heart disease (CHD). We systematically assessed the efficacy and safety of TCM for the adjuvant treatment of patients with CHD and diabetes mellitus and examined the pharmacological effects and potential mechanisms of TCM medication/herbs on diabetes mellitus with CHD. We found that TCM could improve the control effect of conventional treatment on cardiac function, hemorheology, blood glucose, blood lipid, and inflammation, thus reducing the frequency of angina and the incidence of cardiovascular events and all-cause mortality. These findings indicate that TCM may be used as a complementary approach for patients with diabetes mellitus and CHD. Nevertheless, more rigorously designed randomized controlled trials and long-term evaluations are needed to support these findings.

## 1. Introduction

The global population of patients with diabetes mellitus (DM) is approximately 463 million, which is expected to rise to 700 million by 2040 [1]. As an independent risk factor for coronary heart disease (CHD), DM is considered as "coronary heart disease equivalent" [2], which is associated with increased CHD morbidity and mortality [3]. In patients with DM, the risk of mortality due to CHD is twice that of those who do not have DM [4]. Clinical outcomes and complications following coronary interventions are also worse in such high-risk patients [5]. Several medical resources are spent on DM every year. The total global health expenditure due to DM was estimated to be $673 billion in 2015 [6], with cardiovascular diseases attributable to diabetes accounting for the highest proportion (47.9%) of overall costs [7]. However, the need to control cardiovascular diseases is still unmet. At present, the treatment for DM, especially in those with a high atherosclerotic cardiovascular disease risk, mainly focuses on comprehensively controlling multiple risk factors [8]. However, the complexity of multidrug regimens may induce the chance of nonadherence among patients, while long-term use of high-intensity antiplatelet drugs and lipid-lowering statins may increase the incidence of side effects, such as bleeding, muscle complaints, increased liver or muscle enzymes, or various neurological symptoms [9].

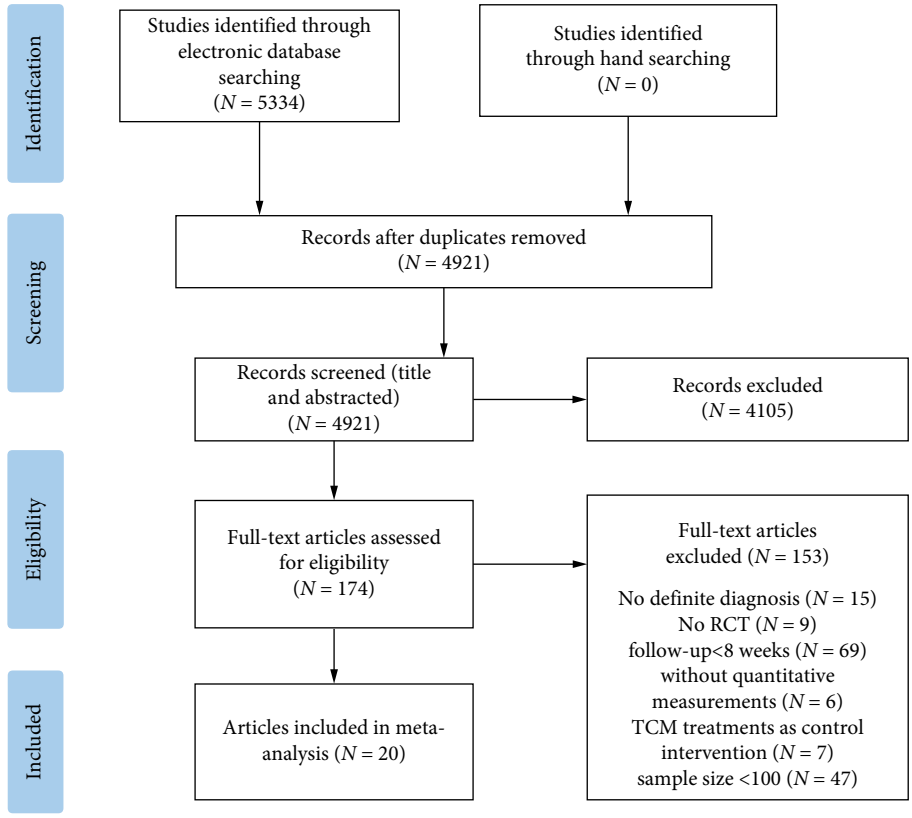


Figure 1: Flow chart of study inclusion.

Therefore, clinicians have begun considering traditional Chinese medicine (TCM) as an adjuvant or alternative treatment for DM.

TCM has been widely used to treat diabetes and angina for 1000 years. The holistic and multitarget approaches of TCM have unique advantages in controlling complex diseases, such as DM [10]. In recent years, large-scale clinical trials have demonstrated that TCM effectively reduces hyperglycemia [11–13] and improves vascular inflammation, lowers lipids, and improves cardiac function [14, 15]. A large-sample prospective cohort study also showed that TCM alone is as efficacious as Western medicines in preventing diabetes from being complicated with coronary artery disease [16]. Moreover, patients who used more types of TCM tended to use much less Western medicine recommended by current guidelines [17]. At the same time, some therapeutic mechanisms underlying TCM's potency have been uncovered with the application of modern science and technology.

A number of clinical trials in China have shown that TCM is effective in treating DM or CHD. However, because of the insufficient sample size, short experimental period, and lack of empirical validity of efficacy evaluation in many studies, the results have been questioned and rejected. To date, there are very few systemic analyses that have focused on the comprehensive regulation effect of TCM adjuvant treatment on both DM and CHD. Considering the aforementioned limitations, we selected more credible studies for a systematic review and meta-analysis to comprehensively evaluate the clinical efficacy and safety of TCM for patients with CHD and DM.

## 2. Materials and Methods

We conducted and reported this systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [18].

*2.1. Search Strategy.* The first three authors independently searched for papers published from January 2005 to May 2021 on the following databases: the China National Knowledge Internet, Wanfang, VIP databases, the China Biology Medicine, Web of Science, Pubmed, Medline; Embase, International Pharmaceutical Abstracts, and the Cochrane Library. Search terms were as follows: ("coronary heart disease" or "cardiovascular disease" or "angina" or "myocardial ischemia" or "myocardial infarction" or "acute coronary syndrome" or "coronary atherosclerosis") and ("diabetes" or "diabetes mellitus" or "Xiaoke syndrome") and ("randomized controlled trial" or "controlled clinical trial" or "random" or "randomly" or "randomized" or "control" or "RCT") and ("TCM" or "traditional Chinese medicine" or "Chinese medicinal herb" or "Chinese herbal medicine" or "decoction" or "formula" or "prescription" or "Chinese patent medicine" or "Chinese patent drug" or "Chinese herbal compound prescription"). The PRISMA flow chart for the study is presented in Figure 1.

Table 1: Summary of included studies.

| Study | Patient group | Participant (M/W) | | Treatment measure | | Duration | Outcomes |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | Control | Treatment | | |
| Chen 2019 | T2DM&CHD 128 | 64 (29/35) | 64 (31/33) | CT | Yanshi Yixin decoction+CT | 3 m | BG, BL, SAA, MMP-9, TBIL |
| Cheng 2019 | T2DM&CHD 118 | 60 | 58 | CT | Supplemented Yuye decoction+CT | 3 m | BG, BL, IM, SOD, MDA, GSH-Px, 8-ios-PGF2a |
| Feng 2020 | T2DM&CHD 130 | 65 (33/32) | 65 (35/30) | CT | Shexiang Baoxin Pill+CT | 3 m | BL |
| Gao 2018 | T2DM&CHD 100 | 50 (27/23) | 50 (24/26) | CT | Shengmai powder+CT | 2 m | BG, BL, FGF-21, Ghrelin |
| Li 2008 | DM&CHD 100 | 50 (33/17) | 50 (32/18) | CT | Jiangtang Shengmai decoction+CT | 2 m | BG, BL, HI |
| Li 2021 | T2DM&CHD 286 | 143 (75/68) | 143 (76/67) | CT | Tongxinluo capsule+CT | 3 m | CF, BL, BG |
| Liu 2008 | T2DM&CHD 260 | 130 (95/35) | 130 (89/41) | CT | Xinshu pill+CT | 2 m | BG, IM, DD, LFTs, KFTs |
| Liu 2019 | T2DM&CHD 108 | 54 (29/25) | 54 (28/26) | CT | Shexiang Yangxin powder+CT | 3 m | BG, BL, HI |
| Ni 2010 | T2DM&CHD 173 | 45 (21/24) | 128 (55/73) | CT | Qizhi Jiangtang capsule+CT | 2 m | BG, dynamic ECG |
| Ning 2017 | T2DM&CHD 150 | 75 (34/41) | 75 (35/40) | CT | Yindan Xinnaotong soft capsule+CT | 3 m | HI, angina frequency |
| Pan 2019 | T2DM&SAP 120 | 60 (29/31) | 60 (28/32) | CT | Yiqi Yangyin Huoxue decoction_CT | 3 m | BG, BL, CRP, Hcy, UMA |
| Qian 2017 | DM&ACS 294 | 147 (97/50) | 147 (102/45) | CT | Xinyue capsule+CT | 6 m | CE, BG, NT-proBNP, CK-MB |
| Qian 2018 | T2DM&CHD 150 | 75 (43/32) | 75 (45/30) | CT | Tongguan Huoxue decoction+Naoxintong capsule+CT | 3 m | BG, BL, HMGB1, Omentin-1 |
| Ren 2020 | T2DM&CHD 320 | 160 (85/75) | 160 (78/82) | CT | Tongguan Huoxue decoction+Naoxintong capsule+CT | 3 m | CF, BG |
| Shi 2020 | T2DM&CHD 120 | 60 (16/44) | 60 (18/42) | CT | Yindan Xinnaotong soft capsule+CT | 3 m | Angina frequency, BL |
| Tao 2017 | T2DM&CHD 118 | 55 (33/22) | 63 (43/20) | CT | Hedan pill+CT | 6 m | BL, IM, SOD, MDA, NO |
| Zhang 2015 | T2DM&CHD 102 | 51 (30/21) | 51 (28/23) | CT | Shuxin Huoxue decoction+CT | 2 m | BL, IM, HI |
| Zhang 2019 | T2DM&SAP 179 | 81 (43/38) | 98 (49/49) | CT | Yiqi Yangyin decoction+CT | 3 m | CE, BG, BL, HI, IM |
| Zhang 2020 | DM&CHD 134 | 67 (36/31) | 67 (34/33) | CT | Xuezhikang+CT | | BG, BL, HI, IM, CF |
| Zhao 2007 | T2DM&CHD 591 | 285 (198/87) | 306 (226/80) | Placebo+CT | Xuezhikang+CT | 4 y | ACM, CE, BL |

ACS: acute coronary syndrome; SAP: stable angina pectoris; CT: conventional treatment; ACM: all-cause mortality; CE: cardiovascular events; CF: cardiac function; HI: hemorrheologic indices; IM: inflammatory marks; BG: blood glucose; BL: blood lipid.

*2.2. Inclusion Criteria and Study Selection.* Clinical studies were included if they satisfied the following criteria: (1) study participants had a definite diagnosis of both DM and CHD and were randomly assigned to receive TCM, contemporary medication, or placebo; (2) the sample size was ≥100; (3) the duration in each study group was ≥8 weeks. Clinical studies with the following features were excluded: (1) nonrandomized trials; (2) the participants had no definite diagnosis; and (3) only symptomatic changes of participants were reported, without objective laboratory measurements or physical examination. As many randomized controlled trials (RCTs) on TCM have been published in Chinese, published reports in both English and Chinese were included. The first two authors (Ding and Wei) independently reviewed all titles and abstracts. If the information was insufficient, the full text was retrieved for further judgment. If the two authors failed to reach a consensus, the senior author (Zhao) made the final judgment.

*2.3. Quality Assessment.* The quality of the included studies was assessed independently by the first two authors (Wei and Ding) as defined by the Cochrane's risk of bias tool [19]. The risk of bias was assigned as low, unclear, or high for the following items: random sequence generation,

Table 2: Composition of TCM in the study.

| Study | Prescription | Medicine | Article |
|---|---|---|---|
| Chen 2019 | Yan's yixin prescription | Dasheng (Radix Codonopsis), Huangqi (Radix Astragali seu Hedysari), Juemingzi (Semen Cassiae), Gegen (Radix Puerariae), Shichangpu (Rhizoma Acori Tatarinowii), Jiangxiang (Lignum Dalbergiae Odoriferae), Danshen (Radix Salviae Miltiorrhizae), Chuanxiong(Rhizoma Ligustici Chuanxiong), Chishao (Radix Paeoniae Rubra), Shenzha (Fructus Crataegi), Fabanxia (Rhizoma Pinelliae Preparatum), Zhiqiao (Fructus Aurantii) | [22] |
| Cheng 2019 | Modified Yuyetang | Shanyao (Rhizoma Dioscoreae), Taizishen (Radix Pseudostellariae), Huangqi (Radix Astragali seu Hedysari), Zhimu (Rhizoma Anemarrhenae), Gegen (Radix Puerariae), Wuweizi (Fructus Schisandrae Chinensis), Tianhuafen (Radix Trichosanthis), Jineijin (Endothelium Corneum Gigeriae Galli), Danshen (Radix Salviae Miltiorrhizae), Sanqi (Radix Notoginseng), Chuanxiong (Rhizoma Ligustici Chuanxiong), Hongqu (fermentum rubrum), Fabanxia (Rhizoma Pinelliae Preparatum), Chenpi (Pericarpium Citri Reticulatae), Gancao (Radix Glycyrrhizae) | [23] |
| Feng 2020 | Shexiang Baoxin Pill | Shexiang (Moschus), Renshen (Radix Ginseng), Niuhuang (Calculus Bovis), Rougui (Cortex Cinnamomi), Anxixiang (Benzoinum), Chansu (Venenum Bufonis), Bingpian (Borneolum Syntheticum) | [46] |
| Gao 2018 | Shengmai powder | Gancao (Radix Glycyrrhizae), Wuweizi (Fructus Schisandrae Chinensis), Huangqi (Radix Astragali seu Hedysari), Renshen (Radix Ginseng), Chuanxiong(Rhizoma Ligustici Chuanxiong), Maidong (Radix Ophiopogonis), Gegen (Radix Puerariae), Danshen (Radix Salviae Miltiorrhizae), Sanqi (Radix Notoginseng) | [24] |
| Li 2008 | Shengmai powder | Renshen (Radix Ginseng), Huangqi (Radix Astragali seu Hedysari), Shanyao (Rhizoma Dioscoreae), Cangzhu (Rhizoma Atractylodis), Xuanshen (Radix Scrophulariae), Danggui (Radix Angelicae Sinensis), Shudi (Radix Rehmanniae Preparata), Maidong (Radix Ophiopogonis), Wuweizi (Fructus Schisandrae Chinensis), Danshen (Radix Salviae Miltiorrhizae), Chuanxiong (Rhizoma Ligustici Chuanxiong), Chishao (Radix Paeoniae Rubra), Yanhusuo (Rhizoma Corydalis), Gancao (Radix Glycyrrhizae) | [25] |
| Li 2021 | Tongxinluo | Renshen (Radix Ginseng), Shuizhi (Hirudo), Quanxie (Scorpio), Chishao (Radix Paeoniae Rubra), Chantui (Periostracum Cicadae), Tubiechong (Eupolyphaga Seu Steleophaga), Wugong (Scolopendra), Tanxiang (Lignum Santali Albi) | [47] |
| Liu 2008 | Xinshu pills | Huangqi (Radix Astragali seu Hedysari), Danshen (Radix Salviae Miltiorrhizae), Gegen (Radix Puerariae), Dasheng (Radix Codonopsis), Shuizhi (Hirudo), Jiezi (Semen Sinapis Albae), Huangjing (Rhizoma Polygonati), Maidong (Radix Ophiopogonis), Wuweizi (Fructus Schisandrae Chinensis) | [26] |
| Liu 2019 | Shexiangyangxin powder | Danshen (Radix Salviae Miltiorrhizae), Xiebai (Bulbus Allii Macrostemonis), Maidong (Radix Ophiopogonis), Chishao (Radix Paeoniae Rubra), Renshen (Radix Ginseng), Guizhi (Ramulus Cinnamomi), Wuweizi (Fructus Schisandrae Chinensis), Sharen (Fructus Amomi Villosi), Tanxiang (Lignum Santali Albi), Bingpian (Borneolum Syntheticum), Shexiang (Moschus) | [27] |
| Ni 2010 | Qizhi jiangtang capsule | Huangqi (Radix Astragali seu Hedysari), Shudi (Radix Rehmanniae Preparata), Huangjing (Rhizoma Polygonati), Shuizhi (Hirudo) | [48] |
| Ning 2017 | Yindan Xinnaotong soft capsule | Yinxingye (Folium Ginkgo), Danshen (Radix Salviae Miltiorrhizae), Xixin (Herba Asari), Jiaogulan (Gynostemma pentaphylla), Shanzha (Fructus Crataegi), Sanqi (Radix Notoginseng), Aiye (Folium Artemisiae Argyi) | [29] |
| Pan 2019 | Yiqiyangyin huoxue recipe | Taizishen (Radix Pseudostellariae), Huangqi (Radix Astragali seu Hedysari), Danggui (Radix Angelicae Sinensis), Shudi (Radix Rehmanniae Preparata), Danshen (Radix Salviae Miltiorrhizae), Guijianyu (Ramuli euonymi), Xuanshen (Radix Scrophulariae), Danpi (Cortex Moutan Radicis), Shanzhuyu (Fructus Corni), Gegen (Radix Puerariae), Sanqi (Radix Notoginseng) | [35] |
| Qian 2017 | Moods capsule | Panax quinquefolium saponin | [32] |
| Qian 2018 | Tongguan huoxue decoction | Shudi (Radix Rehmanniae Preparata), Xiyangshen (Radix Panacis Quinquefolii), Huangjing (Rhizoma Polygonati), Huangqi (Radix Astragali seu Hedysari), Maidong (Radix Ophiopogonis), Gualou (Fructus Trichosanthis), Honghua (Flos Carthami), Chuanxiong (Rhizoma Ligustici Chuanxiong), Dilong (Lumbricus), Chaihu (Radix Bupleuri), Gancao (Radix Glycyrrhizae) | [21] |
| Ren 2020 | Tongxinluo, Jinlida | Renshen (Radix Ginseng), Shuizhi (Hirudo), Quanxie (Scorpio), Chishao (Radix Paeoniae Rubra), Chantui (Periostracum Cicadae), Tubiechong (Eupolyphaga Seu Steleophaga), Wugong (Scolopendra), Tanxiang (Lignum Santali Albi), Cangzhu (Rhizoma | [47, 49] |

Table 2: Continued.

| Study | Prescription | Medicine | Article |
|---|---|---|---|
| Shi 2020 | Yindan Xinnaotong soft capsule | Atractylodis), Baizhu (Rhizoma Atractylodis Macrocephalae), Gegen (Radix Puerariae), Yuzhu (Rhizoma Polygonati Odorati), Fuling (Poria) Yinxingye (Folium Ginkgo), Danshen (Radix Salviae Miltiorrhizae), Xixin (Herba Asari), Jiaogulan (Gynostemma pentaphylla), Shanzha (Fructus Crataegi),Sanqi (Radix Notoginseng), Aiye (Folium Artemisiae Argyi) | [39] |
| Tao 2017 | Hedan tablets | Heye (Folium Nelumbinis), Danshen (Radix Salviae Miltiorrhizae), Shanzha (Fructus Crataegi), Fanxieye (Folium Sennae), Buguzhi (Fructus Psoraleae) | [30] |
| Zhang 2015 | Shuxin huoxue decoction | Huangqi (Radix Astragali seu Hedysari), Maidong (Radix Ophiopogonis), Danshen (Radix Salviae Miltiorrhizae), Huangjing (Rhizoma Polygonati), Shuizhi (Hirudo), Jiezi (Semen Sinapis Albae), Dasheng (Radix Codonopsis), Wuweizi (Fructus Schisandrae Chinensis) | [33] |
| Zhang 2017 | Shexiang Baoxin Pill | Danshen (Radix Salviae Miltiorrhizae), Xiebai (Bulbus Allii Macrostemonis), Maidong (Radix Ophiopogonis), Chishao (Radix Paeoniae Rubra), Renshen (Radix Ginseng), Guizhi (Ramulus Cinnamomi), Wuweizi (Fructus Schisandrae Chinensis), Sharen (Fructus Amomi Villosi), Tanxiang (Lignum Santali Albi), Bingpian (Borneolum Syntheticum), Shexiang (Moschus) | [34] |
| Zhang 2019 | Yiqi yangyin basic prescription | Taizishen (Radix Pseudostellariae), Huangqi (Radix Astragali seu Hedysari), Gegen (Radix Puerariae), Shudi (Radix Rehmanniae Preparata), Danggui (Radix Angelicae Sinensis), Guijianyu (Ramuli euonymi), Danpi (Cortex Moutan Radicis), Shanzhuyu (Fructus Corni) | [31] |
| Zhang 2020, Zhao 2007 | Xuezhikang | Hongqu (fermentum rubrum) | [41, 36] |

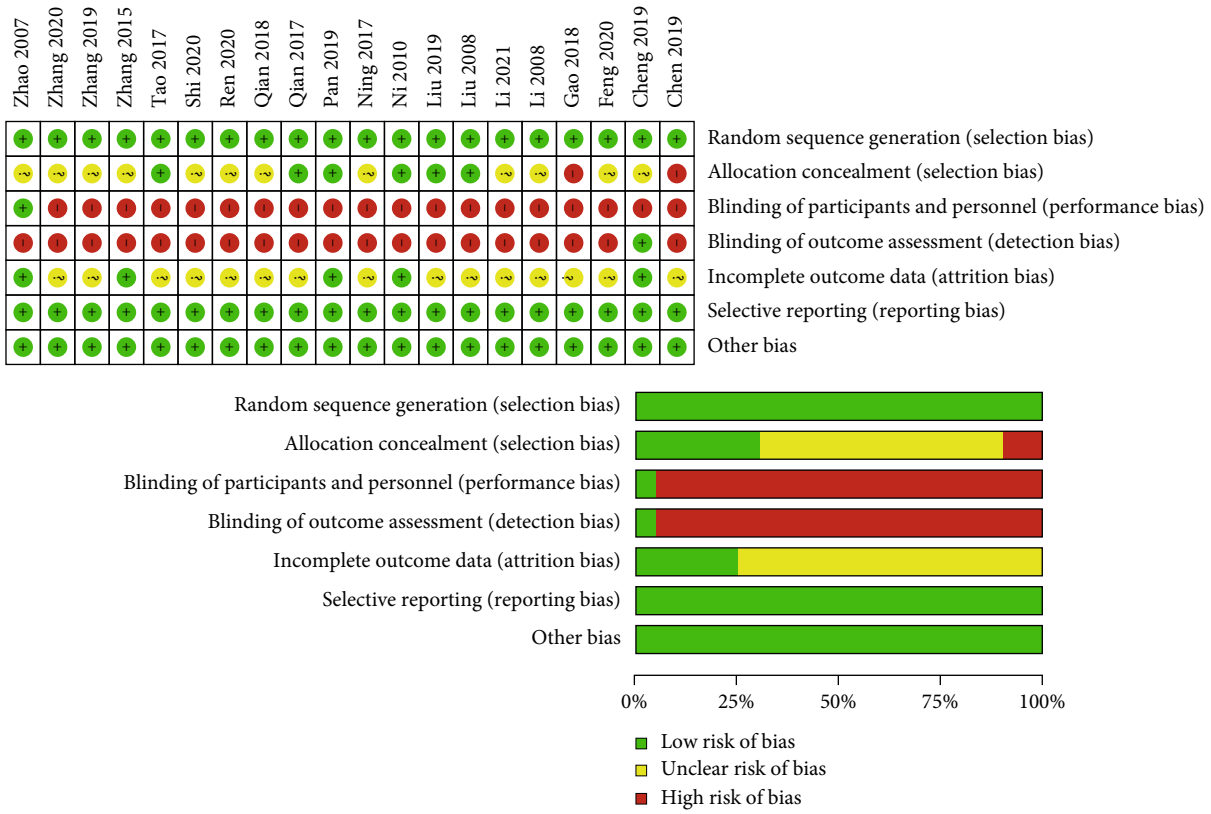


Figure 2: Evaluation of the risk of bias for each included study using the Cochrane Risk of Bias Tool.

allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias.

*2.4. Data Extraction and Analysis.* Data extraction was individually performed by the same authors (Wei and Ding) in an unblended standardized manner. In case of discrepancies,

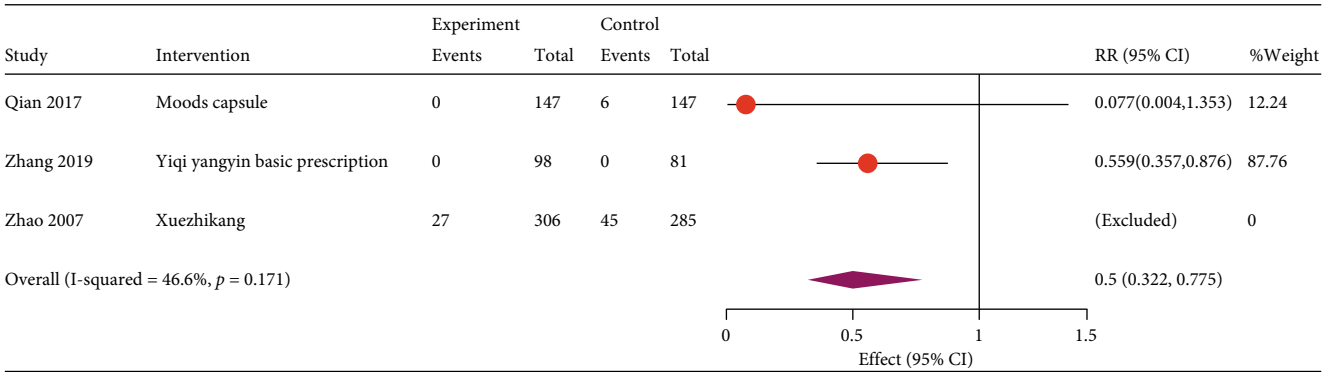


Figure 3: Forest plot of relative risk of all-cause mortality. RR: relative risk; CI: confidence interval.

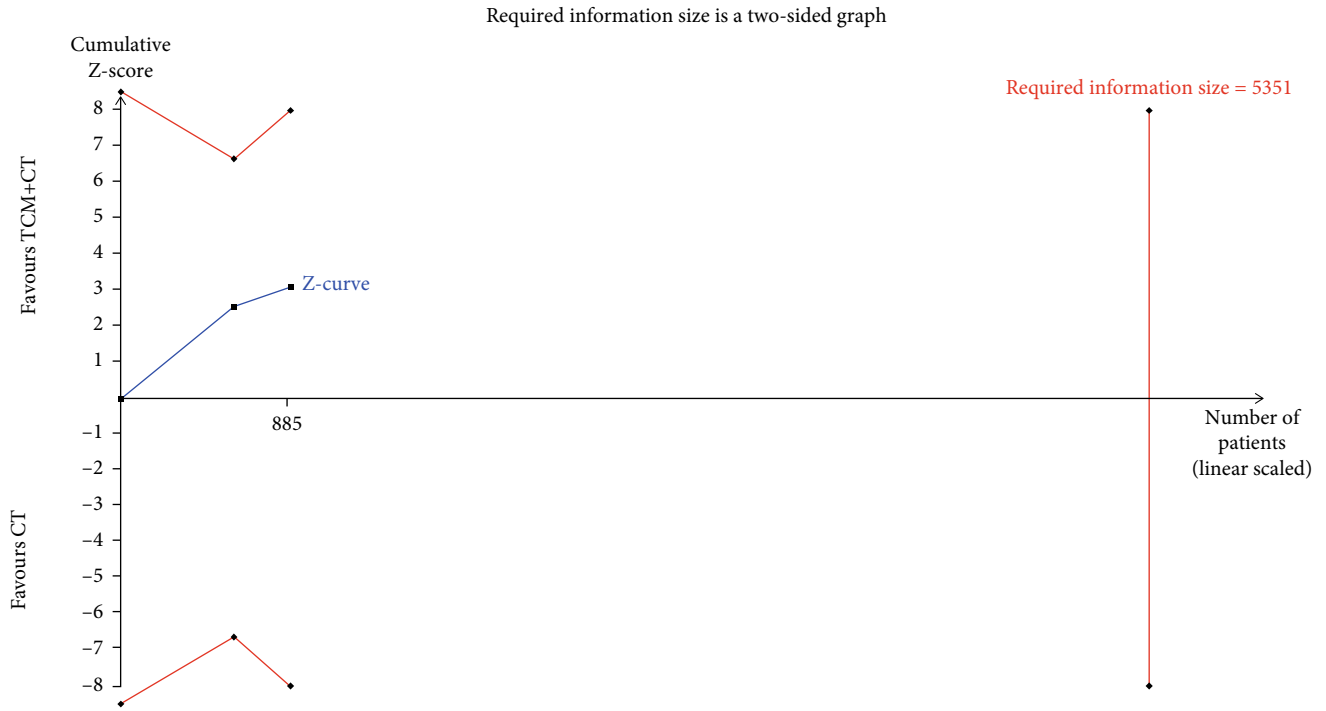


Figure 4: Trial sequential analysis of mortality.

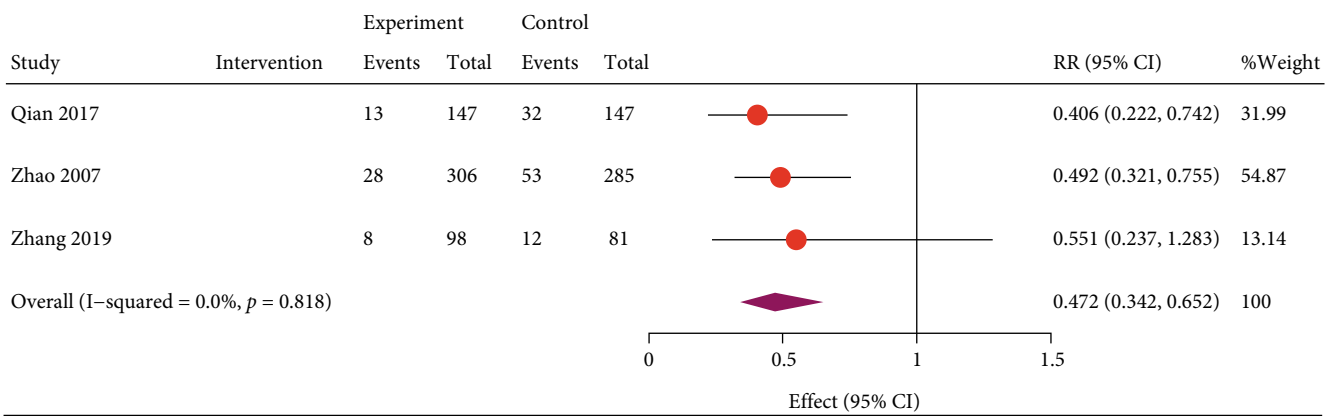


Figure 5: Forest plot of relative risk of incidence of cardiovascular events. RR: relative risk; CI: confidence interval.

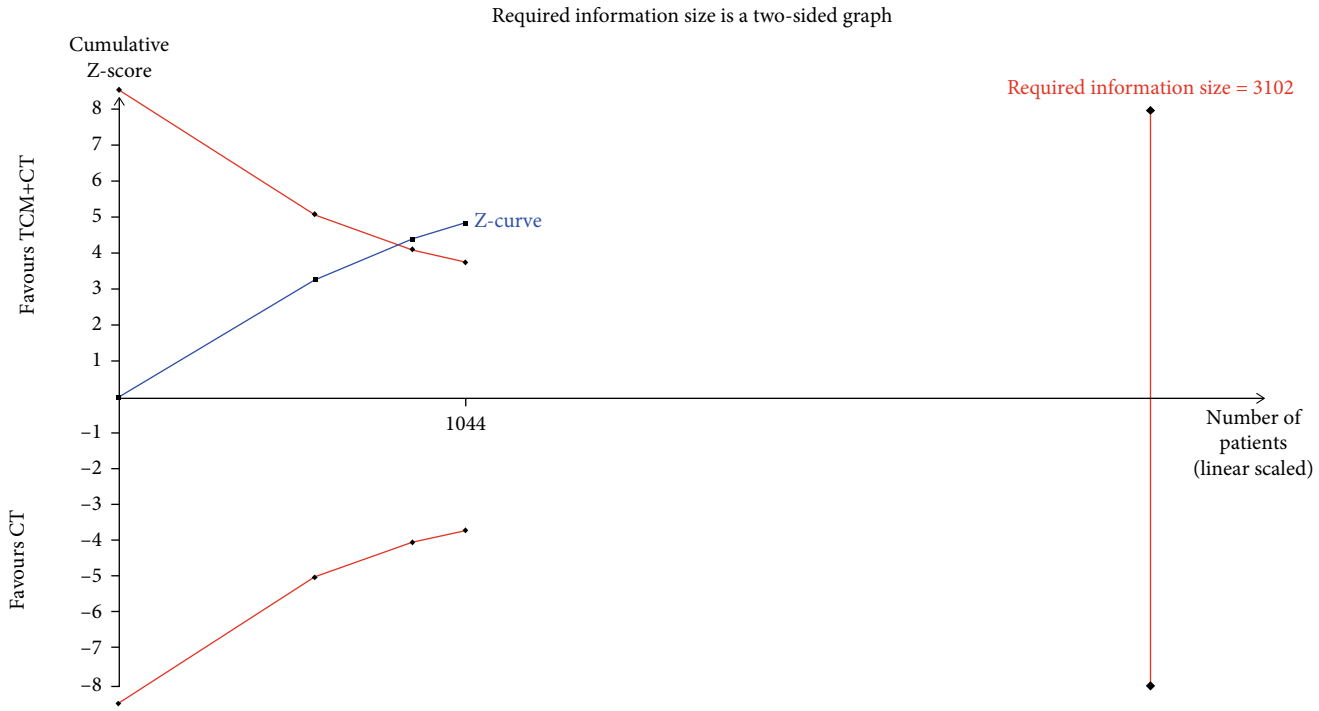


Figure 6: Trial sequential analysis of cardiovascular events.

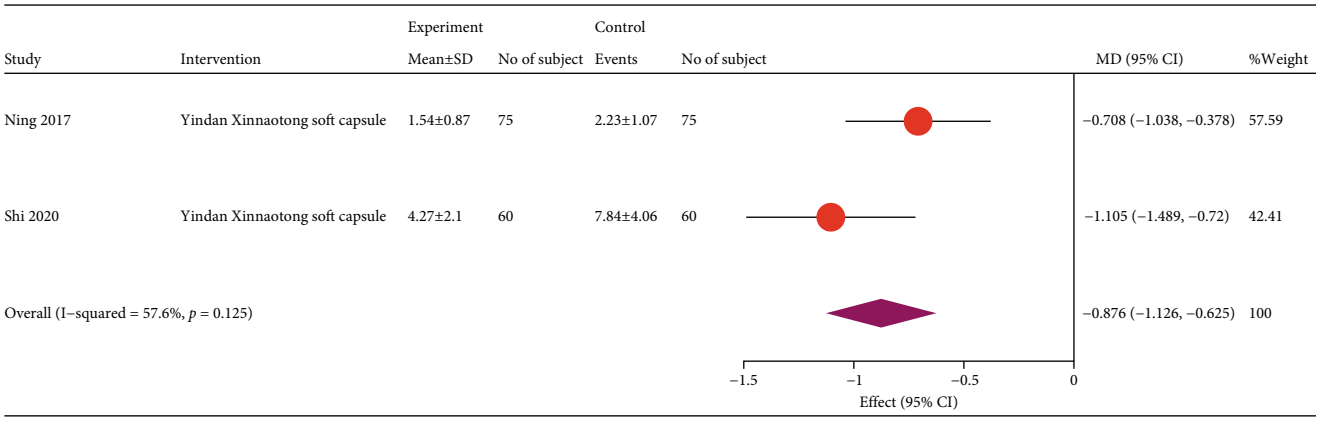


Figure 7: Forest plot of frequency of angina pectoris. MD: mean difference; CI: confidence interval.

the third author (Yeung) was consulted. Relevant reports' information on the author, year, number of participants, intervention (weeks, frequency, and dosage), and passive control condition were extracted and recorded in an excel spreadsheet. The authors of the identified papers were contacted for additional information if necessary. The primary outcomes were all-cause mortality and cardiovascular (CV) events. The frequency of angina contributed to efficacy assessment and was included as a secondary outcome.

Hemorheology indices and cardiac function were also included as secondary outcomes, as abnormalities of these laboratory measurements are associated with CV events. The secondary outcomes also included the levels of fasting blood glucose, postprandial blood glucose (PBG), glycosylated hemoglobin (HbA1c), total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), C-reaction protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor $\alpha$ (TNF-$\alpha$).

RevMan 5.3 (Nordic Cochrane Center, Copenhagen, Denmark) and Stata version 15.0 (Stata Corp., College Station, TX, USA) were used to analyze the data. Continuous outcomes were pooled to find the weighted mean differences (WMDs) accompanied by 95% confidence intervals (CIs), when the outcomes were based on the same scale; otherwise, the standard mean differences (SMDs) were used. Categorical outcomes were pooled to find relative risks (RRs) and were accompanied by 95% CIs. $I^2$ statistics were used to measure heterogeneity. A fixed-effect model was used if $I^2 < 50\%$; otherwise, the random-effect model was used. Publication bias was explored through a funnel-plot analysis.

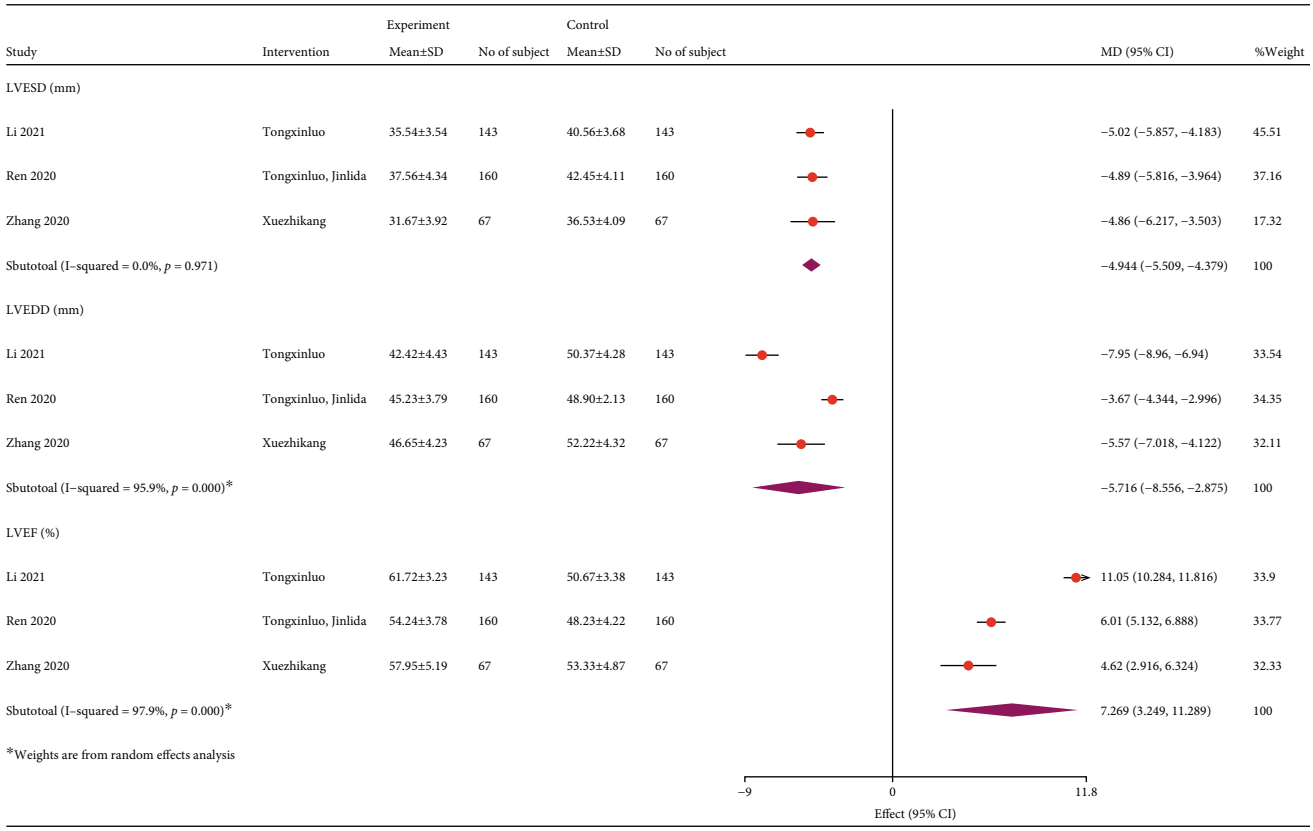


Figure 8: Forest plot of cardiac function. LVEF: left ventricular ejection fraction; LVEDD: left ventricular end diastolic diameter; LVESD: left ventricular end-systolic diameters; MD: mean difference; CI: confidence interval.

*2.5. Trial Sequential Analysis.* In this systematic review and meta-analysis, the curative effect was exaggerated because of random errors, especially when the number of included trials or the total sample size was too small. Thus, the Trial Sequential Analysis (TSA) software version 0.9 Beta (Copenhagen Trial Unit, Copenhagen, Denmark) was used to conduct TSA analysis on the primary outcomes that could be combined and to estimate the meta-analysis sample size and the value strength of the outcome effect. In this study, an overall 5% risk of a type I error was maintained with a power of 80% [20]; the predicted risk reduction was set as 20%, and the relative event rate of the control group was derived from the meta-analysis data.

## 3. Results

*3.1. Characteristics of the Included Trials.* A total of 21 RCTs [21–41] were included in this systematic review. The study by Zhang [34] showed that the HDL levels decreased after treatment, which was contrary to the description of elevated HDL levels. Therefore, this study was excluded from our analysis. Therefore, 20 RCTs were included. One was in English, and the others were in Chinese. The publication years of these studies ranged from 2007 to 2021. The main characteristics of the individual trials are summarized in Table 1. A total of 3565 patients were included; the number of participants in each study ranged from 100 to 591 patients. Only one trial [23] did not indicate the exact number of female and male patients. The overall male to female ratio was approximately 4 : 3. The study period ranged from 8 weeks to 4 years. In all 20 studies, the diagnostic criteria for both DM and CHD were specified. Three studies focused on patients with stable angina pectoris, unstable angina pectoris, or acute coronary syndrome. Chinese medicines used in these studies included both herbal medicines and Chinese patent medicines. The intervention measures in the control group mainly included conventional Western medicines in line with clinical practice and lifestyle modifications, such as exercise and dietary control. Only one study reported that the placebo was delivered to participants in the control group. All 20 studies evaluated multiple outcomes at the end of treatment. Three, two, and the remaining 15 studies reported cardiovascular events, all-cause mortality, and at least two secondary outcome measures, respectively. The specific composition of TCM in the studies is listed in Table 2. Moreover, 12 trials reported adverse events.

*3.2. Methodological Quality.* All 20 trials claimed that the groups were randomized; nine trials [22–24, 26–28, 30, 32, 35] clearly stated the aforementioned using a random number table, while the others did not specify the method used for random sequence generation. No trials described allocation concealment. Only one trial explicitly mentioned blinding the participants and personnel [36]. The blinding of outcome assessment was not specified in any of the trials. Five studies reported cases of loss to follow-up [23, 28, 33,

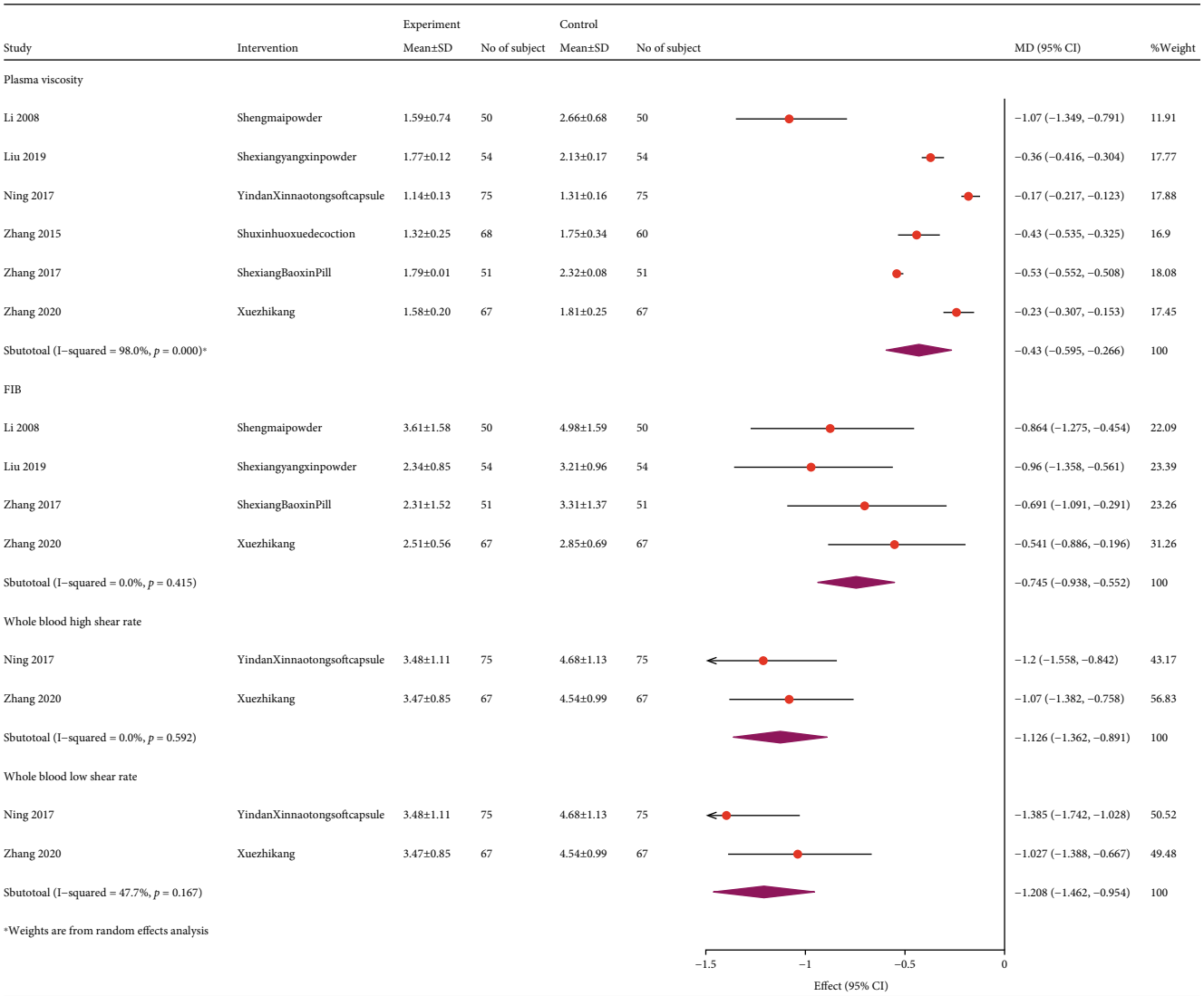


Figure 9: Forest plot of hemorheology. FIB: fibrinogen; MD: mean difference; CI: confidence interval.

35, 36]. Overall, the methodological quality of the included trials was low according to the Cochrane Risk of Bias Tool (Figure 2).

*3.3. Primary Outcomes.* Three studies reported mortality and cardiovascular events. One study found no death events because of the short follow-up period. Compared to patients in the control group, the beneficial effect of TCM participation on mortality was observed (RR, 0.50; 95% CI, 0.32, 0.78; and $P = 0.002$) with low heterogeneity ($I^2 = 47\%$, $P = 0.17$; Figure 3).

However, the TSA result indicated a possibility of a false positive conclusion, and more trials should be included to confirm the efficacy (Figure 4). The results were more reliable when it came to reducing cardiovascular events, showing that TCM adjuvant therapy could effectively reduce the incidence of cardiovascular events (RR, 0.47; 95% CI, 0.34, 0.65; and $P < 0.001$) without heterogeneity ($I^2 = 0\%$; $P = 0.82$; Figure 5). The TSA results also supported this conclusion, as shown in Figure 6. The pooled results ($Z$ curve, blue lines) crossed both the conventional boundary of benefit (dotted line) and the TSA boundary value curve (full line), indicating that a positive conclusion was reached even though the required information size ($n = 3102$) had not yet been reached (Figure 6).

*3.4. Secondary Outcomes*

*3.4.1. Frequency of Angina Pectoris.* Three studies reported data on the change in angina frequency with large heterogeneity. Considering that heterogeneity may be caused by different prescriptions, the pooled effect size analysis was performed according to Ning et al. [29] and Shi et al. [40], with a significant benefit of TCM (MD, -0.88; 95% CI, -1.13, -0.63; and $P < 0.001$), both of which used the Yindan Xinnaotong soft capsules (Figure 7).

*3.4.2. Cardiac Function.* Three studies showed an improvement of cardiac function with TCM. Compared to the control group, TCM decreased the left ventricular end-systolic

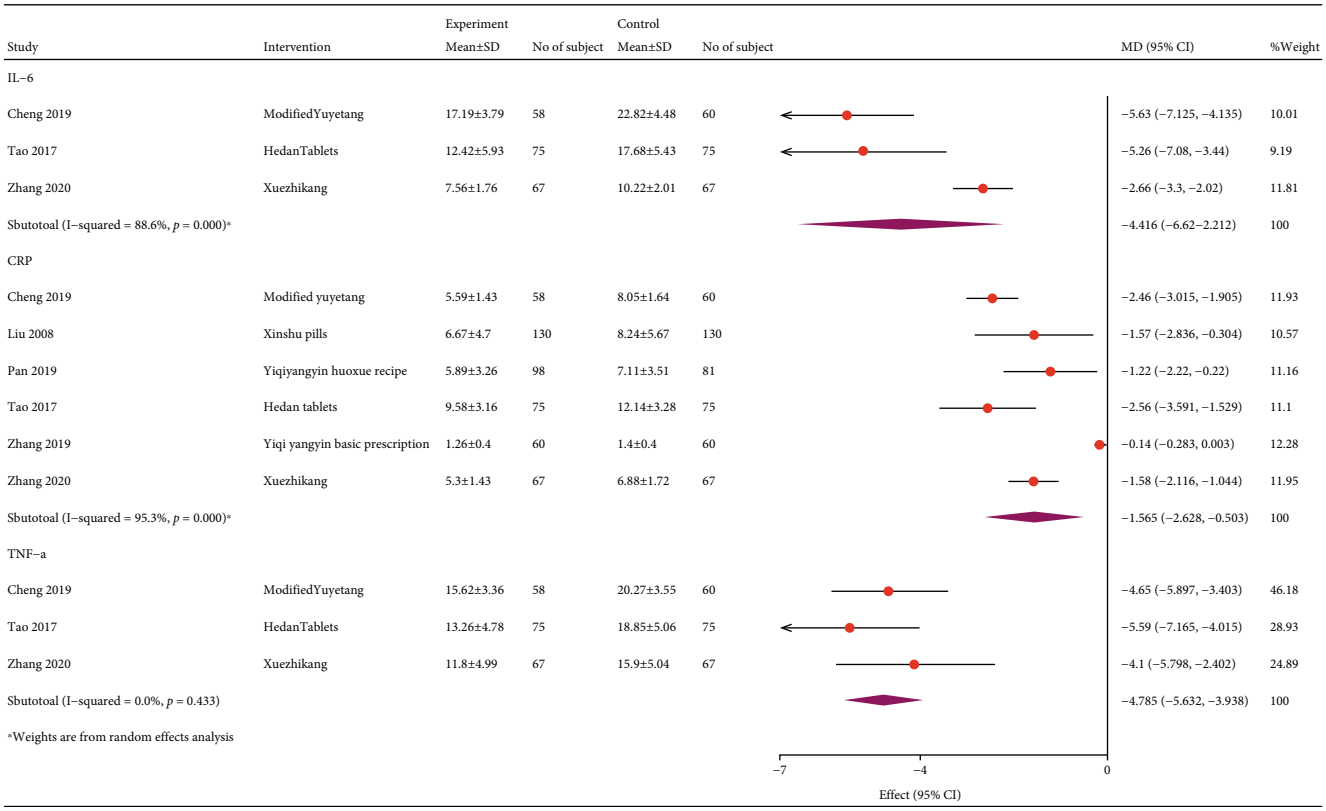


Figure 10: Forest plot of inflammatory factors.

diameters (LVESD) (WMD, -4.94; 95% CI, -5.51, -4.38; $P = 0.971$; and $I^2 = 0$) and left ventricular end-diastolic diameter (LVEDD) (WMD, -5.06; 95% CI, -5.59, -4.54; $P < 0.001$; and $I^2 = 95.9\%$). TCM significantly increased the left ventricular ejection fraction compared to the control group (WMD, 8.43; 95% CI, 7.89, 8.98; $P < 0.001$; and $I^2 = 97.9\%$; Figure 8).

*3.4.3. Hemorheology.* Five articles reported changes in the fibrinogen (FIB) levels. The sensitivity analysis found that Ning et al. [29] reported a large effect on heterogeneity; hence, this study was removed from the analysis, and the remaining studies showed that TCM reduced the FIB levels (SMD, -0.75; 95% CI, -0.94, -0.55; $P = 0.415$; and $I^2 = 0\%$). Six articles reported a decrease in plasma viscosity (WMD, -0.44; 95% CI, -0.46, -0.42; $P < 0.001$; and $I^2 = 98\%$). Two articles reported that TCM had an obvious curative effect on whole blood high shear and whole blood low shear (Figure 9).

*3.4.4. Systematic Inflammatory Factors.* Six studies showed that TCM significantly lowered the TNF-$\alpha$ (WMD, -4.79; 95% CI, -5.63, -3.94; $P = 0.433$; and $I^2 = 0\%$), IL-6 (WMD, -3.32; 95% CI, -3.88, -2.76; $P < 0.001$; and $I^2 = 88.6\%$), and CRP (WMD, -0.43; 95% CI, -2.12, -1.04; $P < 0.001$; and $I^2 = 95.3\%$; Figure 10) levels.

*3.4.5. Blood Lipid Levels.* Fifteen studies reported the blood lipids levels. Eleven studies reported the outcomes of HDL-C. These studies showed that TCM adjuvant therapy improved the HDL-C levels more effectively (WMD, 0.18; 95% CI, 0.15, 0.21; $P < 0.001$; and $I^2 = 98.1\%$). Thirteen studies showed the advantages of TCM adjuvant therapy in reducing TC levels (WMD, -0.83; 95% CI, -0.89, -0.76; $P < 0.001$; and $I^2 = 95\%$). Thirteen studies showed that combined TCM treatment may benefit TG reduction more (WMD, -0.55; 95% CI, -0.6, -0.5; $P < 0.001$; and $I^2 = 89.7\%$). Moreover, 14 studies showed that combined TCM treatment could reduce LDL-C levels more effectively than conventional treatment (WMD, -0.66; 95% CI, -0.7, -0.62; $P < 0.001$; and $I^2 = 95.3\%$; Figure 11). The subgroup analysis of follow-up and prescription is presented in Table 3.

*3.4.6. Blood Glucose Levels.* There were nine studies that reported a change in the HbA1c levels, showing statistically significant differences between the conventional and adjuvant TCM treatments (WMD, -1.29; 95% CI, -1.38, -1.2; $P < 0.001$; and $I^2 = 96.7\%$). Eleven studies showed that adjuvant TCM treatment had advantages in controlling FBC (WMD, -0.49; 95% CI, -0.59, -0.4; $P < 0.001$; and $I^2 = 80.8\%$). Further, 11 studies found that TCM intervention reduced the PBG levels more effectively (WMD, -0.98; 95% CI, -1.1, -0.85; $P < 0.001$; and $I^2 = 96.4\%$; Figure 12). The subgroup analysis of follow-up and prescription is presented in Table 3.

*3.5. Adverse Events.* Among the 12 studies that mentioned adverse events, four declared that no significant adverse

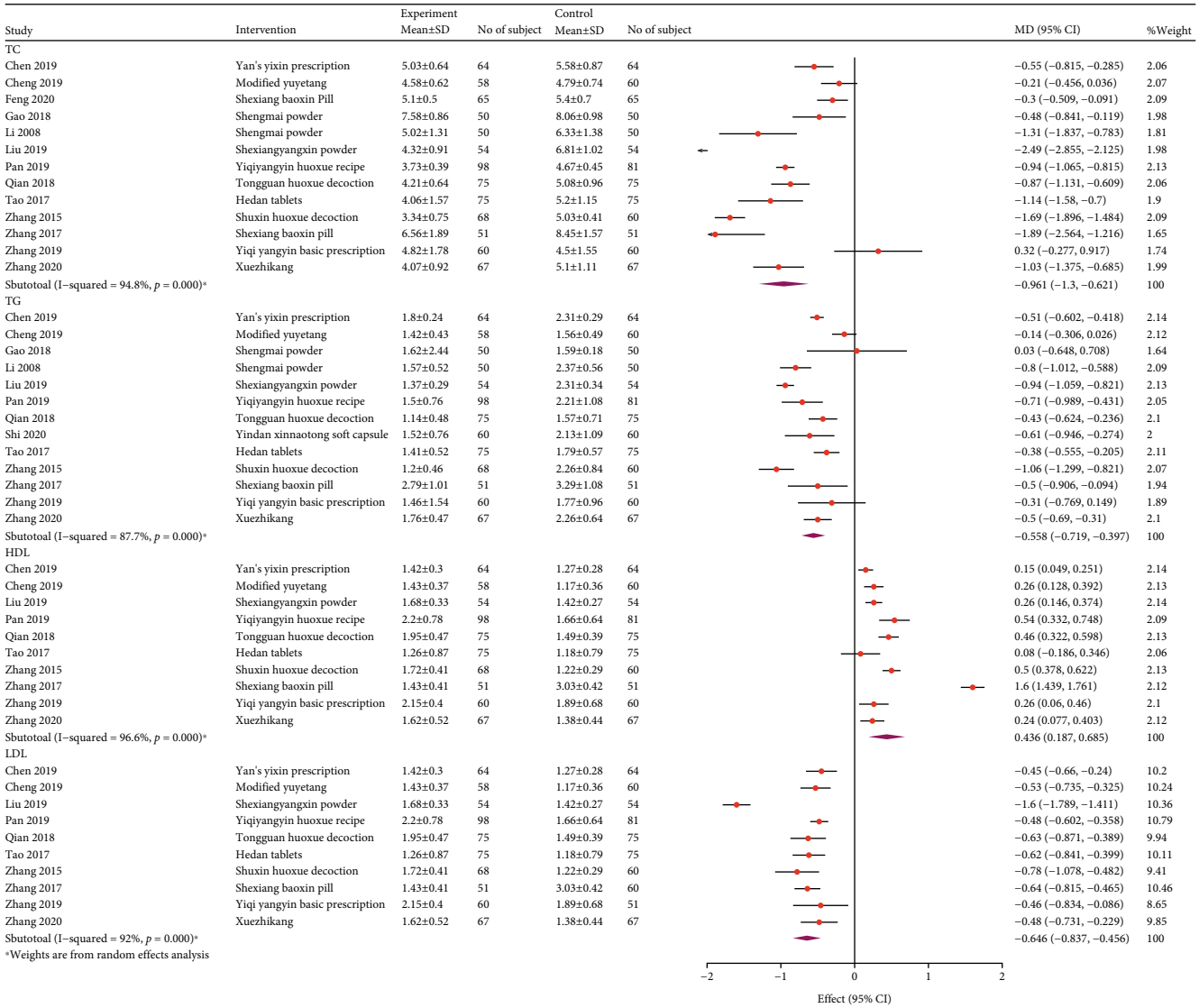


Figure 11: Forest plot of blood lipids.

events occurred with TCM. Gastrointestinal symptoms were reported in the remaining studies; Zhang et al. reported three cases of hypoglycemia, six cases of anorexia, and two cases of headache [41]. Zhao et al. reported two cases of edema, one case of mental-neurological symptoms in the experimental group, and one case of erectile dysfunction in the placebo group [36]. Other adverse events included fever [31], mild headache [25], edema, and itchy skin [21, 30, 31]. There was no statistically significant difference in the incidence of adverse events between the experimental and control groups.

*3.6. Publication Bias.* Funnel plots were drawn in cases where there were >10 articles to examine publication bias regarding the secondary outcomes. Because of the large heterogeneity of the studies, which was difficult to judge by funnel plots alone, Egger's test was performed.

More than 10 studies reported information concerning the TC, TG, LDL, HDL, and 2hPG levels, of which all showed $P$ values > 0.05 by Egger's test, indicating no publication bias in the included studies. The funnel plot and Edger's test results for TC, TG, LDL, HDL, and 2hPG are presented in Supplementary materials (available here).

## 4. Discussion

Diabetes combined with CHD increases the incidence of and mortality due to CV events. This review revealed that the use of TCM in addition to conventional treatment showed potential benefits in the treatment of DM with multiple types of CHD. Compared to the existing integrated management of glucose and cardiovascular risk factors, the addition of many TCM prescriptions formed by the delicate compatibility of herbal ingredients showed better efficacy in reducing cardiovascular events. The Standards of Medical Care for Type 2 Diabetes in China 2019 included a dedicated section focusing on TCM treatment [42].

Table 3: The subgroup analysis of blood lipids and blood glucose.

| Outcomes | No. of studies | No. of experiment | No. of control | MD | 95% CI | *P* value | $I^2$ (%) |
|---|---|---|---|---|---|---|---|
| TC | | | | | | | |
| Total | 13 | 1141 | 1097 | -1.03 | -1.46, -0.60 | <0.001 | 95.2 |
| *Subgroup: follow-up* | | | | | | | |
| 2 months | 3 | 168 | 160 | -1.41 | -2.67, -0.15 | <0.001 | 96.1 |
| 3 months | 8 | 541 | 526 | -1.02 | -1.61,-0.42 | <0.001 | 95.1 |
| 6 months | 1 | 75 | 75 | -0.83 | -1.16, -0.49 | | |
| 4 years | 1 | 285 | 306 | -0.29 | -0.45, -0.13 | | |
| *Subgroup: prescription* | | | | | | | |
| Xuezhikang | 2 | 373 | 352 | -0.63 | -1.34, 0.07 | <0.001 | 92.1 |
| TG | | | | | | | |
| Total | 13 | 1141 | 1097 | -0.93 | -1.32, -0.53 | <0.001 | 94.3 |
| *Subgroup: follow-up* | | | | | | | |
| 2 months | 3 | 168 | 160 | -1.02 | -2.06, -0.03 | <0.001 | 94.8 |
| 3 months | 8 | 541 | 526 | -1.03 | -1.54, -0.53 | <0.001 | 93.3 |
| 6 months | 1 | 75 | 75 | -0.697 | -1.026, -0.367 | | |
| 4 years | 1 | 285 | 306 | -0.062 | -0.223, 0.099 | | |
| *Subgroup: prescription* | | | | | | | |
| Xuezhikang | 2 | 373 | 352 | -0.46 | -1.27,0.35 | <0.001 | 94.2 |
| HDL | | | | | | | |
| Total | 11 | 1068 | 1024 | 0.72 | 0.41, 1.02 | <0.001 | 90.7 |
| *Subgroup: follow-up* | | | | | | | |
| 2 months | 1 | 68 | 60 | 1.39 | 1.01, 1.78 | | |
| 3 months | 8 | 619 | 604 | 0.8 | 0.54, 1.06 | <0.001 | 79.5 |
| 6 months | 1 | 75 | 75 | 0.096 | -0.224, 0.417 | | |
| 4 years | 1 | 306 | 285 | 0.141 | -0.02,0.3 | | |
| *Subgroup:prescription* | | | | | | | |
| Xuezhikang | 2 | 373 | 352 | 0.29 | -0.06, 0.63 | 0.066 | 70.5 |
| LDL | | | | | | | |
| Total | 14 | 1243 | 1199 | -1.20 | -1.61, -0.79 | <0.001 | 95.2 |
| *Subgroup: follow-up* | | | | | | | |
| 2 months | 2 | 110 | 118 | -0.88 | -1.15, -0.6 | 0.711 | 0 |
| 3 months | 10 | 744 | 729 | -1.38 | -1.95, -0.81 | <0.001 | 95.8 |
| 6 months | 1 | 75 | 75 | -0.898 | -1.23, -0.56 | | |
| 4 years | 1 | 306 | 285 | -0.459 | -0.62, -0.3 | | |
| *Subgroup: prescription* | | | | | | | |
| Xuezhikang | 2 | 373 | 352 | -0.49 | -0.64, -0.34 | 0.337 | 0 |
| FBG | | | | | | | |
| Total | 13 | 1143 | 1045 | -0.5 | -0.67, -0.33 | <0.001 | 72.4 |
| *Subgroup: follow-up* | | | | | | | |
| 2 months | 4 | 358 | 275 | -0.44 | -0.73, -0.15 | 0.04 | 64.9 |
| 3 months | 8 | 725 | 710 | -0.49 | -0.7, -0.27 | <0.001 | 76.5 |
| 6 months | 1 | 60 | 60 | -0.886 | -1.26, -0.51 | | |
| *Subgroup: prescription* | | | | | | | |
| Tongxinluo | 2 | 303 | 303 | -0.43 | -0.86, -0.01 | 0.009 | 85.2 |
| HbA1c | | | | | | | |
| Total | 9 | 679 | 664 | -1.159 | -1.85, -0.47 | <0.001 | 96.9 |

Table 3: Continued.

| Outcomes | No. of studies | No. of experiment | No. of control | MD | 95% CI | *P* value | $I^2$ (%) |
|---|---|---|---|---|---|---|---|
| *Subgroup: follow-up* | | | | | | | |
| 3 months | 8 | 619 | 604 | -0.95 | -1.63, -0.28 | <0.001 | 96.6 |
| 6 months | 1 | 60 | 60 | -2.85 | -3.36,-2,3 | | |
| 2hPG | | | | | | | |
| Total | 10 | 948 | 850 | -0.58 | -1, -0.16 | <0.001 | 94.5 |
| *Subgroup: follow-up* | | | | | | | |
| 2 months | 4 | 358 | 275 | -0.42 | -0.72, -0.11 | 0.02 | 68.4 |
| 3 months | 6 | 575 | 590 | -0.69 | -1.37, -0.01 | <0.001 | 96.6 |
| *Subgroup: prescription* | | | | | | | |
| Tongxinluo | 2 | 303 | 303 | -1.42 | -3.33, 0.49 | <0.001 | 99 |

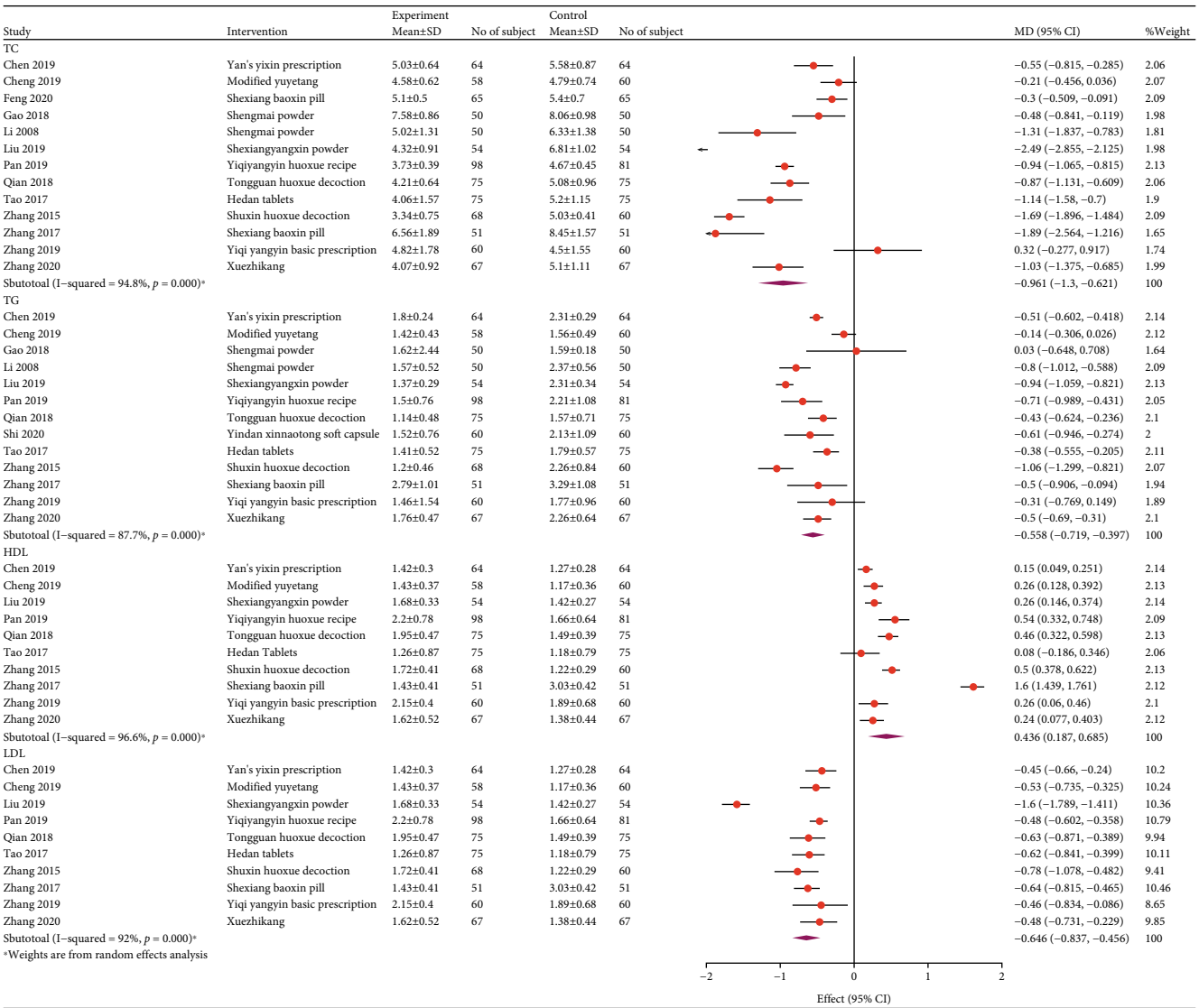


Figure 12: Forest plot of blood glucose levels.

The use of TSA analysis in this paper overcomes the shortcomings of a traditional systematic review or meta-analysis by avoiding hasty conclusions and ignoring the accumulation of random errors. However, it also has certain limitations: it cannot compensate for the defects in the methodological quality of the trial or the errors caused by outcome reporting bias [43].

Articles have compared the efficacy of TCM in the treatment of diabetes and CHD [14], but the outcomes were clinical effectiveness rates, which varied greatly in different studies with no unified standard.

However, combined with the results of previous studies, the use of TCM for the treatment of patients with DM and CHD should be explored further to promote its application.

TCM is characterized by multitargeted effects. The study herein showed beneficial ameliorative effects in the risk factors (blood glucose, lipid, cardiac function, hemorheology, and inflammatory factors) of DM combined with CHD. At present, many Chinese medicines are used to treat cases of DM combined with CHD, but the related clinical studies are insufficient, and the underlying mechanism requires further research. High-quality, randomized, double-blind clinical trials are needed to understand the associated mechanisms.

*4.1. Limitations.* Most of the clinical trials included in this study were high-risk trials. Although all trials claimed to use randomization, only nine described specific randomization methods. Improper randomization often leads to selection bias. At the same time, only one out of 20 studies used a double-blind design, indicating the possibility of potential errors. As the allocation is not concealed, the effect of the intervention may be exaggerated by 30–40% [44]. The reason may be that it is difficult to make a convincing placebo of TCM. This is also a prominent defect of most clinical trials of TCM.

Sensitivity analysis revealed that three studies [25, 29, 36] had a large effect on the heterogeneity of different outcome measures. The work by Zhao et al. [36] was a randomized double-blind trial with intervention for 4 years, which was much longer than the corresponding period in other clinical trials. Interestingly, they reported a clear efficacy on regulating blood lipid indicators [45]. The study suggested that long-term TCM administration may modulate blood lipids better than conventional treatment. The patients included in the study by Li et al. [25] were aged 65–85 (mean age, 74.54) years, which was higher than the corresponding mean ages in other trials. The poor glycemic control, which was possibly attributed to islet cell function, was gradually lost in elderly patients with DM. The patients in the study by Ning et al. [29] received nitrate ester, which can improve hemodynamics and can explain the better control in FIB than other study.

The main sources of heterogeneity were the following: (1) Study protocol designs were not rigorous. (2) The interventions differed greatly in composition, dosage, dosage form, and intervention time. In the theory of TCM, individuals adapted in different formulas for treating DM and CHD are different. Meanwhile, the different dosage forms of TCMs also indicate problems in the quality control of TCMs. (3) Basal interventions differed.

Considering the complicated sources of heterogeneity, we found it difficult to reduce the high heterogeneity through subgroup analysis.

On the one hand, different TCM interventions are main sources of heterogeneity. On the other hand, there are differences in the type of heterogeneity. In addition, although patient baselines were comparable in independent studies, patient conditions, including the severity of CHD, varied between studies.

## 5. Conclusions

Through this systematic review and meta-analysis of adjuvant TCM treatment of CHD in patients with DM, we found that TCM could improve the control effect of conventional treatment on blood glucose, blood lipid, and inflammation, thereby reducing the frequency of angina and the incidence of cardiovascular events and all-cause mortality; this indicates that TCM may be used as a complementary approach to the tertiary prevention of DM. Nevertheless, more rigorously designed RCTs and long-term evaluations are needed to support these findings. The relationship between cardiovascular benefits and TCM control of inflammation, cardiac function, and hemodynamics should be further examined.

## Data Availability

The data used to support the findings of this study are included within the article.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

## Authors' Contributions

Yu Wei and Qiyou Ding, the first two authors, made equal contribution to the work.

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## Supplementary Materials

Supplementary Materials The metaregression analysis of sample, publication year, and sensitivity analysis are available in supplementary materials. *(Supplementary Materials)*

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*Review Article*

# Effects of Resistant Starch on Patients with Chronic Kidney Disease: A Systematic Review and Meta-Analysis

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*Background*. Chronic kidney disease (CKD) is a main health problem associated with increased risk of cardiovascular disease, morbidity, and mortality. Recent studies shown that the progression of CKD may be related to the change of intestinal flora. Resistant starch (RS) is a type of dietary fiber that can act as a substrate for microbial fermentation. Some studies have found that the supplementation of RS can improve the intestinal flora disorder in CKD patients. However, the specific effect of RS on CKD patients remains controversial. *Objective*. We designed this meta-analysis to identify and assess the effects of RS on patients with CKD. *Methods*. A comprehensive search of MEDLINE, Embase, Web of Science, and Cochrane systematic review databases was conducted in January 2020, and all new trials were updated in August 2021. Randomized trials were collected to assess the effects of RS on patients with CKD. The weighted average effect size of the net change was calculated by using the random-effects model. *Results*. The meta-analysis included 8 studies involving 301 participants. RS intake significantly reduced serum indolephenol sulfate (IS), blood phosphorus, IL-6, and uric acid levels in dialysis patients. The mean difference (MD) of serum IS ($P = 0.0002$) in the dialysis subgroup was -12.57 $\mu$mol/L (95% CI: -19.28, -5.86 $\mu$mol/L). The MD of blood phosphorus ($P = 0.03$) was -0.39 mg/dl (95% CI: -0.78, -0.01 mg/dl). The MD of serum uric acid ($P = 0.004$) between the dialysis subgroup and the nondialysis subgroup was -31.58 mmol/L (95% CI: -52.99, -10.17 mmol/L). The mean difference (MD) of IL-6 ($P = 0.02$) in the dialysis subgroup was -1.16 $\mu$mol/L (95% CI: -2.16, -0.16 $\mu$mol/L). However, there was no significant change of RS on hs-CRP, serum creatinine, blood urea nitrogen (BUN), blood paracresol sulfate, and blood lipid. *Conclusions*. The intake of RS reduced the serum IS, serum phosphorus, IL-6, and uric acid levels significantly in dialysis patients, while hs-CRP, serum creatinine, BUN, serum paracresol sulfate, and blood lipid showed no significant changes.

## 1. Introduction

Chronic kidney disease CKD has become a main cause of morbidity and mortality of kidney disease worldwide. CKD affects nearly 16% of the adult population and consumes a disproportionate share of health care resources in both developed and developing countries [1–3]. Patients with CKD experience continuous oxidative stress and inflammation. These conditions are associated with the progression of kidney disease and other complications associated with cardiovascular disease [4]. Recently, some researchers have found that the imbalance of the microbiome that occupies the human gut can be considered a new cardiovascular risk factor in patients with CKD because it is directly associated with inflammation and oxidative stress. With the imbalance of intestinal flora, the structure of intestinal epithelial barrier is destroyed, and the permeability of colon is increased. Some metabolites of bacteria, including uremic toxins (such as IS and precursor of cresol sulfate), enter the blood stream [5, 6]. High levels of sulfate indole of phenol and cresol sulfate are associated with poor prognosis in patients receiving hemodialysis [7].

Resistant starch (RS) is the sum of starch and its degradation products that have not been absorbed by the small intestine. It is defined as a component that is resistant to the hydrolysis of pancreatic amylase in the small intestine and reaches the large intestine. As a dietary fiber, RS can serve as a substrate for microbial fermentation [8, 9]. In addition, some intestinal bacteria promote the fermentation of soluble fiber and RS to produce short chain fatty acids (SCFA). The main function of SCFA is to improve the integrity of the intestinal epithelial barrier and relieve local and systemic inflammation. Otherwise, the increases of SCFA production can decrease the intestinal pH [10–12]. So, the fermentable fiber in patients with chronic kidney disease (CKD) has attracted researchers' interest. An analysis of data from 14,543 participants of the U.S. National Health and Nutrition Examination Survey (III) showed that high dietary fermentable fiber intake was associated with a reduced risk of inflammation and death from kidney disease [13]. A recent systematic review and meta-analysis of 143 CKD participants showed that dietary fiber can reduce BUN and creatinine concentrations and has dose-dependent effects on serum creatinine [14]. Recently, RS has gained attention, and a number of trials have investigated the relationship between RS and serum uremia toxins as well as systemic inflammation and oxidative stress in CKD patients [15–22], but these findings are controversial, for example, Tayebi et al. found that in maintenance hemodialysis patients, a diet rich in RS significantly reduced serum concentrations of paracresol, while there was no significant change in IS levels in the treatment group [20]. Marta et al. found that the plasma level of IS significantly decreased after the addition of RS, and the plasma level of cresol sulfate was not affected [18]. As described above, studies on evaluating the effects of RS in the management of CKD have demonstrated controversial findings, and these results were few systematically reviewed. Therefore, this meta-analysis and systematic review aimed at investigating the effects of resistant starch intake on CKD patients.

## 2. Methods

*2.1. Search Strategy.* A comprehensive search of MEDLINE, Embase, Web of Science, and Cochrane systematic review databases was conducted in July 2020, and all new trials were updated in August 2021. Randomized trials were collected to assess the effects of resistant starch on CKD patients. The retrieval strategy used is as follows: take MEDLINE as an example and set the following retrieval formula: ((high-amylose maize type 2-resistant starch, maize [Mesh] OR Resistant starch OR HAM-RS2 OR Hi-maize 260)) AND (Renal Insufficiency, Chronic [Mesh] OR chronic renal insufficiencies OR chronic kidney insufficiencies OR chronic kidney diseases OR chronic renal diseases OR diabetic nephropathies OR diabetic kidney diseases OR kidney failure OR chronic kidney failure OR kidney disease OR uremia OR dialysis OR continuous ambulatory peritoneal dialysis OR hemodialysis OR renal replacement therapy OR peritoneal dialysis OR Equilibrium dialysis OR extended daily dialysis). The articles are filtered using filters (sensitive search strategies used to ensure the best collection of RCTs in electronic searches). In our study, only randomized controlled trials (RCTs) were identified for inclusion and without any language restriction. In the first step of the retrieval process, the titles and abstracts of each article are carefully filtered to include them. Then, all potentially relevant articles were carefully checked for full text for further identification. References to reviews of the effects of RS on CKD patients were also carefully reviewed for inclusion in potential trials.

*2.2. Selection Criteria*

(1) The study was a crossover or parallel designed RCT test, and the duration time was ≥4 weeks

(2) To study the effect of resistant starch on CKD patients (including dialysis patients)

(3) Adult patients with renal insufficiency or renal failure (over 18 years of age) were eligible for inclusion in the study

(4) The study did not address other factors that may have potentially positive or negative effects on kidney function

The main exclusion criteria are as follows:

(1) Participants under the age of 18

(2) Literature on other results, such as pharmacokinetics and basic research

(3) Kidney transplant patients

(4) Reviews and editorials

*2.3. Data Extraction and Quality Assessment.* Data were collected independently from each included RCT to extract the mean and standard deviation of the primary and secondary outcome indicators. If the published clinical trial report only reported the median, scope, and size of the trial, the mean and standard deviation were estimated by statistical methods [23], and all differences were resolved by consensus. For these included trials, a data extraction table was used to collect the following data: the first author's name, year of publication, number of participants, characteristics of participants (age, gender, dialysis patient, etc.), intervention and control measures, research design, and content and duration of resistant starch (Table 1). The main outcome indicators were serum IS, serum paracresol sulfate, serum creatinine, and serum urea nitrogen, and the secondary outcome indicators were uric acid level, serum phosphorus, hs-CRP, blood lipid, and IL-6.

Methodological quality and risk of bias of each included RCTs were examined carefully using the method described by the Cochrane Collaboration [24]. The items were as follows: (1) random sequence generation, (2) allocation concealment, (3) blinding of participants and personnel, (4) blinding of outcome assessment, (5) incomplete outcome data, (6) selective reporting, and (7) other sources of bias.

Table 1: Basic characteristics of the included research.

| Study, year | Participant | M/F[a] | Control | Intervention | Mean age (S.D. or range), years | Design | Fiber dose (S.D. or range) (g/day) | Duration (weeks) |
|---|---|---|---|---|---|---|---|---|
| Sirich et al. 2014 [15] | 40 HD[b] | 24/16 | Waxy corn starch ($N = 20$) | RS (Hi-maize 260) ($N = 20$) | 60.5 ± 20.5 | R[e].DB[f].P[g] | 15 | 6 |
| Tayebi et al., 2018 [17] | 44 HD | 28/16 | Regular wheat flour ($N = 22$) | HAM-RS2 ($N = 22$) | 57 ± 16 | R.DB.P | 20 (0-4 w)<br>25 (4-8 w) | 8 |
| Marta et al., 2018 [18] | 31 HD | 18/13 | Manioc flour ($N = 16$) | Hi-Maize ® 260 ($N = 15$) | 53.5 ± 11.5 | R.DB.P | 16 | 4 |
| Meng et al., 2019 [19] | 70 type 2 DN[c] | 39/31 | Ordinary staple food ($N = 36$) | High-RS, low-protein flour ($N = 34$) | 53.5 ± 11.5 | R.DB.P | 16 | 4 |
| Tayebi et al., 2019 [20] | 44 HD | N[d] | Waxy corn starch placebo cookie ($N = 21$) | (HAM-RS2) ($N = 23$) | 57.90 ± 13.34 | R.DB.P | 20 (0-4 w)<br>25 (4-8 w) | 8 |
| Laffin et al., 2019 [21] | 20 HD | 13/7 | Fermentable fiber placebo cookies ($N = 11$) | (HAM-RS2) ($N = 9$) | 54.3 ± 12.3 | R.DB.P | 20 (0-4 w)<br>25 (4-8 w) | 8 |
| Marta et al., 2020 (1) [22] | 26 HD | N | Manioc flour ($N = 14$) | Cookies rich in resistant starch ($N = 14$) | 53.5 ± 11.4 | R.TB[h].C[i] | 16 | 4 |
| Marta et al., 2020 (2) [22] | 26 HD | N | Manioc flour ($N = 12$) | Cookies rich in resistant starch ($N = 12$) | 53.5 ± 11.4 | R.TB.C | 16 | 4 |
| Andrade et al., 2021 (1) [26] | 26 PD[h] | 14/12 | Waxy corn starch ($N = 26$) | Unripe banana flour (RS 48%) ($N = 26$) | 55 ± 12 | R.TB.C | 14 | 8 |
| Andrade et al., 2021 (2) [26] | 11 PD | 5/6 | Waxy corn starch ($N = 11$) | Unripe banana flour (RS 48%) ($N = 11$) | 54 ± 15 | R.TB.C | 18.7 | 8 |

Abbreviations: M/F[a]; ratio of male and female; HD[b]: hemodialysis; DN[c]: diabetic nephropathy; N[d]: none; $R$[e]; randomized; DB[f]: double-blind; TB[h]: triple-blind; C[i]: crossover; P[g]: parallel; PD[h]: peritoneal dialysis.

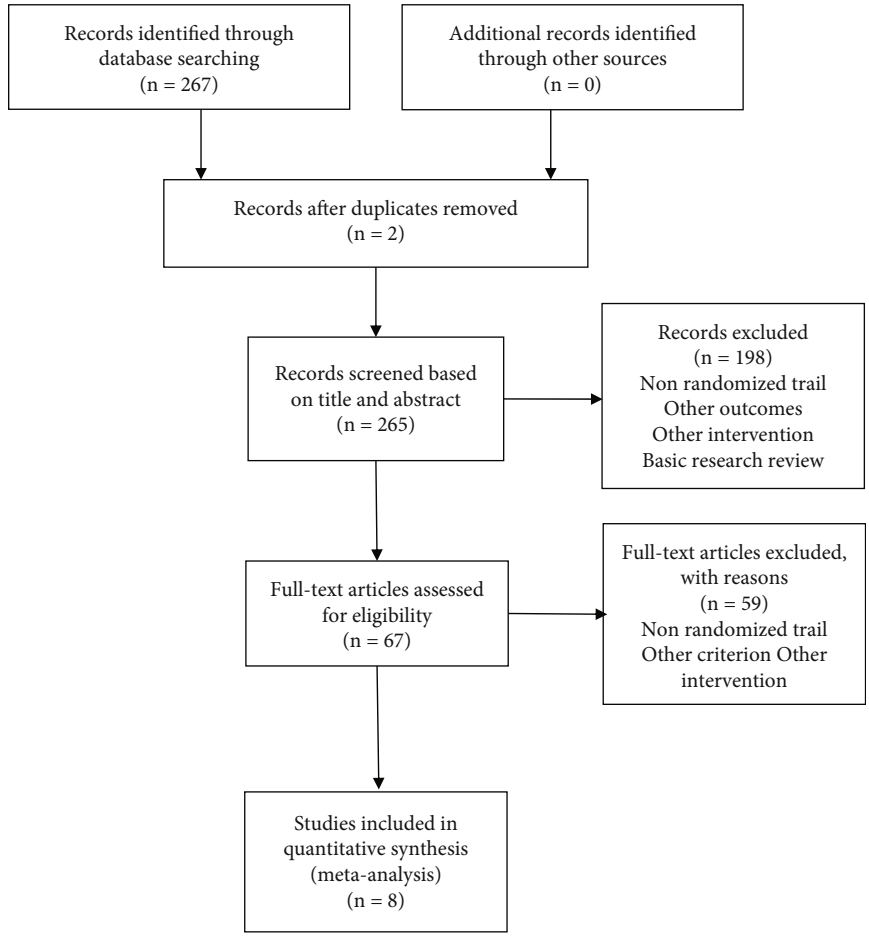


Figure 1: The flow chart of the literature selection process and the reasons for exclusion.

All seven items were classified as "low risk of bias," "high risk of bias," or "unclear risk of bias."

*2.4. Statistical Analyses.* Data from each of the included trials were analyzed using the RevMan version 5.3, Copenhagen: Cochrane center for northern Europe, Cochrane Collaboration, 2017. The treatment effect was expressed as the mean difference between the change and its 95% confidence interval (CI), and the summary effect was calculated by assigning a weight to the reciprocal of each trial variance. We also performed a subgroup analysis to investigate the potential effects of CKD type (hemodialysis or nonhemodialysis) on the outcome. Heterogeneity of treatment is as follows: using the $\chi^2$ test and the $I^2$ test to assess inconsistencies, we used a random-effects model to calculate the pooled effect size. Use of a random-effects model is less likely to produce significant results for pooled effect sizes than use of a fixed-effects model [25]. The $P$ value threshold of statistical significance was set at 0.05, and $P \leq 0.05$ was considered significant and statistically significant.

## 3. Results

*3.1. Trial Flow/Flow of Included Studies.* A search of MEDLINE, Embase, Web of Science, and Cochrane systematic review databases identified 267 trials, of which 200 were excluded at the initial screening. Fifty-nine potentially relevant trials were identified for further review, of which eight met our inclusion criteria. All enrolled studies were randomized controlled trials to evaluate the role of RS in patients with CKD. The detailed process of our study selection is shown in Figure 1.

*3.2. Study Characteristics.* Eight studies (301 participants) were identified to assess the impact of RS on CKD patients. Table 1 lists the specific characteristics of the 8 studies. Since Marta et al. 2020 [22] and Andrade et al. 2021 [26] adopted the treatment of intervention—washout period—reintervention, it was divided into two research groups according to the treatment before and after the washout period. According to the type of CKD (dialysis or nondialysis), we divided the data into different subgroups for combined analysis. Seven of the included studies compared RS and common starch. Among the included studies, 7 articles were targeted at dialysis patients. And only one article was nondialysis patients, which is the study object of Meng et al. 2019 [19] which was patients with early type 2 diabetes. As for follow-up time, the duration of these included studies ranged from 4 weeks to 12 weeks.

*3.3. Quality of Included Studies.* The quality of the included studies varied according to the standard methodology

recommended by the Cochrane Collaboration for assessing the risk of bias in Figure 2.

### 3.4. Quantitative Data Synthesis

*3.4.1. Indolephenol Sulfate.* A total of five trials were conducted to evaluate the effect of RS intake on CKD. For dialysis patients treated with RS, blood IS was reduced in the RS group compared with the control group, which has statistically significant, and the estimated change of combined effect was -12.57 $\mu$mol/L ((95% CI: -19.28, -5.86 $\mu$mol/L), $P = 0.0002$), using the random-effects model (Figure 3). In addition, seven trials were insufficient to detect publication bias.

*3.4.2. Paracresol Sulfate.* A total of four trials were conducted to evaluate the effect of RS intake on CKD. Compared with the control group, there was no significant difference in the change of serum p-cresol sulfate in the RS subgroup, and the combined estimated change was 1.16 $\mu$mol/L ((95% CI: -12.38, 14.71 $\mu$mol/L), $P = 0.87$) (Figure 4). Using the random-effects model, six trials were insufficient to detect publication bias.

*3.4.3. Blood Uric Acid.* A total of four trials were included, which were divided into two subgroups according to whether they received dialysis or not. Compared with the control group, the serum uric acid level of the dialysis subgroup after RS treatment was significantly reduced, and the combined estimated change was -30.04 mmol/L ((95% CI: -57.65, -2.43 mmol/L), $P = 0.03$). The nondialysis subgroup included only one trial in which the serum uric acid level was reduced, with an estimated change of -33.90 mmol/L ((95% CI: -67.81, 0.01 mmol/L), $P = 0.05$). The total estimated change was -31.58 mmol/L ((95% CI: -52.99, -10.17 mmol/L), $P = 0.004$) (Figure 5). The above random-effects model was used, and four experiments were not enough to detect publication bias.

*3.4.4. Blood Phosphorus.* There were 4 trials, all of which were dialysis combined with RS. Compared with the control group, the blood phosphorus level significantly decreased after treatment, which was statistically significant. The estimated change of the combination was -0.39 mg/dl ((95% CI: -0.78, -0.01 mg/dl), $P = 0.05$) (Figure 6). Using the random-effects model, five trials were insufficient to detect publication bias.

*3.4.5. Blood Urea Nitrogen.* Five tests were identified to assess the impact of RS intake on CKD, and they were divided into two subgroups according to whether or not they received dialysis. The dialysis and RS subgroups included four tests. Compared with the control group, there was no significant difference in blood urea nitrogen change, and the combined estimated change was -4.94 mg/dl ((95% CI: -10.81, 0.93 mg/dl), $P = 0.10$). Only one trial was included in the nondialysis subgroup, in which there was no significant difference in blood urea nitrogen between the nondialysis and RS subgroups, and the combined estimated change was -0.28 mg/dl ((95% CI: -1.86, 1.30 mg/dl), $P = 0.73$) (Table 2). Using the random-effects model, five trials were insufficient to detect publication bias.

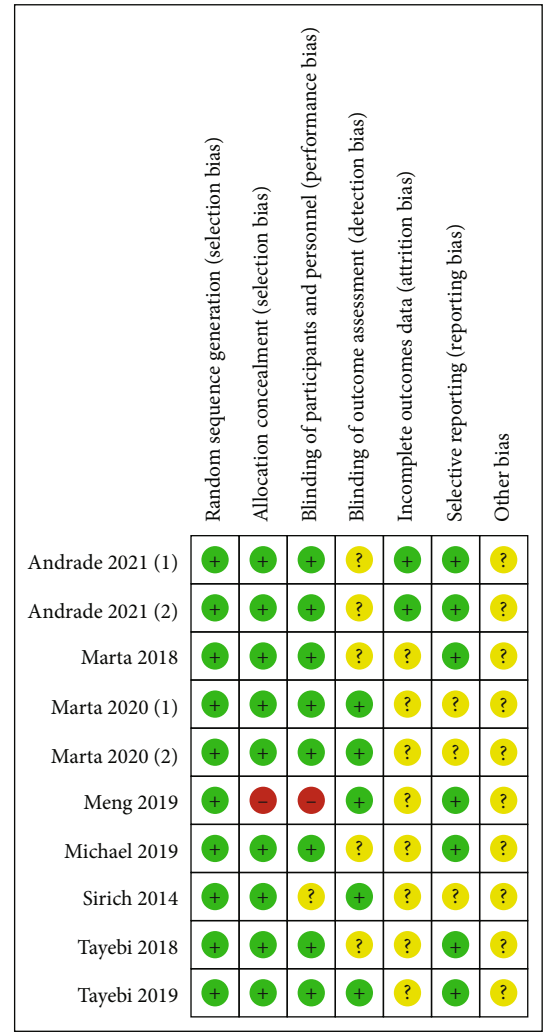


Figure 2: Quality evaluation of included studies and risk of bias summary.

*3.4.6. Serum Creatinine.* Four tests were determined to evaluate the effect of RS intake on CKD. The patients were divided into two subgroups according to whether they received dialysis or not. Compared with the control group, there was no significant difference in serum creatinine changes between the dialysis and RS subgroups, and the combined estimated changes were -44.94 $\mu$mol/L ((95% CI: -171.99, 82.12 $\mu$mol/L), $P = 0.69$). The nondialysis subgroup included only one test, in which there was no significant difference in serum creatinine between the nondialysis and RS subgroups, with an estimated change of 4.0 $\mu$mol/L ((95% CI: -3.49, 11.49 $\mu$mol/L), $P = 0.30$) (Table 2) using the random-effects model. In addition, 3 trials were insufficient to detect publication bias.

*3.4.7. hs-CRP.* This result was reported in only four trials. No significant difference was observed in the change of hs-CRP after the RS intervention compared with the

| Study or Subgroup | Intervention Mean | SD | Total | Control Mean | SD | Total | Weight | Mean Difference IV, Random, 95% Cl |
|---|---|---|---|---|---|---|---|---|
| 4.81 RS | | | | | | | | |
| Andrade 2021 (1) | 63 | 26 | 26 | 72.7 | 25.875 | 26 | 22.6% | −9.70 (−23.80, 4.40) |
| Andrade 2021 (2) | 72.25 | 29.878 | 11 | 83 | 32.107 | 11 | 6.7% | −10.75 (−36.67, 15.17) |
| Marta 2018 | 90.33 | 15.52 | 15 | 100.575 | 18205 | 16 | 31.9% | −10.25 (−22.13, 1.64) |
| Marta 2020 (1) | 87.98 | 39.59 | 14 | 131.7 | 68.32 | 14 | 2.6% | −43.72 (−85.08, −2.36) |
| Marta 2020 (2) | 66.85 | 35.42 | 12 | 71.99 | 44.57 | 12 | 4.3% | −5.14 (−37.35, 27.07) |
| Sirich 2014 | 115.44 | 55.73 | 20 | 123.4 | 47.77 | 20 | 4.4 | −7.96 (−40.13, 24.21) |
| Tayebi 2019 | 154.97 | 21.32 | 23 | 171.98 | 21.94 | 21 | 27.5 | −17.01 (−29.82, −4.20) |
| Subtotal (95% Cl) | | | 121 | | | 120 | 100% | −12.57 (−19.28, −5.86) |
| Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 3.25$, df = 6 (P = 0.78); $I^2 = 0\%$ | | | | | | | | |
| Test for overall effect: Z = 3.67 (P = 0.0002) | | | | | | | | |
| Total (95% Cl) | | | 121 | | | 120 | 100.0% | −12.57 (−19.28, −5.86) |
| Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 3.25$, df = 6 (P = 0.78); $I^2 = 0\%$ | | | | | | | | |
| Test for overall effect: Z = 3.67 (P = 0.0002) | | | | | | | | |
| Test for subgroup diffrences: Not applicable | | | | | | | | |

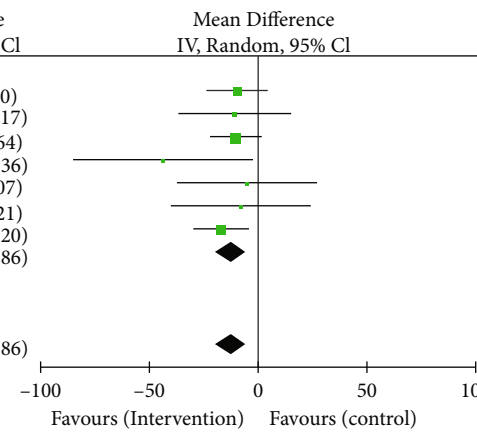


Figure 3: Pooled estimated effect of RS intake on IS ($\mu$mol/L) in patients with CKD, with estimated MD and 95% CIs.

| Study or Subgroup | Intervention Mean | SD | Total | Control Mean | SD | Total | Weight | Mean Difference IV, Random, 95% Cl |
|---|---|---|---|---|---|---|---|---|
| 4.5.1 RS | | | | | | | | |
| Andrade 2021 (1) | 171.5 | 33 | 26 | 164 | 33.5 | 26 | 56.1% | 7.50 (−10.58, 25.58) |
| Andrade 2021 (2) | 130.75 | 25.703 | 11 | 134.25 | 26.695 | 11 | 34.1% | −3.50 (−26.71, 19.71) |
| Marta 2018 | 482.465 | 176.415 | 15 | 572.89 | 126.89 | 16 | 1.5% | −90.43 (−199.22, 18.37) |
| Marta 2020 (1) | 490.97 | 342.34 | 14 | 530.92 | 286.61 | 14 | 0.3% | −39.95 (−273.82, 193.92) |
| Marta 2020 (2) | 488.84 | 250.8 | 12 | 379.38 | 262.11 | 12 | 0.4% | 109.46 (−95.79, 314.71) |
| Sirich 2014 | 154.09 | 85.01 | 20 | 164.72 | 74.39 | 20 | 7.5% | −10.63 (−60.14, 38.88] |
| Subtotal (95% Cl) | | | 98 | | | 99 | 100.0% | 1.16 (−12.38, 14.71) |
| Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 4.76$, df = 5 (P = 0.45); $I^2 = 0\%$ | | | | | | | | |
| Test for overall effect: Z = 0.17 (P = 0.87) | | | | | | | | |
| Total (95% Cl) | | | 98 | | | 99 | 100.0% | 1.16 (−12.38, 14.71) |
| Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 4.76$, df = 6 (P = 0.45); $I^2 = 0\%$ | | | | | | | | |
| Test for overall effect: Z = 0.17 (P = 0.87) | | | | | | | | |
| Test for subgroup diffrences: Not applicable | | | | | | | | |

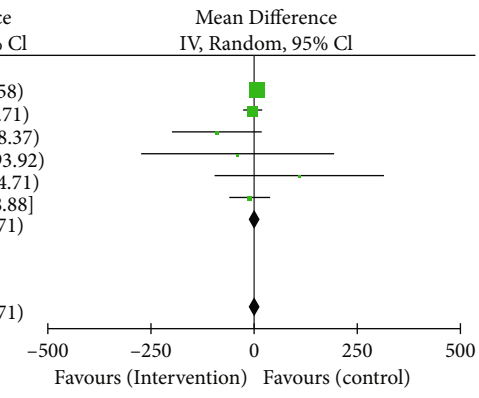


Figure 4: Pooled estimated effect of RS intake on paracresol sulfate ($\mu$mol/L) in patients with CKD, with estimated MD and 95% CIs.

| Study or Subgroup | Intervention Mean | SD | Total | Control Mean | SD | Total | Weight | Mean Difference IV, Random, 95% CI |
|---|---|---|---|---|---|---|---|---|
| 4.1.1 Dialysis patients | | | | | | | | |
| Michael 2019 | 400.435 | 74.375 | 9 | 422.45 | 73.185 | 11 | 10.8% | −22.01 (−87.07, 43.04) |
| Tayebi 2018 | 392.1 | 59.5 | 22 | 422.45 | 69.45 | 22 | 31.4% | −30.35 (−68.56, 7.86) |
| Tayebi 2019 | 392.1 | 64.26 | 23 | 426.45 | 73.185 | 11 | 17.9% | −34.35 (−84.95, 16.25) |
| Subtotal (95% CI) | | | 54 | | | 44 | 60.1% | |
| Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 0.09$, df = 2 (P = 0.96); $I^2 = 0\%$ | | | | | | | | |
| Test for overall effect: Z = 2.13 (P = 0.03) | | | | | | | | |
| 4.1.2 Non-Dialysis patients | | | | | | | | |
| Meng 2019 | 307.3 | 58.2 | 34 | 341.2 | 84.8 | 36 | 39.9% | −33.90 (−67.81, 0.01) |
| Subtotal (95% CI) | | | 34 | | | 36 | 39.9% | −33.90 (−67.81, 0.01) |
| Heterogeneity: Not applicable | | | | | | | | |
| Test for overall effect: Z = 1.96 (P = 0.05) | | | | | | | | |
| Total (95% CI) | | | 88 | | | 80 | 100.0% | −31.58 (−52.99, −10.17) |
| Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 0.12$, df = 3 (P = 0.99); $I^2 = 0\%$ | | | | | | | | |
| Test for overall effect: Z = 2.89 (P = 0.004) | | | | | | | | |
| Test for subgroup differences: $Chi^2 = 0.03$, df = 1 (P = 0.86), $I^2 = 0\%$ | | | | | | | | |

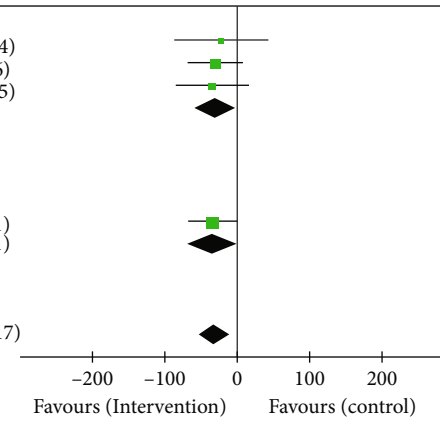


Figure 5: Pooled estimated effect of RS intake on blood uric acid (mmol/L) in patients with CKD, with estimated MD and 95% CIs.

control, with the combined estimated change of -0.05 mg/dl ((95% CI: -0.15, 0.05 mg/dl), $P = 0.31$) (Table 2). Using the random-effects model, four trials were insufficient to detect publication bias.

*3.4.8. IL-6.* There were four trials, which were divided into two subgroups according to whether they received dialysis or not. Compared with the control group, there was significant change in IL-6 levels after RS treatment in the dialysis

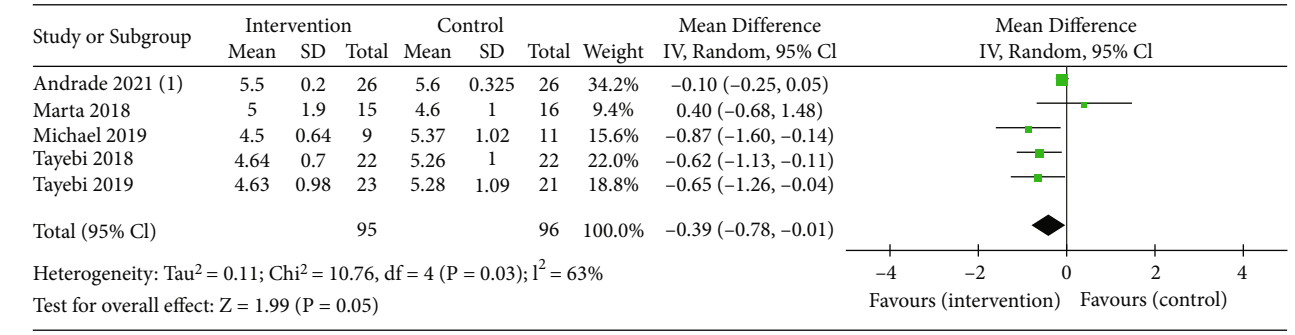


Figure 6: Pooled estimated effect of RS intake on blood phosphorus (mg/dl) in patients with CKD, with estimated MD and 95% CIs.

Table 2: Summary of the effects of RS intake in patients with CKD compared with control.

| Outcome | No. of studies | No. of populations (intervention/control) | Test for $I^2$ | Heterogeneity ($P$) | Analysis model | Overall effect ($P$) | Mean difference, 95% CI |
|---|---|---|---|---|---|---|---|
| SCR[a] (mmol/L) | 4 | 98/99 | 75% | 0.007 | Random-effects | 0.78 | -28.88 (-101.11, 43.35) |
| HD[b] | 3 | 64/63 | 77% | 0.01 | Random-effects | 0.69 | -44.94 (-171.99, 82.12) |
| NHD[c] | 1 | 34/36 | — | — | — | 0.30 | 4.00(-3.49, 11.49) |
| hs-CRP[a] (mg/dl) | 4 | 84/83 | 1% | 0.39 | Random-effects | 0.31 | -0.05(-0.15, 0.05) |
| IL-6[a] (pg/mL) | 4 | 84/89 | 88% | <0.00001 | Random-effects | 0.12 | -0.77 (-1.75, 0.21) |
| HD[b] | 3 | 50/53 | 79% | 0.008 | Random-effects | 0.02 | -1.16 (-2.16, -0.16) |
| NHD[c] | 1 | 34/36 | — | — | — | 0.21 | 0.30 (-0.17, 0.77) |
| BUN[a] (mg/dl) | 5 | 118/119 | 0% | 0.64 | Random-effects | 0.44 | -0.60 (-2.12,0.93) |
| HD[b] | 4 | 84/83 | 0% | 0.97 | Random-effects | 0.10 | -4.94 (-10.81,0.93) |
| NHD[c] | 1 | 34/36 | — | — | Random-effects | 0.73 | -0.28 (-1.86,1.30) |
| TC (mmol/L)[a] | 3 | 79/79 | 0% | 0.82 | Random-effects | 0.21 | 0.20 (-0.11, 0.51) |
| HD[b] | 2 | 45/43 | 0% | 0.86 | Random-effects | 0.19 | -0.29 (-0.14, 0.72) |
| NHD[c] | 1 | 34/36 | — | — | — | 0.21 | 0.10 (-0.35, 0.55) |
| TG (mmol/L)[a] | 3 | 79/79 | 0% | 0.86 | Random-effects | 0.70 | 0.05(-0.20,0.29) |
| HD[b] | 2 | 45/43 | 0% | 0.72 | Random-effects | 0.57 | 0.10 (-0.25,0.46) |
| NHD[c] | 1 | 34/36 | — | — | — | 1 | 0.00 (-0.33, 0.33) |
| HDL (mmol/L)[a] | 3 | 79/79 | 0% | 0.68 | Random-effects | 0.16 | 0.06 (-0.03, 0.15) |
| HD[b] | 2 | 45/43 | 0% | 1 | Random-effects | 0.76 | 0.02 (-0.11, 0.15) |
| NHD[c] | 1 | 34/36 | — | — | — | 0.1 | 0.10 (-0.02, 0.22) |

[a]The total effect. HD[b]: hemodialysis subgroup; NHD[c]: nonhemodialysis subgroup; TC: total cholesterol; TG: triglycerides; HDL: high-density lipoprotein; SCR: serum creatinine; BUN: blood urea nitrogen.

subgroup, and the combined estimated change was -1.16 pg/mL ((95% CI: -2.16, -0.16 pg/mL), $P = 0.02$). There was only one trial in the nondialysis subgroup, in which there was no significant change in blood IL-6 level, with an estimated change of 0.30 pg/mL ((95% CI: -0.17, 0.77 pg/mL), $P = 0.21$). The total estimated change was -0.77 pg/mL ((95% CI: -1.75, 0.21 pg/mL), $P = 0.12$) (Table 2). Using the random-effects model, four trials were insufficient to detect publication bias.

*3.4.9. Blood Lipids.* This result was reported in 3 trials, which were divided into two subgroups according to whether they received dialysis or not. Compared with the control group, there was no significant change in lipid level after RS treatment in the dialysis subgroup, and the combined estimated change was total cholesterol -0.29 mmol/L ((95% CI: -0.14, 0.72 mmol/L), $P = 0.19$), triglyceride 0.10 mmol/L ((95% CI: -0.25, 0.46 mmol/L); $P = 0.57$), and high-density lipoprotein 0.02 mmol/L ((95% CI: -0.11, 0.15 mmol/L); $P = 0.76$). The nondialysis subgroup included a trial, in which there was no significant change in lipid level, and the change was estimated to be 0.10 mmol/L of total cholesterol ((95% CI: -0.35, 0.55 mmol/L), $P = 0.21$), triglyceride 0.00 mmol/L ((95% CI: -0.33, 0.33 mmol/L; $P = 1$), and high-density lipoprotein 0.10 mmol/L ((95% CI: -0.02, 0.22 mmol/L); $P = 0.1$) (Table 2). Using the random-effects model, 3 trials were insufficient to detect publication bias.

## 4. Discussion

Nutritionists and clinicians have long explored whether different dietary interventions can control or improve the

CKD. A recent systematic review and meta-analysis of 143 CKD participants showed that dietary fiber can reduce BUN and creatinine concentrations and has dose-dependent effects on serum creatinine [14]. However, there is an obvious lack of tests to assess other uremia retention solute (such as IS and paracresol sulfate) and inflammatory indicators and only one Meijers' study [27]. In addition, the effects of different fiber types on the CKD were not discussed separately. Recently, RS have gained attention, and a number of trials have investigated the relationship between RS and serum uremia toxins as well as systemic inflammation and oxidative stress in CKD patients [15–22]. As these findings are controversial, further analysis is needed. For example, Tayebi et al. found that in maintenance hemodialysis patients, a diet rich in RS significantly reduced serum concentrations of paracresol, while there was no significant change in IS levels in the treatment group [20]. Marta et al. found that the plasma level of IS significantly decreased after the addition of resistant starch, and the plasma level of cresol sulfate was not affected [18]. Meijers et al. supplemented the diet of hemodialysis patients with inulin in the form of a fructose-rich inulin. P-cresol sulfate production and plasma levels were reduced by 20%, but there was no effect on IS [27].

Some studies have suggested that RS and other dietary fiber components have beneficial effects on patients with CKD, which may involve multiple mechanisms. One is that an RS-rich diet can promote the growth of SCFA-producing bacteria. According to the study of Laffin et al., Faecalibacterium diversity in feces of patients with end-stage renal disease increased significantly after supplementing ham-RS2. The diversity of Faecalibacterium is related to disease state and is also the main bacterium producing butyric acid, whose content is lower in western people, enteritis, and obese people [21]. Vaziri also proved this by finding that diets rich in RS and other fermentable fibers can promote the production of short-chain fatty acids, and the increased production of short-chain fatty acids leads to the reduction of intestinal pH, thus reducing the formation of proinflammatory and prooxidative uremia toxins in the colon [10–12]. SCFA can enable beneficial microorganisms to reproduce and survive, prevent the entry and adhesion of opportunistic bacteria, and reduce the accumulation of toxic substances, thus maintaining the integrity of intestinal epithelium [10–12]. The shortening of colon transport time may also be one of the mechanisms by which RS plays a role. In people with constipation, stool accumulates in the intestines for a long time, and the fermentation of proteins in the intestines leads to the production of toxins. RS can trap water in the intestine, prevent dry stool, help prevent constipation, and reduce fluid overload [10, 27]. In animal experiments, CKD creatinine clearance was significantly improved compared with rats fed the RS diet, with decreased oilfield fibrosis and inflammation, renal tubular damage and reduced NF-KB activation, and increased antioxidant enzyme production, whereas experimental models on the low-fiber diet showed opposite effects [10]. Many studies have shown that metabolic syndrome is closely related to overall health and chronic diseases such as cardiovascular disease, diabetes, and chronic kidney disease. By ingestion of resistant starch, cholesterol and triglycerides are reduced, and insulin sensitivity is improved, which can greatly reduce the incidence of metabolic syndrome. Patients with CKD may also benefit from better glucose metabolism, lipid levels, and better weight management.

As mentioned above, CKD patients have an imbalance of intestinal flora, resulting in changes in intestinal permeability. Some byproducts produced by bacteria metabolizing aromatic amino acids (e.g., tryptophan and tyrosine) include uremia toxins (e.g., IS and precursors of para-cresol sulfate) that enter the blood [5, 6]. CKD patients have high levels of uremia toxins, both because of their increased production and because the damaged kidney cannot be cleared from the bloodstream by urine [28] and because of their low binding rate to plasma proteins and low dialysis clearance [29, 30]. Through the analysis of seven included studies, we found that the intake of RS significantly reduced serum IS in dialysis patients, but there was no significant change in serum p-cresol sulfate. Sirich et al. proposed possible reasons for this inconsistency. In his study, the free fractions of IS and paracresol sulfate in the RS group showed a decreasing trend compared with the control group, while the total solute level decreased less than the free solute level [15]. Since the indices of IS and paracresol sulfate in the included literature were both measured at the total solute level, the free solute level may be a better measurement index, which, of course, needs to be proved by more long-term large-scale RCTS in the future.

Resistant starch also has beneficial effects on the intestinal environment, including increased Ruminococcus bromide. Ruminococcus brucei, one of the main members of Firmicutes, is a major resistant starch fermentation strain. Through its special activity against resistant starch, Ruminococcus brucei releases energy from starch to evade digestion by host enzymes. In addition, the intake of foods rich in resistant starch has been shown to increase intestinal short-chain fatty acid levels, regulate microbial metabolites, and improve glucose homeostasis and insulin sensitivity. Interestingly, the increase in butyric acid levels after taking resistant starch depends on each person's unique gut flora. The effects of resistant starch on intestinal environment indicate that resistant starch has positive effects on the physiological function of intestinal flora, including metabolic activity, nutritional effect on intestinal epithelial and immune structure and function, and protection of host from pathogen invasion.

In this meta-analysis, we found a significant reduction in serum phosphorus concentration, which may be clinically significant because hyperphosphatemia is a problem that is often difficult to solve in patients with predialysis CKD, and the reduction may be due to reduced phosphate intake and reduced intestinal absorption. Phosphorus from plant sources is not well absorbed because much of it is present as a phytic acid and cannot be absorbed well [31].

We also observed a significant reduction in uric acid levels in the RS group. Some studies have shown that dietary fiber can reduce serum uric acid levels by reducing dietary adenine absorption [32, 33].

Some studies have reported that the RS can decrease the serum creatinine and blood urea nitrogen, such as Tayebi

[20], but in our meta-analysis, serum creatinine and urea nitrogen levels were no obvious changes, and the duration of the possible reason is that it is included in the study which is too short range (4 weeks to 12 weeks), not very obvious changes in serum creatinine and blood urea nitrogen, and needs more long-term RCT to clarify in the future.

In addition, increased uremia toxins in the blood have been shown to exacerbate inflammation and oxidative stress, as well as endothelial dysfunction and atherosclerotic processes [33–35]. Marta et al. found in their study that the average mRNA Nrf2 expression increased after RS treatment, and Nrf2 is considered to be one of the most important factors for cell defense against oxidative stress and inflammation, demonstrating that cookies rich in resistant starch may reduce the level of indoles sulfate from intestinal flora and reduce inflammation in hemodialysis patients [18]. Tayebi et al. also demonstrated significant reductions in serum TNF-a, IL-6, and malondialdehyde levels in the RS group [17]; however, in our meta-analysis, IL-6 had significant changes in the dialysis group, and there was no significant change in some inflammatory indicators such as hs-CRP in the RS group. One possible reason is still the short duration of the study included, and RS is a longer term process to improve inflammatory markers. Although no significant changes in CRP and serum paracresol sulfate were detected, many articles have shown the RS can reduce inflammation index and dialysis patients' formation of oxidizing uremic toxins. This needs to be elucidated by future large long-term RCTS, too.

Fermentation of the fibers in the colon produces gases including hydrogen and methane, which can cause flatulence and abdominal discomfort. The potential advantage of resistant starch over fructose-rich inulin and other oligosaccharides is that the slow fermentation due to its high molecular weight limits flatulence and other gastrointestinal side effects, and the consumption of RS is well tolerated and significantly ameliorates the prevalence of constipation in CKD patients [17, 27, 34–36].

There are some limitations in our research. First, most of the clinical trials identified with the meta-analysis were relatively short duration and were involved a small number of patients, and the number of trials selected in the meta-analysis for relevant indicators was small; so, changes in aggregate estimates may have been influenced by some studies, particularly those with higher weights. Second, although most of our results showed relatively little or no heterogeneity between studies, there were some differences in the type and amount of RS products used in the intervention and significant differences in the control diet regimens in each study, which may have an impact on the results. Finally, the system review included a crossover study whose data could affect the accuracy of the results because of the washout period. Despite these limitations, based on our meta-analysis, we believe that RS is beneficial to CKD patients, especially hemodialysis patients.

## 5. Conclusions

This meta-analysis indicates that RS reduced the serum IS, serum phosphorus, and uric acid levels significantly in dialysis patients, while hs-CRP, serum creatinine, BUN, serum paracresol sulfate, and blood lipid showed no significant changes.

## Abbreviations

CKD: Chronic kidney disease
RS: Resistant starch
IS: Indolephenol sulfate
MD: Mean difference
BUN: Blood urea nitrogen
SCFA: Short chain fatty acids
RCTs: Randomized controlled trials.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

## Authors' Contributions

Gao CL designed this article and supported the research. Du XY and Wu J reviewed the search results, conducted the primary data abstraction, performed the statistical analysis, and drafted the review. Tan QQ proofread the manuscript. Gao CL and Xu Y critically revised the article. All authors read and approved this article. Xinyi Du and Jing Wu contributes equally to this work.

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*Research Article*

# 2′-O-Methylperlatolic Acid Enhances Insulin-Regulated Blood Glucose-Lowering Effect through Insulin Receptor Signaling Pathway

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*Purpose*. Insulin receptor (InsR) sensitizers represent a new type of therapeutic agent for the treatment of diabetes, with 2′-O-methylperlatolic acid (2-O-M) being a potential InsR targeting drug. The purpose of this study was to determine whether 2-O-M functions as an activator of the insulin signaling pathway, regulating glucose hemostasis through the InsR and exerting a glucose-lowering effect in an animal model of diabetes. *Methods*. SPR-based analyses were used to detect the binding of different concentrations of 2-O-M to the InsR. The protein levels of IR-$\beta$, p-IR, AKT, and p-AKT in Hepa and C2C12 cell lines and liver and muscle tissues were determined by western blotting. Glucose uptake capacity was determined in C2C12 cells. Streptozotocin-induced diabetic mice were randomly divided into four groups: the control, insulin treated, 2-O-M treated, and combined insulin and 2-O-M treated. Mice were injected with 2-O-M or normal saline and the average blood glucose concentration after 120 min, and the serum levels of insulin, glucagon, and C-peptide were measured. Next, qRT-PCR was performed to detect the mRNA expression of genes involved in lipid and glucose metabolism in the liver and muscle tissues. *Results*. 2-O-M binds to the extracellular domain of the InsR. Moreover, combination treatment with 2-O-M and insulin resulted in significant activation of the insulin signaling pathway *in vitro* and significant stimulation of the glucose uptake capacity of C2C12 myotubes. In mice with streptozotocin-induced diabetes, 2-O-M significantly prolonged the blood glucose-lowering effect of insulin, significantly reduced the secretion of exogenous insulin, and reduced the blood glucose concentration *in vivo*. In addition, treatment with 2-O-M alone significantly enhanced the phosphorylation of AKT in muscle tissue, which enhanced glucose uptake in C2C12 myotubes. Further, 2-O-M significantly increased glucagon secretion and enhanced liver gluconeogenesis to prevent hypoglycemia. *Conclusion*. 2-O-M enhances the hypoglycemic effect of insulin through the insulin signaling pathway and can be used as a complement to insulin. This synergetic effect may lower the required dose of insulin and protect $\beta$ cells.

## 1. Introduction

The incidence of diabetes has increased at an alarming rate, making it a major global public health concern. A review of population health in 110 countries from 1980 to 2014 identified 366 million patients with diabetes in 2011, and this number is expected to increase to 552 million by 2030 [1]. There are four types of diabetes: type 1 diabetes (insulin deficiency), type 2 diabetes (insulin resistance), specific-type diabetes, and gestational diabetes [2]. High circulating concentrations of

glucose caused by diabetes can lead to various chronic diseases, including retinopathy, diabetic nephropathy, and chronic cardiovascular disease [3–5]. Diabetes also increases the risk of cancer [6]. In addition, diabetes imposes a heavy economic burden on individuals and households as well as on healthcare systems [7].

Effective pharmacological glycemic control is the key to treating diabetes [8]. Currently, the main drugs used to treat diabetes include insulin, insulin analogues, metformin, sodium-glucose cotransporter-2 (SGLT2) inhibitors, and natural compounds [9–11]. Medicinal use of natural compounds in the treatment and prevention of diseases, including diabetes, has a long history compared to conventional medicines. Moreover, herbal medications may be used as effective and sustainable alternatives to treat diabetes [10]. Many plants have proven antidiabetic activity, with their main ingredients being polyphenols [12]. Polyphenol compounds found in many plants can enhance insulin sensitivity and reduce blood glucose in animal models of diabetes [13–17]. Therefore, research into new antidiabetic drugs from natural compounds is becoming increasingly relevant in the search for novel treatments for diabetes.

The InsR is one of the most important targets for diabetic drug discovery [18, 19]. InsR sensitizers can bind to the InsR to activate the insulin pathway independent of insulin; thus, InsR sensitizers have the potential to alleviate insulin resistance and minimize the risk of hypoglycemia [19]. InsR sensitizers increase insulin sensitivity for patients with type 2 diabetes and lower glucose levels for patients with type 1 disease. Given these attributes, InsR sensitizers represent an opportunity to develop a new type of therapeutic drug to treat diabetes. However, only two InsR sensitizers, TLK19781 [19] and TLK16998 [20, 21], have been identified. Because insulin sensitizers offer so many treatment advantages, finding new examples of these drugs in natural compounds may play a major role in improving the quality of life of patients with diabetes.

In this study, surface plasmon resonance (SPR) analysis was used to determine the binding affinities between natural compounds and the extracellular domain of the InsR. The polyphenolic compound, 2′-O-methylperlatolic acid (2-O-M), is a monoamine oxidase B inhibitor extracted from *Pertusaria parasommerfeltii*, and it directly binds with the InsR. The combination of 2-O-M with insulin enhances the function of insulin-induced glucose-lowering effect through activation of insulin signaling pathway in both type 1 diabetes mice and type 2 diabetes mice models. In this study, the streptozotocin- (STZ-) induced diabetic mice represent a model of type 1 diabetes, while *db*/*db* mice represent a model of insulin resistance induced type 2 diabetes. We found that 2-O-M regulates glucose homeostasis by stimulating the insulin signaling pathway in liver and muscle tissues, which synthesize glycogen, lipids, and gluconeogenesis genes.

## 2. Materials and Methods

*2.1. Animals.* All animal care and animal experiments in this study were approved by the animal ethics committee of Yunnan Agricultural University (no. 202010057). Mature and healthy BALB/c mice and *db*/*db* mice aged 6–8 weeks were purchased from Cawens Lab Animal Co. (Changzhou, China). The mice were individually housed in an environmentally controlled room (ventilated, 22°C, relative humidity of 55% ± 5%) with a 12-hour light-dark cycle. Mice were given free access to water and food.

Two weeks before commencing the modeling experiment, the BALB/c mice were fed a high-fat diet (D12492, research diet) with fat making up 60% of the total caloric intake. These mice were fasted overnight and then injected with 55 mg/kg of streptozotocin (Sigma-Aldrich, St. Louis, USA) for 3 consecutive days. After 7 days, blood glucose was measured. If the blood glucose was ≥15 mmol/L, the type 2 diabetes model was considered successfully established [22]. Diabetic mice were randomly divided into four groups (4-8 mice in each group): the control group, the insulin group, the 2-O-M group, and the combined (insulin+2-O-M) treatment group. Insulin was injected subcutaneously at a dose of 0.5 U/kg, and 2-O-M was injected into the tail vein at a concentration of 1 mg/kg. We purchased 2-O-M (95.0% purity) from BioBioPha (Kunming, China), dissolved in dimethyl sulfoxide (DMSO) to make a 10 mmol/L stock solution, and stored at −20°C in the dark until the tail vein injections were administered.

Diabetic *db*/*db* mice were randomly divided into four groups (6 mice in each group): the control group, the insulin group, the 2-O-M group, and the combined (insulin+2-O-M) treatment group. The *db*/*db* mice fasted overnight before the experiment. Insulin was injected subcutaneously at a dose of 1 U/kg, and 2-O-M was injected into the tail vein at a concentration of 1 mg/kg.

*2.2. Cell Culture and Treatments.* A mouse hepatocyte cell line, Hepa 1-6, and a skeletal muscle cell line, C2C12, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, USA) and 1% penicillin-streptomycin liquid (Solarbio Life Science, Beijing, China). For differentiation, C2C12 cells were placed in 2% horse serum in DMEM for 6–7 days and incubated at 37°C under humidified conditions of 95% air and 5% $CO_2$. Before each experiment began, the cells were washed with phosphate-buffered saline (PBS) buffer and were maintained in DMEM without FBS for at least 4 h. For measurement of protein phosphorylation, the cells were treated with 1 nM insulin or 4 $\mu$M 2-O-M for 20 min before cell lysates were collected for western blotting. Insulin was used as the positive control.

*2.3. Cell Viability Assay.* The effects of 2-O-M on cell growth were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Briefly, Hepa 1-6 cells were seeded at $1.5 \times 10^4$ cells/well in a 96-well plate, cultured overnight, and then treated with different concentrations of 2-O-M (1–16 $\mu$M) or 1 nM insulin for 24 h. After the supernatant was removed, DMSO was added to the wells, and the optical density at 492 nm was measured with a microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, USA). Cell viability was normalized to that of control cells.

Table 1: Gene sequences of primers.

| Gene name | Forward primer | Reverse primer |
|---|---|---|
| $\beta$-Actin | 5′-GAGACCTTCAACACCCCAGC-3′ | 5′-ATGTCACGCACGATTTCCC-3′ |
| Gys | 5′-ATCTTCTTCGTCTTCCGCATC-3′ | 5′-GACACTGAGCAGGGCTTTTCC-3′ |
| G6pase | 5′-AAAAAGCCAACGTATGGATTCCG-3′ | 5′-CAGCAAGGTAGATCCGGGA-3′ |
| Pepck | 5′-TTTGATGCCCAAGGCAACTT-3′ | 5′-ATCGATGCCTTCCCAGTAAA-3′ |
| Fas | 5′-CTGGCATTCGTGATGGAGTC-3′ | 5′-TGTTTCCCCTGAGCCATGTA-3′ |
| Acc1 | 5′-CGCTCGTCAGGTTCTTATTG-3′ | 5′-TTTCTGCAGGTTCTCAATGC-3′ |
| $\alpha$-Tubulin | 5′-CTCTACCCTCACATCCACTTCC-3′ | 5′-ATAGGCAGCAAGCCATGTATTT-3′ |

*2.4. Real-Time Quantitative Polymerase Chain Reaction Analysis.* TRIzol (TransGen Biotech, Beijing, China) was used to extract total RNA from muscle and liver tissues. Next, cDNA was synthesized in accordance with the manufacturer's instructions using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan). PCR detection and quantification were performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) and the Roche 480 (Roche, Basel, Switzerland) fast real-time PCR system. Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to $\beta$-actin expression in liver tissue and normalized to $\alpha$-tubulin expression in muscle tissue. All primer sequences are listed in Table 1.

*2.5. Western Blotting Analysis.* RIPA buffer and supporting PMSF (Solarbio Life Science, Beijing, China) were used to extract total protein content from Hepa cells, C2C12 cells, and liver and muscle tissues. Protein concentration was measured using the BCA protein determination kit (Beyotime Biotechnology, Shanghai, China). An equal mass (60 $\mu$g) of each protein sample was loaded into each well of an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were then transferred to a 0.45 $\mu$m PVDF membrane, and nonspecific binding to the membrane was blocked by incubation in 5% ($w/v$) skimmed milk powder in1X TBST for 1 h. Membranes were then incubated with the indicated primary antibodies at 4°C overnight with shaking: anti-insulin receptor $\beta$, anti-phospho-insulin receptor $\beta$, anti-Akt, anti-phospho-Akt, and anti-$\beta$-tubulin. Antibodies were recovered, and membranes were washed three times for 5 minutes each with Tris buffer containing Tween 20 (TBST). The washed immunoblots were incubated with horseradish peroxidase-conjugated secondary antibody diluted in 5% skimmed milk powder in 1X TBST at room temperature with shaking for 1 h. Immunoblots were then visualized using the UltraSignal Ultra-Sensitive ECL Chemiluminescence Substrate (4A Biotech, Beijing, China), and $\beta$-tubulin staining was used as the loading control.

*2.6. SPR Studies.* SPR experiments were performed using a Biacore S200 instrument (Biacore, GE Healthcare) at 25°C. The extracellular domain of the InsR (10 $\mu$g/mL in 10 mM sodium acetate, pH 4.5) was immobilized on an S CM5 sensor chip (GE Healthcare) using an amine coupling kit. The analytes (2-O-M at concentrations of 6.25–100 $\mu$M, twofold dilution) were passed over the immobilized InsR on the sensor surface. The flow rate was set to 30 $\mu$L/min with a binding time of 90 s and a dissociation time of 90 s. Kinetic and affinity analyses were performed using the Biacore S200 evaluation software (version 1.1, GE Healthcare).

*2.7. Glucose Uptake Assay.* C2C12 cells were treated with 4 $\mu$M 2-O-M and with 1 nM insulin, and the glucose uptake capacity of the cells was measured with a glucose uptake kit (Promega, USA). The glucose uptake assay is based on the 2-deoxyglucose-6-phosphate (2DG6P) assay for the detection of glucose uptake by mammalian cells. When 2-deoxyglucose is added to the cells, it is transported into cells *via* the membrane GLUT2/GLUT4 protein and is rapidly phosphorylated in the same way as glucose. However, enzymes that further modify glucose-6-phosphate cannot modify 2-deoxyglucose-6-phosphate, and the reactants cannot penetrate the membrane, so the membrane-impermeable analyte accumulates in the cell. The culture media were removed from the cells, and the cells were lysed using acid culture. The detection of 2-deoxyglucose-6-phosphate produced after lysis positively reflected the glucose uptake capacity of the cells.

*2.8. Detection of Serum Hormones.* Diabetic mice were randomly divided into four groups as specified previously. After appropriate treatment, the blood glucose levels were tested at 120 min. Subsequently, the mice were euthanized, and the blood was collected from the canthus into microcentrifuge tubes with heparin sodium or an anticoagulant tube containing EDTA-2Na and aprotinin. The blood samples were centrifuged at 3,000 g for 20 min at 4°C, and the serum was kept frozen at −80°C until analysis. The levels of insulin, glucagon, and C-peptide were measured and analyzed using the relevant kits (Crystal Chem, USA) in accordance with the manufacturer's protocols.

*2.9. Statistical Analysis.* Statistical analysis of the experimental data was performed using SPSS 17.0 and GraphPad Prism 6. Data are presented as the mean ± SEM. Differences between groups were analyzed using one-way ANOVA; $P < 0.05$ was considered significant.

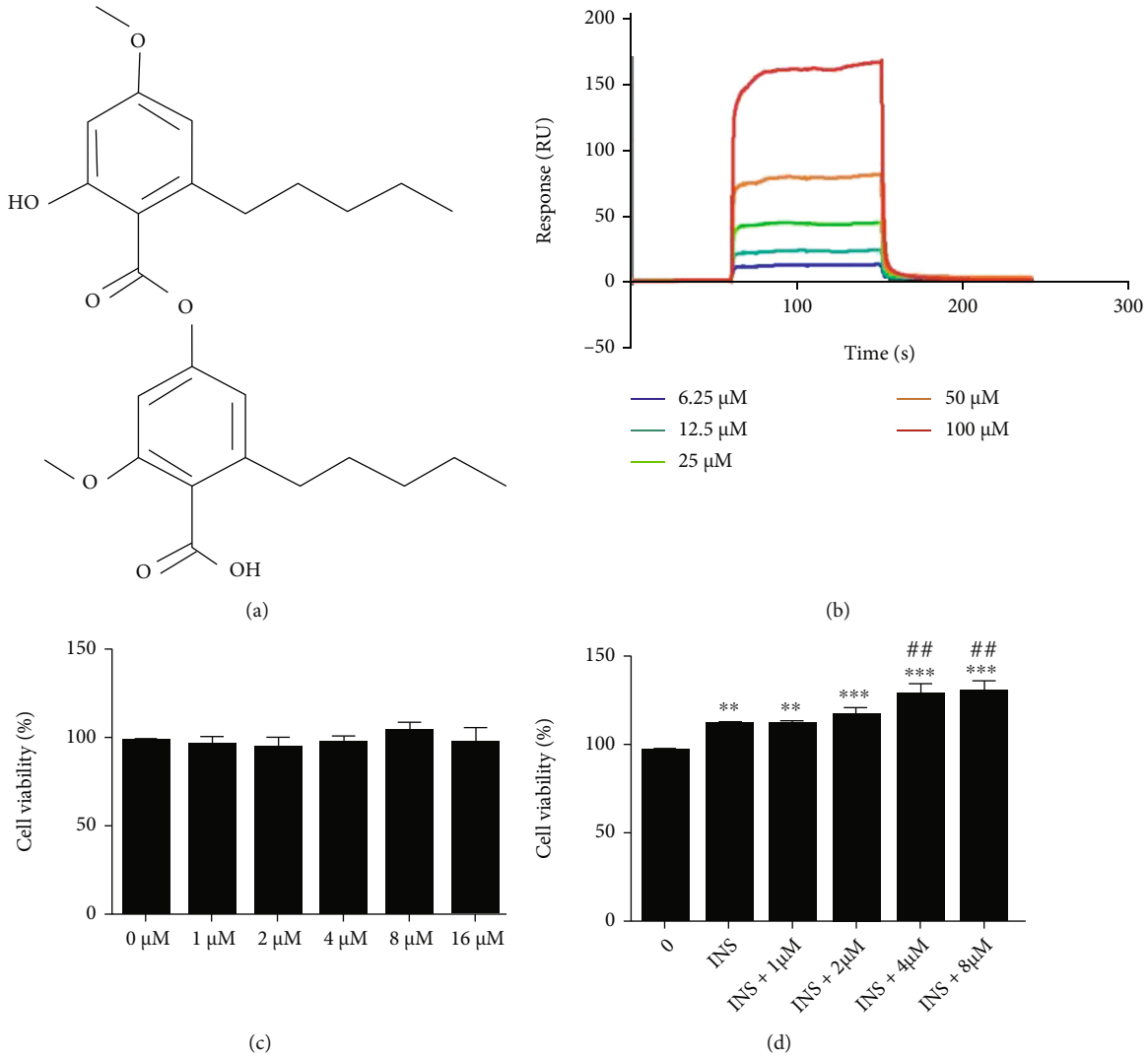


Figure 1: 2′-O-Methylperlatolic acid (2-O-M) binds to the InsR. (a) Chemical structural of 2-O-M. (b) Binding affinity of 2-O-M to InsR. (c) Cytotoxic effects of 2-O-M on cells. The change in cell viability caused by 2-O-M in the presence of insulin. Data are shown as the mean ± SEM ($n = 6/7$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus insulin.

## 3. Results

*3.1. 2-O-M Binds to InsR.* To determine whether 2-O-M (Figure 1(a)) interacts with the InsR, SPR-based analysis was used to detect the binding of 2-O-M to the receptor at different concentrations. The chemical structure of 2-O-M is shown in Figure 1(a). Different concentrations of 2-O-M (6.25–100 $\mu$M) flowed over the InsR protein, which was immobilized on the CM5 chip. The result showed that 2-O-M may bind to the InsR, with a $K_D$ of 88.72 $\mu$M (Figure 1(b)).

To confirm the working concentration of 2-O-M on cells, we determined the cytotoxicity of 2-O-M on Hepa 1-6 cells, and the experiment revealed that the cell viabilities were not affected by 2-O-M (Figure 1(c)). To determine whether 2-O-M affected cell proliferation in the presence of 1 nM insulin, which mimics the concentration in circulation, Hepa 1-6 cells were incubated in a medium containing different concentrations of 2-O-M (0–8 $\mu$M) and 1 nM insulin. In the presence of 1 nM insulin, 4 $\mu$M and 8 $\mu$M 2-O-M significantly enhanced cell viability; however, lower concentrations of 2-O-M (1 $\mu$M and 2 $\mu$M) did not improve cell viability (Figure 1(d)).

*3.2. 2-O-M Enhanced Insulin-Activated Insulin Signaling Pathway and Stimulated Cellular Glucose Uptake.* Adverse effects from high doses of insulin include weight gain and excessive hypoglycemia. Low-dose insulin, combined with other medications, has become the primary treatment for diabetes [23]. To examine insulin receptor phosphorylation, Hepa 1-6 cells and C2C12-differentiated myocytes were both treated with insulin (1 nM) and 2-O-M (4 $\mu$M). The autophosphorylation of InsRs and the phosphorylation of AKT significantly increased in the insulin (1 nM) group compared to the control group. Autophosphorylation of the receptor and the phosphorylation of AKT were significantly higher in the combined (insulin+2-O-M) treatment group compared to the insulin group (Figures 2(a) and 2(b)).

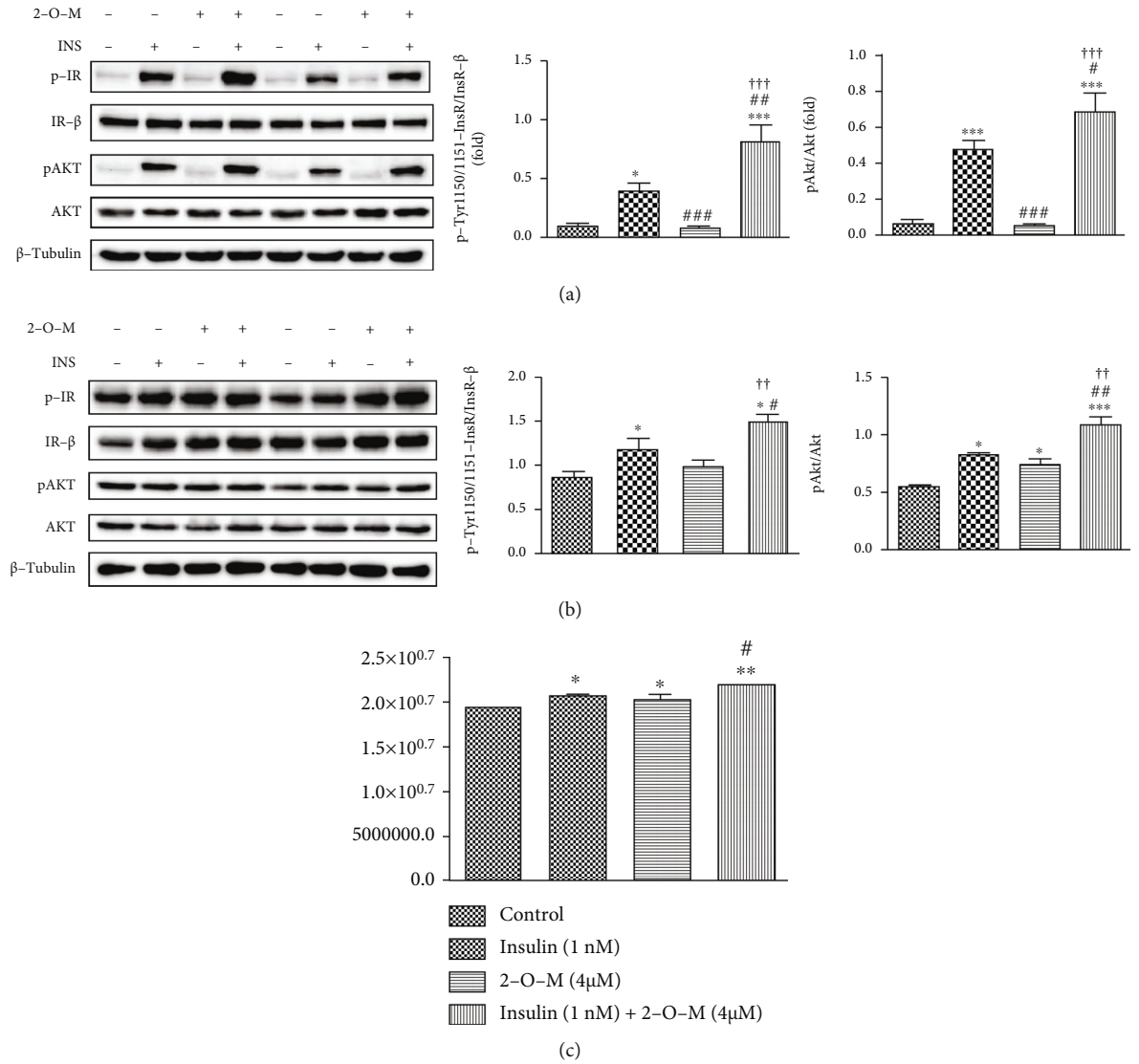


FIGURE 2: The combination of 2′-O-methylperlatolic acid (2-O-M) and insulin activates the insulin signaling pathway and stimulates cellular sugar uptake. (a) Expression of key proteins of the insulin signaling pathway in different groups of 2-O-M and insulin in Hepa 1-6 cells. (b) The expression of key proteins in the insulin signaling pathway in different groups of 2-O-M and insulin in C2C12 cells. (c) The effect of 2-O-M combined with insulin on glucose uptake capacity in C2C12 cells. Data are shown as the mean ± SEM ($n = 6/7$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus insulin. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, 2-O-M versus insulin+2-O-M.

The goal of insulin signaling pathway activation is the promotion of the glucose uptake capacity of the cells. We determined the ability of the combination of insulin and 2-O-M to enhance glucose uptake. This combination significantly enhanced the glucose uptake capacity in C2C12 cells compared to the insulin-only treatment to cells (Figure 2(c)), and this result was consistent with the western blotting results shown in Figure 2(b).

*3.3. 2-O-M Enhanced Insulin-Activated Hypoglycemic Effects in Diabetic Mice.* The 6–8-week-old BALB/c mice were fed a high-fat diet (containing 60% fat kcal) for 2 weeks before attempting to establish the streptozotocin-induced model of diabetes. Mice were injected with 55 mg/kg of STZ for 3 consecutive days. Blood glucose levels ≥ 15 mmol/L were indicative of a successfully established disease model [22]. Changes in body weight and fasting blood glucose concentration are shown in Figure S1. Body weight decreased significantly in mice following STZ injection (Figure S1A), and blood glucose levels increased significantly 7 days after STZ injection (Figure S1B).

To explore the hypoglycemic effect of 2-O-M *in vivo*, 2-O-M and insulin were injected into STZ-induced diabetic mice, and blood samples and tissues were collected for blood glucose analysis for up to 120 min after injection. Blood glucose levels revealed that the average blood glucose concentration was significantly lower at 60–120 min in the group that received both insulin and 2-O-M compared to the insulin and the control groups. Moreover, blood glucose in the insulin group had returned to its initial level (Figure 3(a)).

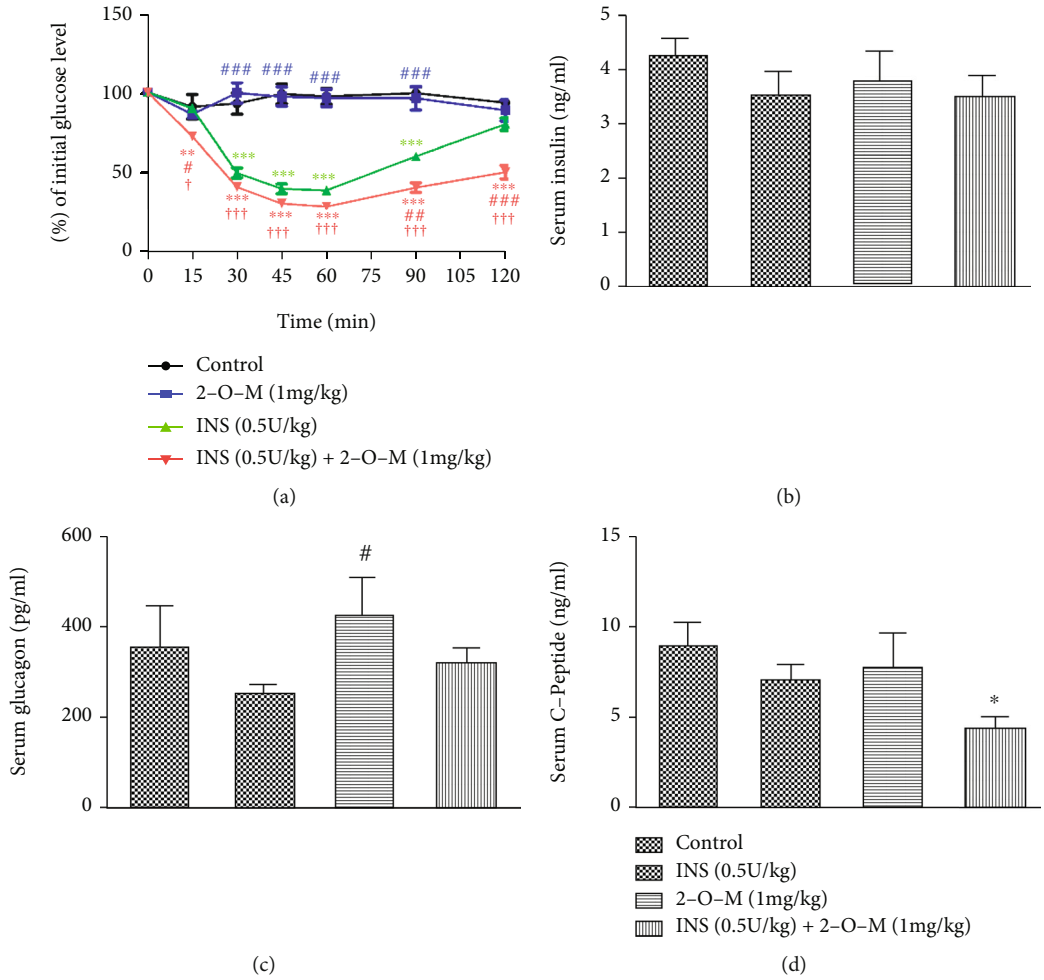


Figure 3: 2′-O-Methylperlatolic acid (2-O-M) enhances the insulin-activated hypoglycemic effect in mice with STZ-induced diabetes. (a) Blood glucose change within 120 minutes. (b) Serum insulin level at 120 minutes. (c) Serum glucagon level at 120 minutes. (d) Serum C-peptide level at 120 minutes. Data are shown as the mean ± SEM ($n = 4 - 8$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus insulin.

Additionally, we injected 2-O-M and insulin into *db*/*db* mice and determined the blood glucose levels for 120 min. Our data revealed that the average blood glucose concentration was significantly lower at 90 and 120 min in the group that received both insulin and 2-O-M compared to the insulin-only group (Figure 4(a)).

We detected the serum levels of insulin, glucagon, and C-peptide in each group of STZ-induced diabetic mice and found no difference in serum insulin levels among the groups (Figure 3(b)). The average glucagon level in mice in the 2-O-M group was significantly higher than that in the insulin group (Figure 3(c)). The serum level of C-peptide in the combined treatment group was significantly lower than the level in the control group (Figure 3(d)), and there were no significant differences among the control, insulin, and 2-O-M groups (Figure 3(e)). In the same way, the levels of insulin, glucagon, and C-peptide in the serum of *db*/*db* mice were detected. There were no differences in the levels of serum insulin in each group (Figure 4(b)). The level of serum glucagon in the 2-O-M group was significantly higher than that in the 2-O-M group. The C-peptide level of the 2-O-M combined with the insulin group was also significantly lower than that of the control group.

The combination of 2-O-M and insulin can significantly enhance the hypoglycemic effect of insulin in diabetic mice and warrants additional exploration to reveal the specific mechanism(s) and the fate of glucose *in vivo*. Therefore, we investigated the expression of proteins related to the insulin signaling pathway in the liver and muscle tissues of the mice 120 min after treatment. The phosphorylation of the InsR and AKT in the combined treatment group was significantly lower in the liver tissues than in the insulin group (Figure 5(a)). However, compared to the insulin group, phosphorylation of the InsR and AKT in the combined treatment group was significantly increased in muscle tissues (Figure 5(b)). In addition, phosphorylation of AKT in the 2-O-M group was significantly higher than in the other three groups (Figure 5(b)). In *db*/*db* mice, the key proteins of the insulin signaling pathway in muscles were detected after injection of 2-O-M. We found that the combination of 2-O-M and insulin could significantly activate InsR and AKT phosphorylation. Further, phosphorylation of AKT in the

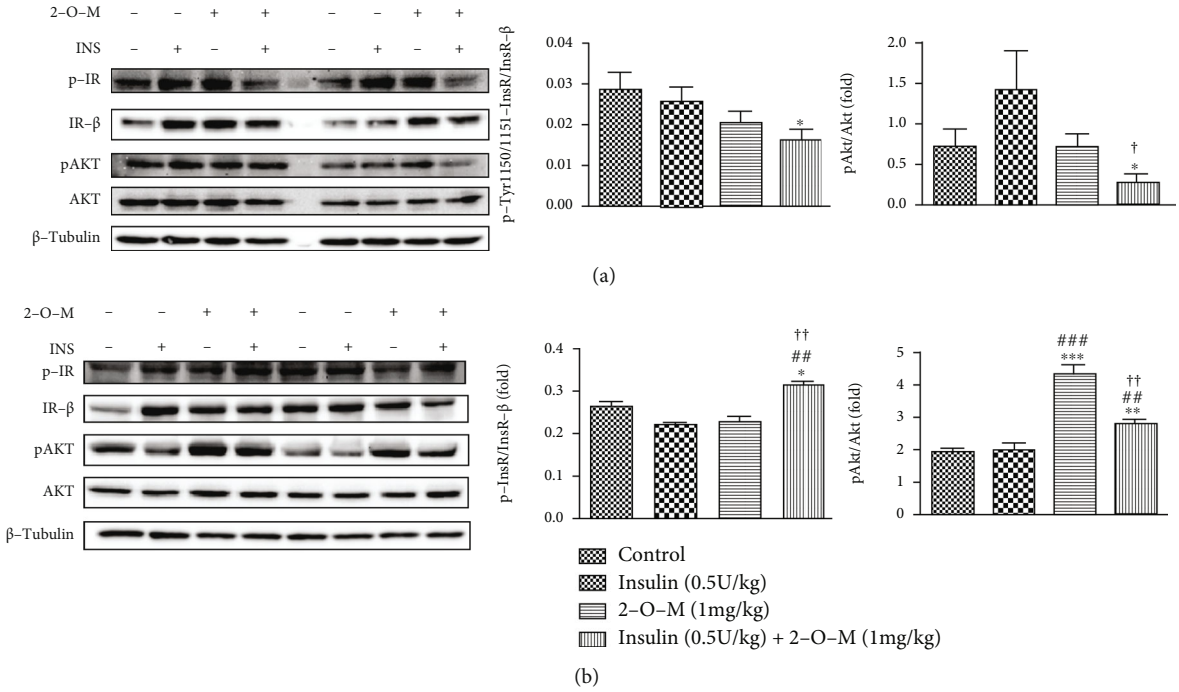


Figure 4: 2′-O-Methylperlatolic acid (2-O-M) enhanced the insulin-activated hypoglycemic effect in *db*/*db* mice. (a) Blood glucose change within 120 minutes in *db*/*db* mice. (b) Serum insulin level at 120 minutes. Serum C-peptide level at 120 minutes. Serum glucagon level at 120 minutes. Expression of key proteins of the insulin signaling pathway in muscle. Data are shown as the mean ± SEM ($n = 6$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$ versus insulin. $^{\dagger}p < 0.05$, 2-O-M versus insulin+2-O-M.

2-O-M group was also significantly higher than that of the control group and the insulin group.

*3.4. Effect of 2-O-M on Glycogen Synthesis, Fat Synthesis, and Gluconeogenesis Genes in Diabetic Mice.* To explore whether 2-O-M weakened the effect of insulin on lipid metabolism and glucose metabolism in the liver and muscle tissues of mice, we determined the mRNA expression of genes related to lipid metabolism and glucose metabolism after 2 h of treatment with 2-O-M. In the liver tissues, expressions of lipid synthesis genes *Fas* and *Acc1* were significantly decreased in the combination group with the insulin group, whereas insulin tended to enhance the expression of fat synthesis genes (Figures 6(a) and 6(b)). In the *db*/*db* mouse model, 2-O-M significantly enhanced *Fas* gene expression and inhibited *Acc1* gene expression (Figures 7(a) and 7(b)). The detection of glycogen synthesis gene expression revealed that the relative expression level of *Gys2* was significantly reduced in the combination group, and the expression levels of *Gys2* were similar between the control and the 2-O-M groups (Figure 6(c)). However, in *db*/*db* mice, there was no difference in *Gys2* expression among all groups (Figure 7(c)). In addition, the genes involved in gluconeogenesis (*G6pase* and *Pepck*) in two mouse models of diabetes were significantly enhanced in the 2-O-M group (Figures 6(d), 6(e), 7(d), and 7(e)). In the muscle tissues, the expression of fat synthesis genes *Fas* was significantly enhanced in the 2-O-M group. Compared to the insulin group, the expression of *Acc1* was significantly decreased in the combination group. Expression of *Fas* and *Acc1* in the combined group was significantly higher compared to other groups (Figures 7(f) and 7(g)). In both diabetic mice models, the expression of *Gys1* in the combination group was significantly enhanced, and the difference was that in the STZ-induced model, the expression of *Gys1* in the combined group was also significantly enhanced.

## 4. Discussion

The InsR is an important target for glycemic control [18, 19]. A previous review suggests that all InsR domains have the potential to be drug targets [20, 21]. Insulin sensitizers represent a new type of therapeutic drug for diabetes patients. Insulin sensitizers bind allosterically to InsR and sensitize insulin action to alleviate insulin resistance and minimize the hypoglycemia risk. However, insulin, insulin mimics, or orthosteric InsR activators may increase the risk of hypoglycemia in patients with diabetes, potentially leading to worse glycemic control in patients. Therefore, these factors represent limitations for the wider adoption of insulin products or InsR activators. Until now, only two InsR sensitizers, TLK19781 [19] and TLK16998 [20, 21], have been reported. Therefore, it would be beneficial to discover new small molecular natural compounds that can act as insulin sensitizers for use as compliments to insulin for patients with diabetes.

In this study, we screened 400 small molecular natural compounds using SPR and found that 2-O-M binds to the extracellular domain of the InsR with the highest response value (results not shown). Further studies found that 2-O-M has the potential to bind to the extracellular domain of

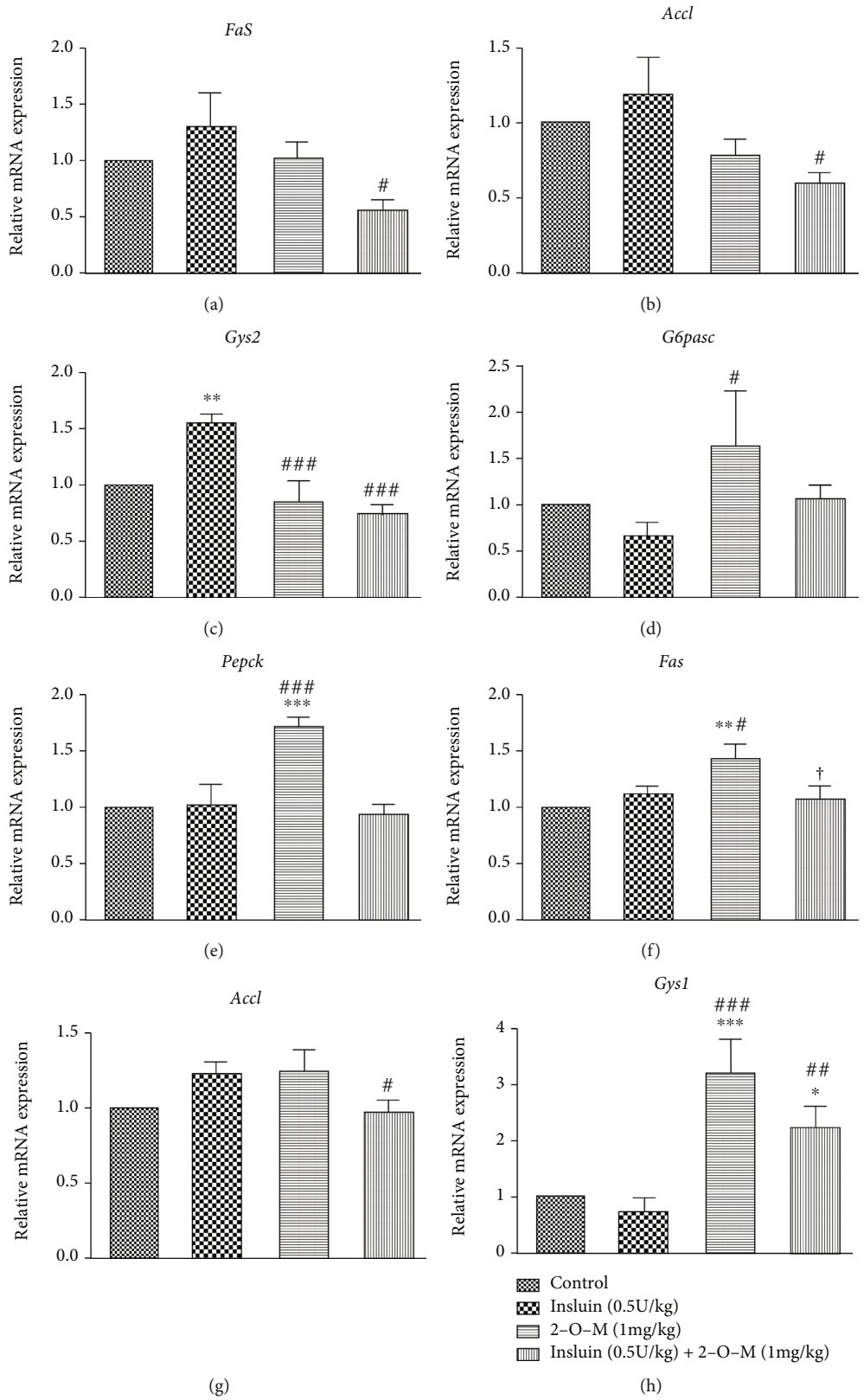


FIGURE 5: Effect of 2′-O-methylperlatolic acid (2-O-M) on the insulin signal pathway in muscle and liver tissues of diabetic mice. (a) Expression of key proteins of insulin signaling pathway in liver. (b) Expression of key proteins of insulin signaling pathway in muscle. Data are shown as the mean ± SEM ($n = 4 - 8$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus insulin. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, 2-O-M versus insulin+2-O-M.

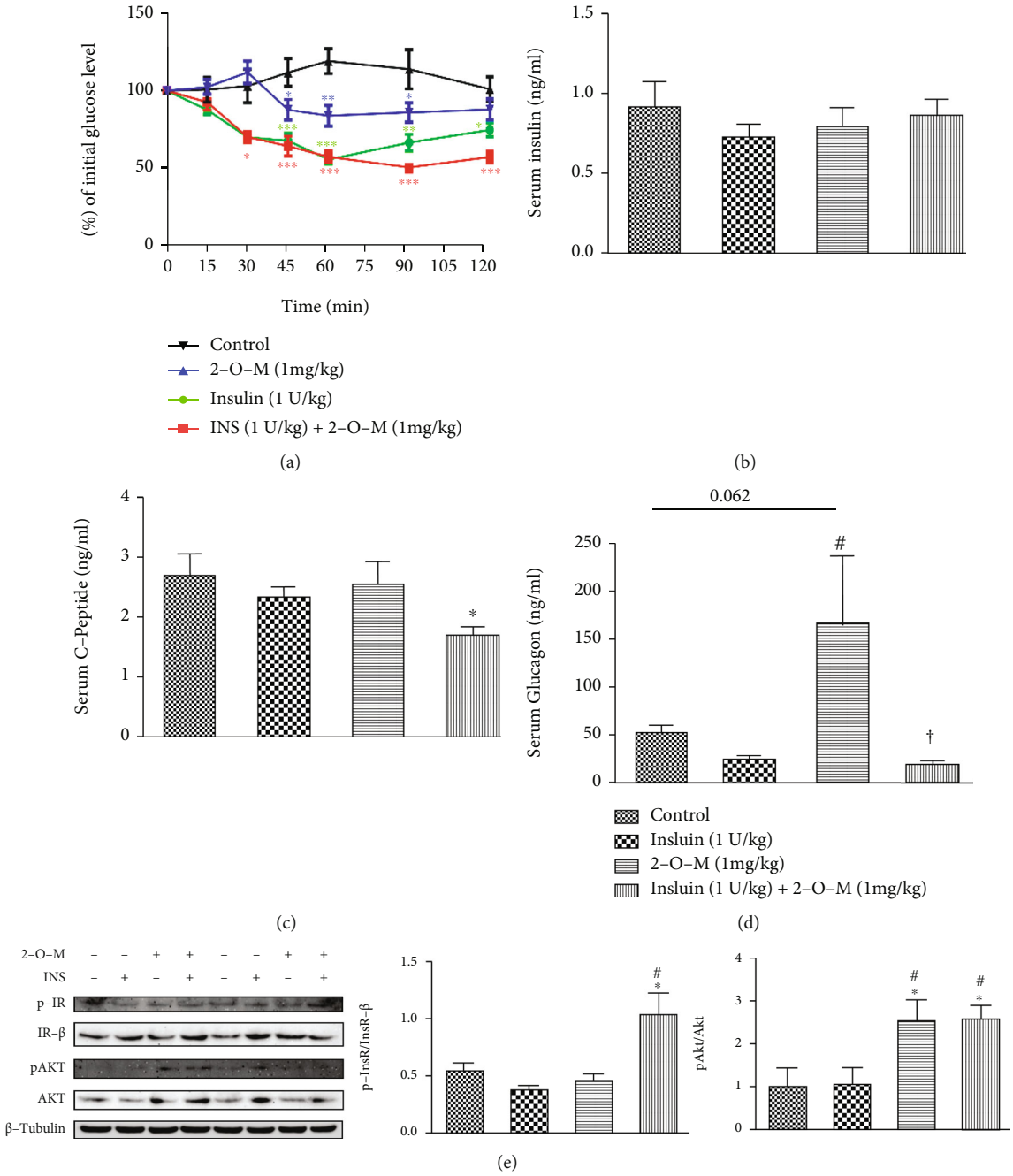


Figure 6: The effect of 2′-O-methylperlatolic acid (2-O-M) on glycogen synthesis, fat synthesis, and gluconeogenesis genes *in vivo*. (a–e) Genes in the liver. Genes in the muscle. Data are shown as the mean ± SEM ($n = 4 - 8$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus insulin. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, 2-O-M versus insulin+2-O-M.

the InsR protein with a $K_D$ value of 88.72 $\mu$M (Figure 1(b)) and that the dose range of 2-O-M used in the experiment was not toxic to cells (Figure 1(c)). In C2C12 and Hepa 1-6 cell lines, we used insulin as a positive control, and the insulin signaling pathway was significantly activated following treatment with 1 nM 2-O-M for 20 min. We also found that 2-O-M cannot activate the insulin signaling pathway but could enhance the function of insulin, significantly increasing the expression of the insulin signaling pathway and improving glucose uptake (Figure 2). These results indicate that 2-O-M directly binds to the InsR but cannot activate the insulin signaling pathway alone and that 2-O-M can significantly enhance the insulin-activated insulin signaling pathway to substantially enhance the glucose uptake capacity of cells. Furthermore, in this study, the combination of insulin with 2-O-M resulted in improved glucose-lowering effects compared to insulin alone, even though 2-O-M alone did not appear to exert a glucose-lowering effect (Figure 3(a)). Thus, 2-O-M acts as an insulin sensitizer, assisting insulin to activate the insulin signaling cascade.

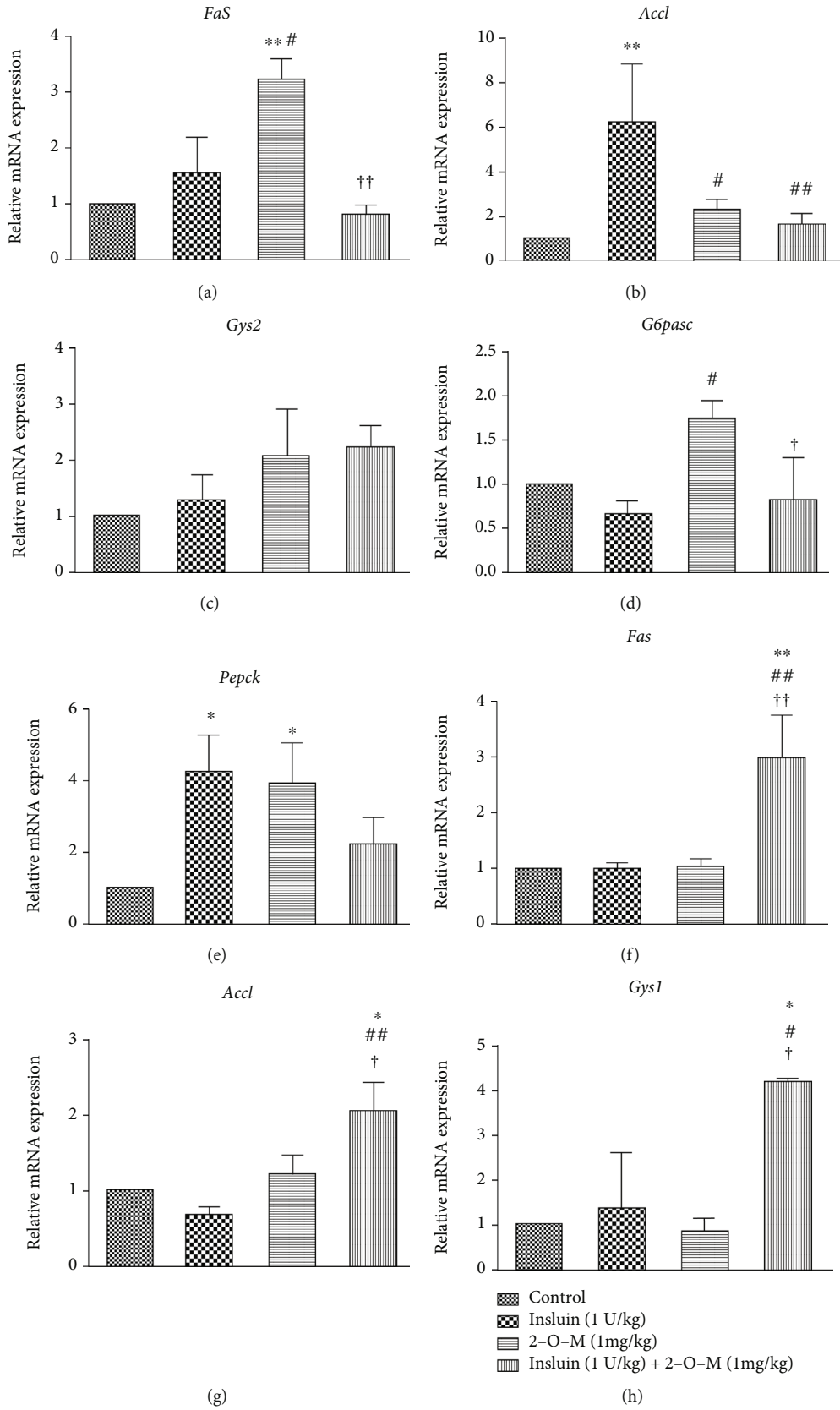


Figure 7: The effect of 2′-O-methylperlatolic acid (2-O-M) on glycogen synthesis, fat synthesis, and gluconeogenesis genes in *db*/*db* mice. (a–e) Genes in the liver. (f–h) Genes in the muscle. Data are shown as the mean ± SEM ($n = 6$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$ versus control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ versus insulin. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, 2-O-M versus insulin+2-O-M.

Previous studies have reported that several InsR sensitizers, such as TLK16998, TLK19781, and dicholine succinate, did not activate insulin signaling but could enhance the action of insulin on InsR phosphorylation [19, 21]. Because of their potential to alleviate insulin resistance and minimize the risk of hypoglycemia, insulin sensitizers represent a complementary therapeutic approach for diabetes [19].

In this study, the combined injection of insulin and 2-O-M into diabetic mice showed that 2-O-M could effectively prolong the glucose-lowering effect of insulin *in vivo* (Figure 3(a)). An examination of the serum concentrations of insulin, C-peptide, and glucagon 120 min after their injection into mice revealed that average insulin levels were similar among groups, but the average level of C-peptide was significantly reduced in the combined treatment group (Figures 3(b) and 3(c)). Exogenous insulin can significantly inhibit the secretion of endogenous insulin [24]. This response was observed in the mice treated with insulin only. Conversely, in the mice treated with both insulin and 2-O-M, the inhibition of endogenous insulin secretion was observed, in contrast to the insulin response in the mice treated with insulin alone (Figure 3). Serum levels of insulin, glucagon, and C-peptide in *db*/*db* mice were detected, and the conclusion was the same as that in STZ-induced diabetic mice (Figures 4(b). These results indicate that 2-O-M can improve the impact of exogenous insulin *in vivo* and further reduce endogenous insulin secretion. Therefore, combined administration of 2-O-M with insulin not only exerts a better glucose-lowering effect, minimizing the risk of hypoglycemia, but also decreases the secretion of endogenous insulin to protect $\beta$ cell function.

As shown in Figure 3(a), the blood glucose level of the insulin group had returned to the initial level at 120 min, so no difference was found in the levels of phosphorylated AKT and InsR in liver and muscle tissues isolated from the insulin group (Figure 5). Protein expression analysis in liver tissues revealed that levels of phosphorylated AKT in the combined group and in the 2-O-M-treated group were significantly reduced (Figure 5(a)). However, in muscle tissue, the insulin signaling pathway was significantly activated in the combined group and significantly enhanced the accumulation of glycogen in the muscle tissues of diabetic mice (Figure 5(b)). In the muscle tissue of *db*/*db* mice, the levels of phosphorylated AKT and InsR in the 2-O-M combined with insulin group were significantly increased, showing that 2-O-M treatment directly activates muscle tissue AKT phosphorylation. In addition, 2-O-M played very different roles in the liver and muscle tissues of diabetic mice, demonstrating tissue heterogeneity that might reflect the apparent heterogeneity of InsR structure in different tissues [25].

In STZ-induced diabetic mice, the expression of *Acc1* and *Fas* was increased in the insulin-treated group. While this was not significant, the expression of these genes was significantly inhibited in the liver tissues in the combined treatment group (Figures 6(a) and 6(b)). However, the expression of *Acc1* and *Fas* increased significantly in the 2-O-M group in muscle tissues, which was consistent with the high activation of AKT in muscles (Figure 5(b)). These results showed that the combination of insulin with 2-O-M could inhibit fat synthesis in both liver and muscle tissues, which might help reduce lipid accumulation *in vivo*. Therefore, further studies should focus on the mechanisms of inhibition of insulin-induced lipogenesis by 2-O-M. A previous study has shown that increased expression of phosphorylated AKT causes membrane transfer of the glucose transporter and promotes glucose uptake [26]. In *db*/*db* mice, 2-O-M combined with insulin still significantly inhibited the expression of *Fas* and *Acc1* in the liver. In muscle, however, 2-O-M combined with insulin enhanced the expression of *Fas* and *Acc1*. This may be related to different mechanisms of lipid metabolism in the two animal models. In the two diabetes model mice, *Gys1* in the muscle of the mice in the combination group was significantly enhanced, indicating that the combination of 2-O-M and insulin can significantly enhance the glycogen synthesis of the muscle in both models. But the difference is that 2-O-M treatment alone can also significantly increase the expression of *Gys1* gene in muscle in STZ-induced diabetic mice, indicating that 2-O-M exhibits different mechanisms of action in the two model mice 7(h)).

In this study, we found that 2-O-M activates phosphorylation of AKT in muscle tissues, but not through activation of the InsR (Figure 5(b)). In addition, 2-O-M showed no effect on lowering blood glucose in diabetic mice (Figure 3(a)). These results imply that other mechanisms may exist to drive the 2-O-M activation of AKT phosphorylation. We also found that the concentration of serum glucagon in mice in the 2-O-M group was significantly higher than the concentration in other groups (Figures 3(c) and 6(d)). We determined the mRNA expression of genes involved in gluconeogenesis (*G6pase* and *Pepck*) in the liver tissues of STZ-induced diabetic mice and found that the expression of these genes significantly increased in the 2-O-M group (Figures 6(d) and 6(e)). We also found the same results in *db*/*db* mice (Figures 7(d) and 7(e)). These data suggest that 2-O-M increases glucagon secretion and induces gluconeogenesis in the liver tissues of diabetic mice. Glucagon and insulin are completely competitive antagonists *in vivo* [27]. Therefore, 2-O-M may activate both AKT phosphorylation and gluconeogenesis *in vivo*; this hypothesis is supported by the lack of a glucose-lowering effect by 2-O-M *in vivo*.

## 5. Conclusions

SPR experiments demonstrate that 2-O-M binds to the extracellular domain of InsR. The combination of 2-O-M and insulin significantly activates the insulin signaling pathway *in vitro* and significantly stimulates the glucose uptake capacity of C2C12 myotubes. Further, 2-O-M significantly reduces the secretion of endogenous insulin and reduces the blood glucose concentration *in vivo*. In addition, 2-O-M alone significantly enhances the phosphorylation level of AKT in muscle tissue, which also enhances glucose uptake in C2C12 myotubes. In contrast, 2-O-M significantly enhances the secretion of glucagon and enhances liver gluconeogenesis to mitigate the risk of hypoglycemia. Therefore, 2-O-M can be used as a supplement to insulin, allowing the injection of lower doses of insulin, which may reduce

the potential adverse effects of injected insulin (e.g., hypoglycemia and obesity) to protect $\beta$ cell function.

## Data Availability

The data analyzed during the study are not publicly available. Rigorous analysis of the data in order to ensure the objective authenticity of the results.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

## Authors' Contributions

Sheng Jun, Wang Xuanjun, and Wu Xiaoyun designed this study; Wang Yinghao and Guan Qiaoli performed most of the experiments and data analysis; Liu Guanfu performed part of the animal experiments and western blotting; and Wu Xiaoyun and Wang Yinghao wrote the paper. Wang Yinghao and Guan Qiaoli contributed equally to this work.

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## Supplementary Materials

Figure S1: change in body weight (A) and blood glucose concentration (B) of mice after STZ injection. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus day 0. *(Supplementary Materials)*

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*Review Article*

# Diabetic Heart Failure with Preserved Left Ventricular Ejection Fraction: Review of Current Pharmacotherapy

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Diabetes is associated with several diabetic-related abnormalities which increase the risk of onset or worsening of heart failure. Recent studies showed that the majority of diabetic patients present with heart failure with preserved ejection fraction (HFpEF), and the prevalence of HFpEF in diabetics is alarming. Moreover, outcomes in HFpEF are poor and could be compared to those of heart failure with reduced ejection fraction (HFrEF), with 1-year mortality ranging between 10 and 30%. In contrast to HFrEF, there is very limited evidence for pharmacologic therapy in symptomatic patients with preserved ejection fraction, and therefore, the optimal selection of treatment for diabetic HFpEF remains challenging. This narrative review article summarizes the currently available data on the pharmacological treatment of HFpEF in patients with diabetes.

## 1. Introduction

Cardiovascular diseases (CVD) and heart failure represents an important clinical problems in patients with type 2 diabetes (T2D), with reported prevalence of CVD 6.9–40.8%, reported prevalence of heart failure 4.3-21.0%, and in-hospital CVD-related mortality of 5.6–10.8% [1]. A previous screening of the prevalence of heart failure in outpatients with T2D showed a 2.4% prevalence of heart failure with reduced left ventricular ejection fraction (HFrEF) and a 17.5% prevalence of heart failure with preserved ejection fraction (HFpEF) in screened population [2]. Thus, the majority of T2D patients present with HFpEF, at least at early stages, and the prevalence of HFpEF in diabetics is alarming. In contrast to HFrEF [3, 4], there is very limited evidence for pharmacologic therapy in symptomatic patients with preserved ejection fraction, and therefore, the optimal selection of treatment for diabetic HFpEF (DHFpEF) remains challenging. This narrative review article summarizes the currently available data on the pharmacological treatment of DHFpEF.

## 2. Diabetic Heart Failure with Preserved Ejection Fraction as a Challenging Clinical Problem

Diabetes is associated with several diabetic-related abnormalities, such as ischemia from either coronary artery atherosclerosis, or microvascular dysfunction, myocardial hypertrophy, dysfunction of mitochondria, dysfunction of autonomic nervous system, proinflammation, and increased retention of sodium (upregulation of sodium-glucose cotransporters) which increase the risk of onset or worsening of heart failure [5, 6]. Unfortunately, outcomes in HFpEF are poor and could be compared to those of HFrEF, with 1-year mortality ranging between 10 and 30% [7]. A subanalysis of I-Preserve Trial (Irbesartan in Heart Failure With Preserved Ejection Fraction) showed (Table 1) that, in HFpEF, patients with diabetes have more signs of congestion, worse quality of life, higher levels of heart failure biomarkers (N-terminal pro-B-type natriuretic peptide: NT-proBNP), and a poorer prognosis [8]. In addition, comparing in-patient costs of heart failure admissions,

Table 1: Summary of studies on heart failure with preserved ejection fraction.

| Study | Study size | Patient population | Dedication on diabetes | Studied drug and comparator | Main outcomes | Conclusion |
|---|---|---|---|---|---|---|
| CHARM–Preserved [23] | 3023 patients | NYHA functional class II-IV CHF and LVEF > 40% | No (857 patients with diabetes; subanalysis was not reported) | Candesartan (1514 patients, target dose 32 mg once daily) or matching placebo (1509 patients) | 22% patients in the candesartan vs. 24% in the placebo group experienced the primary outcome ($p = 0.051$); no reduction of CV death; fewer patients with candesartan group admitted to hospital for CHF | Moderate impact in preventing admissions for CHF |
| I–Preserve [24] | 4128 patients | At least 60 years of age, NYHA class II-IV HF and LVEF ≥ 45% | No (1134 patients with diabetes; subanalysis was not reported) | 300 mg of irbesartan (2067 patients) or placebo (2061 patients) | Primary event rates with irbesartan 100.4 vs. 105.4 per 1000 patient-years with placebo ($p = 0.35$); no reduction of CV death or CVD-related hospitalization | No impact on outcomes |
| PEP–CHF [25] | 850 patients (207 patients finished follow-up) | At least 70 years of age, HF with diastolic dysfunction, LVEF > 40% | No (175 patients with diabetes; subanalysis was not reported) | 4 mg of perindopril (424 patients; 100 evaluated) or placebo (426 patients, 107 evaluated) | Reduction in the primary outcome, HF-related hospitalizations, NYHA class and 6-MWT distance | Significant impact on outcomes; however, insufficient power of study for primary endpoint |
| PARAGON–HF [33] | 4822 patients | NYHA class II-IV HF, LVEF ≥ 45%, elevated level of natriuretic peptides, evidence of structural heart disease | No (2062 patients with diabetes; subanalysis was not reported) | Sacubitril-valsartan (2407 patients, target dose, 97 mg of sacubitril with 103 mg of valsartan twice daily) or valsartan (2389 patients, target dose, 160 mg twice daily) | 894 primary events in 526 patients in the sacubitril-valsartan group and 1009 primary events in 557 patients in the valsartan group ($p = 0.06$); no reduction of CV death or HF-related hospitalization | No impact on outcomes |
| TOPCAT [37] | 3445 patients | Symptomatic HF and LVEF ≥ 45% | No (1118 patients with diabetes; subanalysis was not reported) | Spironolactone (1722 patients, 15 to 45 mg daily) or placebo (1723 patients) | Primary outcome occurred in 18.6% of patients with spironolactone vs. 20.4% of patients with placebo ($p = 0.14$); no reduction of CV death or hospitalization (for any reason) | No impact on outcomes |
| J–DHF [42] | 245 patients | At least 20 years of age, HF and LVEF >40% | No (75 patients with diabetes; subanalysis was reported) | Carvedilol (120 patients, uptitrated from 1.25 mg twice daily to the target dose of 10 mg twice daily) or no carvedilol (125 patients) | Primary endpoint occurred in 29 patients in the carvedilol group vs. in 34 patients in the control group ($p = 0.68$); no reduction of CV death, HF-related or CVD-related hospitalization (no difference according to diabetes status) | No impact on outcomes (similar in patients with and without diabetes) |
| EDIFY [47] | 179 patients | NYHA class II-III, in sinus rhythm with HR of ≥70 b.p.m., NT-proBNP level of ≥220 pg/mL (or BNP ≥ 80 pg /mL) and LVEF ≥ 45% | No (78 patients with diabetes; subanalysis was not reported) | Ivabradine (95 patients, titrated to 7.5 mg b.i.d.) or placebo (84 patients) | No evidence of improvement was found in any of the three coprimary endpoints (Doppler $E/e'$ ratio, distance on the 6MWT, and decrease of plasma NT-proBNP levels) | No impact on outcomes |

Table 1: Continued.

| Study | Study size | Patient population | Dedication on diabetes | Studied drug and comparator | Main outcomes | Conclusion |
|---|---|---|---|---|---|---|
| EMPEROR Preserve [55, 57] | 5988 patients | NYHA class II-IV HF and LVEF > 40% | No (2934 patients with diabetes; subanalysis was reported) | Empagliflozin (2997 patients, 10 mg once daily) or placebo (2991 patients) | Primary outcome event occurred in 13.8% of patients with empagliflozin vs. in 17.1% of patients with placebo ($p < 0.001$); the effect mainly related to a lower risk of HF-related hospitalization (no difference according to diabetes status) | Significant reduction of the combined risk of CV death or hospitalization for HF(similar in patients with and without diabetes) |
| DELIVER [56] | 6263 patients | At least 40 years of age, CHF and LVEF > 40% | No (patients with or without diabetes enrolled; subanalysis will be probably reported) | Dapagliflozin (10 mg once daily) or placebo | Ongoing study | Ongoing study |

6MWT: 6 min walking test; BNP: B-type natriuretic peptide; (C)HF: (chronic) heart failure; CV(D): cardiovascular (disease); HR: heart rate; NYHA: New York Heart Association.

patients with diabetes have the highest cost, and cost per day alive appears to be the highest for HFpEF patients with diabetes [9]. As already mentioned, the data for heart failure pharmacotherapy in HFpEF are so far very limited, and no trial has achieved convincing morbidity/mortality endpoints to date. This probably reflects the disease complexity, as there are multiple pathophysiologic mechanisms in HFpEF, such as impaired diastolic function and impaired systolic reserve, impaired longitudinal ventricular systolic and atrial function, impaired autonomic heart function, and peripheral mechanisms such as endothelial and skeletal muscle dysfunction [6, 10–13]. Now, the question is: "How should we treat patients with DHFpEF?"

## 3. "Established Heart Failure Drugs" in the Treatment of DHFpEF

*3.1. Angiotensin-Converting Enzyme Inhibitors, Angiotensin Receptor Blockers, and Angiotensin Receptor/Neprilysin Inhibitors.* Angiotensin-converting enzyme inhibitors (ACEi) reduce morbidity and mortality in patients with HFrEF and represent currently the standard of HFrEF pharmacotherapy [3, 4]. The evidence for the use of ACEi in HFrEF is coming from multiple randomized controlled trials [14–16]. Angiotensin receptor blockers (ARB), drugs directly blocking angiotensin receptor 1, have similar hemodynamic effect to ACEi, with a lower risk of cough and angioedema (side effects frequently limiting the tolerability of ACEi therapy). ARB have been shown to reduce morbidity and mortality in patients with HFrEF, especially in those not tolerating ACEi [17–19]. Considering these data, one could assume that ACEi or ARB should be preferred for HFpEF. This assumption might be supported with beneficial effects of these agents, such as reduction of afterload, reduction of myocardial fibrosis and myocardial remodeling [20, 21], anti-inflammatory effect, and improvement of endothelial function [22]. Nevertheless, looking more closely to this issue, the current evidence for the use of ACEi or ARB in the treatment of HFpEF is limited to that coming from CHARM (Candesartan in Heart failure: Assessment of Reduction in Mortality and morbidity)-Preserved Trial [23]. This trial (Table 1) enrolled 3023 patients with symptomatic heart failure and left ventricular ejection fraction >40% who were randomly assigned to candesartan ($n = 1514$, target dose 32 mg once daily) or matching placebo ($n = 1509$). The primary outcome of this trial was cardiovascular death or need for in-hospital admission for heart failure; patients were followed for a median of 36.6 months. In this trial, cardiovascular death did not differ between candesartan-treated patients and controls, but candesartan therapy reduced the need for in-hospital admissions (230 versus 279, $p = 0.017$). Although 28.7% of patients in candesartan group and 28.0% of patients in placebo group had a history of diabetes, the subanalysis of trial results in diabetic patients was not reported. On the other side, in the I-Preserve Trial [24], irbesartan therapy failed to improve the outcomes of HFpEF. In this trial enrolling 4128 patients with HFpEF, who were followed for a median of 49.5 months, the primary outcome occurred in 742 patients in the irbesartan group and in 763 patients in the placebo group, which was not statistically significant. Overall rates of death were 52.6 and 52.3 per 1000 patient-years, respectively ($p = 0.98$); rates of hospitalization for cardiovascular causes were 70.6 and 74.3 per 1000 patient-years, respectively ($p = 0.44$). The study groups did not differ significantly in diabetes status (27% of patients in irbesartan group and 28% of patients in placebo group), and similarly with previous trial, a subanalysis of study results in patients with diabetes was not specifically reported. Additionally, the Perindopril in Elderly People with Chronic Heart Failure Trial (PEP-CHF) was a randomized, placebo controlled, double-blind trial, which aimed to compare perindopril (dosed 4 mg/day) versus placebo in patients aged ≥70 years who were diagnosed to have heart failure (on diuretics) and an echocardiographic finding suggesting diastolic dysfunction and excluding substantial LV systolic dysfunction (LV EF > 40%) or valve heart disease [25]. The primary endpoint of this trial was a composite of all-cause mortality and unplanned heart failure-related hospitalization with a minimum follow-up of 1 year. In this trial, by the 1 year of follow-up period, reductions in the primary outcome (hazard ratio: HR 0.692, 95% confidence interval: CI 0.474-1.010; $p = 0.055$) and hospitalization for heart failure (HR 0.628, 95% CI 0.408-0.966; $p = 0.033$) were observed with perindopril. Furthermore, a functional class ($p < 0.030$) and 6-minute corridor walk distance ($p = 0.011$) had improved in those assigned to perindopril. Nevertheless, the enrollment and event rates in this study were lower than anticipated, which significantly reduced the power of the study to show a difference in the primary endpoint (to 35%). A significant amount of patients withdrew from study drugs (28% of patients taking perindopril and 26% of patients taking placebo) after 1 year and started taking open-label ACEi. The authors concluded that although improved symptoms and exercise capacity and fewer hospitalizations for heart failure in the first year were observed on perindopril, the study had insufficient power for its primary endpoint. The subanalysis of patients with diabetes was not reported (Table 1). Summarizing these data, currently, there is no clear evidence supporting the use of ACEi or ARB for the treatment of DHFpEF (as there are no data on global benefit of these agents in HFpEF from CHARM-Preserved and I-Preserve trials); however, looking on possible benefits, the therapy (preferring candesartan) could be probably considered in those patients with hypertension, left ventricular hypertrophy, prior myocardial infarction, in case of microalbuminuria/proteinuria, and diabetic kidney disease [4, 26]. Angiotensin receptor/neprilysin inhibitors, an ARB combined with a neprilysin inhibitor, commercially available as sacubitril/valsartan molecule, adds additional effect on heart failure by inhibition of enzyme which degrades natriuretic peptide, adrenomedullin, and other vasoactive peptides, leading to vasodilation and decreased retention of sodium [27]. The benefit of ARNI (compared to ACEi enalapril) in patients with HFrEF was demonstrated in PARADIGM-HF (Prospective Comparison of Angiotensin Receptor–Neprilysin Inhibitor with Angiotensin-Converting–Enzyme Inhibitor to Determine Impact on Global Mortality and Morbidity in Heart Failure) trial [28] which showed

significant (by 20%) reduction of the composite endpoint of cardiovascular death or heart failure hospitalization with ARNI. ARNI therapy seemed promising in patients with HFpEF, considering the data from phase 2 clinical trial [29] which showed in patients with HFpEF a greater reduction of heart failure biomarkers with ARNI than with valsartan alone, and promising data from studies on animal models [30, 31] or data regarding beneficial effect of ARNI on left ventricular diastolic function coming from small postmarketing study [32]. However, these promises were not verified in PARAGON-HF (The Prospective Comparison of ARNI with ARB Global Outcomes in HF with Preserved Ejection Fraction) trial [33], a randomized trial comparing the effect of ARNI (sacubitril/valsartan) and valsartan alone in patients (with or without diabetes) with HFpEF (defined as left ventricular ejection fraction ≥ 45%) on a composite end-point of total hospitalizations for heart failure and death from cardiovascular causes. The study randomized totally 4822 patients with symptomatic HFpEF (left ventricular ejection fraction ≥ 45%), from whom 42.2% had diabetes. In this trial (Table 1), ARNI administration did not result in a significantly lower rate of total hospitalizations for heart failure and was not connected with significantly lower cardiovascular mortality (a trend towards benefit in reduction of primary events was observed, but the differences did not reach statistical significance: 894 primary events in the ARNI group versus 1009 primary events in the valsartan group; HR 0.87; 95% CI: 0.75-1.01; $p = 0.06$). Unfortunately, the subanalysis of trial outcomes in diabetic patients was not reported, and there is no other study reporting the efficacy and safety of ARNI for the treatment of DHFpEF. Undoubtedly, such a subanalysis/study could be off clinical interest, as ARNI could have in individuals with diabetes positive effect on glycaemic control [34] and renal function [35], suggesting a possible pleiotropic benefit for diabetic patients with heart failure. Nevertheless, the administration of ARNI still remains reserved for patients with HFrEF.

*3.2. Mineralocorticoid Receptor Antagonists and Diuretics.* Mineralocorticoid (aldosterone) receptor antagonists (MRA)—spironolactone and eplerenone—are recommended in all symptomatic patients with HFrEF to reduce mortality and heart failure-related hospitalizations [3]. The role of these agents in HFpEF is being intensively studied, with conflicting results. First, Deswal et al. reported in their small randomized study enrolling 44 patients with HFpEF that eplerenone therapy lead to a significant reduction in markers of collagen turnover and to an improvement in left ventricular diastolic function. However, in this trial, eplerenone did not improve exercise capacity during 6-minute walking test [36]. The TOPCAT (Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist) trial [37] examined the effect of spironolactone (dosed 15 to 45 mg daily) versus placebo on a composite endpoint of death from cardiovascular causes, aborted cardiac arrest, and hospitalization for heart failure. The study (Table 1) randomized totally 3445 patients with symptomatic heart failure and left ventricular ejection fraction ≥ 45%; from these patients, 32.5% had diabetes. Although spironolactone therapy in this study failed to improve primary composite endpoint (18.6% in spironolactone group versus 20.4% in placebo group, $p = 0.14$), patients treated with spironolactone had significantly lower incidence of hospitalizations for heart failure (12.0% versus 14.2%; $p = 0.04$). Based on these data, the American College of Cardiology formed its current recommendation on the use of MRA in HFpEF which states that MRA therapy might be considered in appropriately selected patients with HFpEF (left ventricular ejection fraction ≥ 45%, elevated markers of heart failure, heart failure-related hospitalization within 1 year, estimated glomerular filtration rate > 30 mL/min., and blood potassium level < 5 mmol/L) to decrease heart failure-related hospital admissions [4]. Looking on DHFpEF, a subanalysis of TOPCAT trial [38] showed that diabetic patients enrolled in this trial had higher levels of cardiac, profibrotic, and proinflammatory biomarkers. The administration of spironolactone in patients with diabetes appeared to improve the markers of extracellular matrix remodeling in an antifibrotic fashion. Additionally, Brandt-Jacobsen et al. [39] reported that the addition of high-dose eplerenone in patients with T2D and high risk for cardiovascular diseases was associated with a clear reduction in left ventricular mass and a clear reduction of NT-proBNP levels. Based on these data, MRA therapy could be probably recommended in selected patients with DHFpEF; however, due to diabetic-related kidney impairment, it might be more difficult to select appropriate DHFpEF patients for this therapy. Loop diuretics (furosemide, bumetanide, and torasemide) are recommended to reduce signs and symptoms of congestion in patients with HFrEF and HFpEF [3], but their effects on mortality and morbidity have not been studied in randomized trials. This recommendation should be probably applied also to DHFpEF patients, as there is no other pharmacologic approach to relieve signs and symptoms of congestion in HFpEF. However, the risk of side effects of the therapy, mainly the risk of orthostatic hypotension, worsening of renal function, and the risk of loss of minerals, could be higher in patients with diabetes [40].

*3.3. Beta Blockers, Ivabradine, and Digoxin.* A previous subanalysis of already mentioned I-PRESERVE trial showed that in this trial, heart rate (in sinus rhythm) was an independent predictor of adverse clinical outcomes. Each standard deviation (12.4 beats per minute) increase in heart rate was associated with an increase in risk of 13% for cardiovascular death or heart failure hospitalization ($p = 0.002$ ). Considering the data, "optimal" heart rate achieved with beta blockers or If-inhibition with ivabradine might be a "therapeutic target" in HFpEF. However, data regarding the use of beta blockers for HFpEF are inconsistent. The SENIORS (Study of Effects of Nebivolol Intervention on Outcomes and Rehospitalization in Seniors With Heart Failure) trial showed an overall benefit of beta blockage with nebivolol compared to placebo in 2128 heart failure patients > 70 years of age. The primary outcome was similar in the impaired and preserved left ventricular ejection fraction groups. However, the preserved left ventricular ejection

fraction (HFpEF) was in this trial defined as left ventricular ejection fraction > 35% (and impaired left ventricular ejection fraction as ejection fraction ≤ 35%) [41]. On the other side, in the Japanese Diastolic Heart Failure Study (J-DHF), carvedilol therapy did not improve the prognosis of HFpEF patients (Table 1) [42]. In contrast, data from the Croatian heart failure registry showed a higher overall survival rate, improvement in ejection fraction, and NYHA class in HFpEF patients on long-term (at least 4 years) carvedilol therapy [43]. None of these studies was dedicated on patients with DHFpEF, and there is no other study examining the effect of beta blockage on clinical outcomes in those with DHFpEF. Therefore, routine beta blockage in patients with DHpEF is currently not recommended. However, beta blockers might be considered in patients with DHFpEF and arterial hypertension or known coronary artery disease [4, 26]. If-inhibition with ivabradine improved vascular stiffness, left ventricular contractility, and diastolic function in a mouse model of HFpEF [44]. Moreover, ivabradine therapy improved diastolic left ventricular function in an observational study performed by Cacciapuoti et al. [45] and significantly reduced the need of hospital admissions for worsening heart failure in a previous randomized placebo-controlled study enrolling patients with HFrEF [46]. The effect of ivabradine therapy on clinical outcomes in patients with HFpEF was specifically examined in the EDIFY (prEserveD left ventricular ejectIon fraction chronic heart Failure with ivabradine study) trial [47]. This randomized, placebo-controlled trial (Table 1) included 179 patients with symptomatic (NYHA classes II and III) HFpEF (left ventricular ejection fraction 45%), who were in sinus rhythm (with heart rate of ≥70 beats per minute) and had an elevation of heart failure biomarkers (NT-proBNP of ≥220 pg/mL or BNP ≥ 80 pg/mL). Patients were randomized to ivabradine (uptitrated to 7.5 mg b.i.d.) or placebo and followed for 8 months for the incidence of primary end-point defined as improvement in left ventricular diastolic function (assessed by echocardiography), distance on the 6-minute walking test, and change of plasma NT-proBNP levels. In this trial, ivabradine did not improve any of the three clinical outcomes (although there was a significant decrease in heart rate by ivabradine therapy). 43.2% of patients randomized to ivabradine and 44.1% of patients randomized to placebo had diabetes; the subanalysis of outcomes among patients with diabetes was not reported. Additionally, there is no other study examining the efficacy/safety profile of ivabradine in patients with DHFpEF. Summarizing, although several small studies pointed on a possible positive effect of heart rate reduction with ivabradine in patients with HFpEF, based on the results of EDIFY trial, ivabradine is not indicated for the treatment of HFpEF/DHFpEF. Thus, ivabradine should not be administrated in diabetic patients with HFpEF, unless there is other indication for ivabradine therapy (such as symptomatic angina despite beta blocker therapy). Digoxin might be considered as a treatment option in patients with HFrEF in sinus rhythm who remain symptomatic despite ACEi (or ARB), a beta blocker, and MRA therapy to reduce the risk of hospitalizations, or in patients with HFrEF and atrial fibrillation to slow a rapid ventricular rate [3]. Currently, there is no randomized study examining the efficacy/safety profile of digoxin in the treatment of HFpEF/DHFpEF. Furthermore, in a previously published analysis of a heart failure registry, the initialization of digoxin therapy in patients with HFpEF requiring heart failure-related hospitalization prior their discharge was not associated with lower rates of rehospitalizations or all-cause mortality [48]. Moreover, data from another observational multicentre study suggested that digoxin therapy in patients with HFpEF might be associated with increased mortality and/or heart-failure-related readmission, especially in patients with lower heart rate [49]. Therefore, digoxin is not indicated in patients with HFpEF, and this could be probably applied also to patients with DHFpEF.

## 4. Novel Antidiabetic Drugs: Promising Agents for the Treatment of DHFpEF?

*4.1. Sodium-Glucose Cotransporter 2 Inhibitors.* The data regarding significant benefit of sodium-glucose cotransporter 2 (SGLT-2) inhibition on heart failure-related mortality and heart failure-related hospitalizations came firstly from studies which were not specifically dedicated on heart failure patients. The EMPA-REG OUTCOME (Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients) trial [50] randomized a total of 7020 patients with T2D at high cardiovascular risk (left ventricular ejection fraction was not reported) to receive 10 mg or 25 mg of empagliflozin or placebo once daily. The primary composite outcome was death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke, as analyzed in the pooled empagliflozin group versus the placebo group. The primary outcome occurred in 10.5% of patients in the pooled empagliflozin group and in 12.1% of patients in the placebo group ($p = 0.04$ for superiority). In the empagliflozin group, there were significantly lower rates of death from cardiovascular causes (3.7%, versus 5.9%; 38% relative risk reduction), hospitalization for heart failure (2.7% versus 4.1%, 35% relative risk reduction), and death from any cause (5.7% versus 8.3%, 32% relative risk reduction). The study showed cardiovascular benefit of SGLT2 inhibition in T2D patients; there was no specific mention of HF or left ventricular ejection fraction status in this study. Similar data in patients with T2D and higher risk of cardiovascular diseases were subsequently published with dapagliflozin [51] and with canagliflozin [52]. The EMPEROR-Reduced (EMPagliflozin outcomE tRial in Patients With chrOnic heaRt Failure With Reduced Ejection Fraction) trial [53] tested the effect of empagliflozin (10 mg once daily) on a composite endpoint of cardiovascular death or hospitalization for worsening heart failure in patients with symptomatic HFrEF (left ventricular ejection fraction ≤ 40%). This study randomized totally 3730 patients (with or without diabetes) who were followed for a median of 16 months. The primary outcome was less frequent in the empagliflozin group (19.4% versus 24.7%, $p < 0.001$). The effect of empagliflozin on the primary outcome was consistent in patients regardless of the presence or absence of diabetes. The total number of hospitalizations for heart failure was lower in the

empagliflozin group than in the placebo group. In addition, empagliflozin-treated patients had lower risk of serious renal outcomes. The study confirmed significant cardiovascular benefit of SLGT2 inhibitor therapy in a cohort of patients with HFrEF, regardless of T2D status. Another robust evidence for the use of SGLT-2 inhibition in patients with HFrEF comes from DAPA-HF (Study to Evaluate the Effect of Dapagliflozin on the Incidence of Worsening Heart Failure or Cardiovascular Death in Patients With Chronic Heart Failure) trial [54]. This randomized, placebo-controlled trial enrolled 4744 patients (with or without diabetes) with symptomatic heart failure and reduced left ventricular ejection fraction (≤40%) who were randomized to receive either dapagliflozin (10 mg once daily) or placebo, in addition to standard of care therapy. Patients were followed for a median of 18.2 months for the incidence of primary outcome defined as a composite of worsening heart failure (hospitalization or an urgent visit resulting in intravenous therapy for heart failure) or cardiovascular death. During the follow-up period, the primary outcome occurred in 16.3% of patients in the dapagliflozin group and in 21.2% of patients in the placebo group ($p < 0.001$). A first worsening heart failure event occurred in 10.0% of patients in the dapagliflozin group and in 13.7% of patients in the placebo group; cardiovascular disease-related death occurred in 9.6% of patients in the dapagliflozin group and in 11.5% of patients in the placebo group, respectively. In addition, results in diabetic patients were similar to those in patients without diabetes. Although promising, the results of upper-mentioned studies cannot be directly applied to patients with DHFpEF. Therefore, it is not surprising that EMPEROR Preserved (EMPagliflozin outcomE tRial in Patients With chrOnic heaRt Failure With Preserved Ejection Fraction) trial [55] and DELIVER (Dapagliflozin Evaluation to Improve the LIVEs of Patients With PReserved Ejection Fraction Heart Failure) trial [56], studies directly dedicated on patients with (D)HFpEF, have been designed. The DELIVER (Dapagliflozin Evaluation to Improve the LIVEs of Patients With PReserved Ejection Fraction Heart Failure) trial [57] is designed as an international, multicentre, randomized, placebo-controlled study which aims to test the effect of SGLT-2 inhibition with dapagliflozin (10 mg daily) versus placebo, in addition to standard of care, in patients with HFpEF (left ventricular ejection fraction ≥ 40%). Study (Table 1) plans to enroll patients with or without diabetes (6263 patients), with preserved ejection fraction, with signs and symptoms of heart failure, elevation in natriuretic peptides, and evidence of structural heart disease. The primary endpoint will be the time-to-first cardiovascular death or worsening heart failure event (heart failure hospitalization or urgent heart failure visit). Finally, the results of the EMPEROR-Preserved (EMPagliflozin outcomE tRial in Patients With chrOnic heaRt Failure With Preserved Ejection Fraction) trial [55] have been recently reported [57]. The study was designed to test the SGLT-2 inhibition with empagliflozin in patients with HFpEF. The study (Table 1) enrolled 5988 patients with class II–IV heart failure and left ventricular ejection fraction > 40%, with and without T2D, who were randomized to receive empagliflozin (10 mg daily) or placebo, in addition to standard therapy. In this study, 2997 patients received empaliflozin and 2991 patients received placebo. The primary end-point of this study was a composite of cardiovascular death or hospitalization for heart failure. In a median of 26.2 months of clinical follow-up, the primary outcome occurred in 13.8% in the empagliflozin group and in 17.1% in the placebo group (hazard ratio, 0.79; 95% confidence interval [CI]: 0.69-0.90; $p < 0.001$). This effect was mainly related to a lower risk of hospitalization for heart failure. The efficacy of empagliflozin was consistent in patients with or without T2D. The total number of hospitalizations for heart failure was lower in the empagliflozin group than in the placebo group (407 with empagliflozin versus 541 with placebo; HR 0.73; 95% CI: 0.61-0.88; $p < 0.001$). Looking on the side effects of the therapy, unsurprisingly, uncomplicated genital and urinary infections and hypotension were reported more frequently with empagliflozin. The results of the EMPEROR-Preserved trial confirmed the efficacy of empagliflozin in patients with HFpEF (with and without T2D) and will probably establish SGLT-2 inhibition as a first evidence-based and clinical practice guideline-recommended pharmacologic therapy for HFpEF and also for DHFpEF.

To summarize, empagliflozin is the first agent with evidence-based efficacy data for the treatment of HFpEF (irrespectively on diabetes status). Based on a robust evidence in T2D patients with higher cardiovascular risk, in patients with HFrEF, as well as in those with HFpEF, SGLT-2 inhibitors should be administrated in patients with DHFpEF (unless contraindicated) to treat diabetes (to safely improve glycaemic control), to reduce the risk of heart failure-related hospitalizations and cardiovascular mortality and to improve renal outcomes.

*4.2. Glucagon-Like Peptide 1 Receptor Antagonists.* Glucagon-like peptide 1 receptor antagonists (GLP-1 RA), such as liraglutide, semaglutide, or dulaglutide, are novel antidiabetic agents with the evidence for reduction of cardiovascular events [26]. In previous randomized controlled trials in patients with T2D, liraglutide reduced the rate of the first occurrence of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke [58], semaglutide significantly reduced the rate of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke [59], and dulaglutide reduced the primary outcome defined as the first occurrence of the composite endpoint of nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes [60]. Nevertheless, the cardiovascular benefit of GLP-1 RA was mostly due to reduction of adverse vascular events and reduced cardiovascular mortality and not due to reduced incidence of heart failure or reduced rate of heart failure-related hospitalizations. Additionally, none of these studies were dedicated on a population of patients with diabetic heart failure. Withaar et al. previously demonstrated in a study using HFpEF animal model that treatment with liraglutide improved the cardiometabolic dysregulation and cardiac function, reduced cardiac hypertrophy, reduced myocardial fibrosis, and improved atrial weight, natriuretic peptide levels, and lung congestion [61]. In another animal

model, liraglutide improved pressure-overload induced cardiac hypertrophy and cardiac apoptosis [62], and liraglutide administration had favorable effects on BNP and left ventricular diastolic function, but not on left ventricular ejection fraction, in a small-sample clinical study in patients with T2D and preserved left ventricular ejection fraction performed by Yagi et al. [63]. Nonetheless, there is no clinical study examining the effect of GLP-1 RA therapy on clinical outcomes in patients with DHFpEF (reported or on-going). Thus, GLP-1 RA cannot be considered as a treatment option for DHFpEF. However, it is probably rational to administer GLP-1 RA in patients with T2D and known cardiovascular disease (including heart failure), especially in those with known coronary artery disease or other known atherosclerotic vascular diseases, to treat diabetes (to safely improve glycaemic control), and to prevent future vascular events.

## 5. Conclusion

How to treat DHFpEF? Right now, there is no conclusive answer to this question. Obviously, there is a need to treat the cause of the HFpEF (if treatable) and manage comorbidities as is currently recommended in HF guidelines (T2D is a common comorbidity in HFpEF) [3, 64]. In addition, considering the discussed data, it is probably reasonable to administer loop diuretics in those patients with signs or symptoms of congestion; ARB in patients with left ventricular hypertrophy, hypertension, or concomitant diabetic kidney disease and SGLT-2 inhibitors in all reliable patients with DHFpEF to treat diabetes, reduce the risk of heart failure-related hospitalizations and cardiovascular mortality, and improve renal outcomes. Additionally, selected patients with DHFpEF could benefit from beta blocker and MRA therapy. Moreover, it is probably rational to administer GLP-1 RA in patients with T2D and known cardiovascular (especially atherosclerotic) disease to treat diabetes and prevent future vascular adverse events. Nevertheless, the issue of optimal pharmacotherapy for DHFpEF is still open for future research.

## Data Availability

All the source data are available at the corresponding author upon a reasonable request.

## Ethical Approval

This research was done according to ethical standards.

## Consent

Formal consent for this type of study is not required

## Conflicts of Interest

Jakub Benko, Matej Samoš, Tomáš Bolek, Dana Prídavková, Jakub Jurica, Martin Jozef Péč, Peter Galajda, and Marián Mokáň have no conflicts of interest to declare

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*Research Article*

# A Randomized Controlled Clinical Trial of Lifestyle Intervention and Pioglitazone for Normalization of Glucose Status in Chinese with Prediabetes

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*Aims*. Prediabetes has been proved as an important risk factor of both diabetes and cardiovascular disease (CVD). Previous studies have shown that both lifestyle intervention and pioglitazone may delay the development of diabetes in patients with prediabetes. However, no study has ever explored whether these interventions could revert prediabetes to normal glycemic status as the primary outcome. Interventions that may revert prediabetes back to normal glucose status would be of great clinical importance. *Materials and Methods*. We conducted a randomized, multicenter, $2 \times 2$ factorial designed study to examine whether intensive lifestyle intervention and/or pioglitazone could revert prediabetes to normal glucose tolerance. The participants were followed up for three years unless they reverted to normal glucose state or developed diabetes at the annual oral glucose tolerance test (OGTT). Reversion to normal glucose tolerance was confirmed on the basis of the results of OGTT. *Results*. In our study, 1945 eligible patients were ultimately randomized into four groups. In this three-year follow-up study, overall, 60.0%, 50.3%, 56.6% and 65.1% reverted back to normoglycemic state over 3 years of follow-up in the conventional lifestyle intervention plus placebo, intensive lifestyle intervention plus placebo, conventional lifestyle intervention plus pioglitazone, and intensive lifestyle intervention plus pioglitazone groups, respectively. Compared to the conventional lifestyle intervention plus placebo group, all the other three groups did not show any significant benefit in terms of reverting back to normoglycemic state. *Conclusion*. In our study, for patients with prediabetes, neither intensive lifestyle intervention nor pioglitazone had led to a higher reversion rate to normal glucose state. *Trail registration*. http://www.chictr.org.cn: ChiCTR-PRC-06000005.

## 1. Introduction

Diabetes is one of the major components of the burden of disease in China, while a significant proportion of the population has prediabetes. The most recent data showed that among adults in China, the estimated overall prevalence of diabetes was 10.9% and that for prediabetes was 35.7% [1]. Patients with prediabetes are not only at high risk to develop diabetes but also the high risk population to develop cardiovascular disease (CVD) [2, 3].

Da Qing study was the first intervention study for prediabetes all over the world, which was also a prevention study in Chinese population [4]. After a 23-year follow-up, it showed that the majority of deaths (74.7%; 130 of 174)

occurred in those who developed diabetes. Progression to type 2 diabetes was associated with a 73% higher risk of death in this study. In China, the risk of mortality is increased in people with prediabetes; this excess risk might be explained by the development of type 2 diabetes [5]. Numerous clinical trials had showed that early intervention based on individualized prevention model may be beneficial in delaying the progression to type 2 diabetes in high-risk populations [6].

It is possible that interventions which could revert prediabetes to normal or could delay the development of diabetes may also be effective to decrease the risk of developing long-term complications of hyperglycemia or death. During the last 30 years, many studies have shown that lifestyle modification [4, 7–13]; the use of metformin [14], acarbose [10, 15], and thiazolidinediones [11, 12, 16]; and bariatric surgery may delay the progression of type 2 diabetes in patients with impaired glucose tolerance [17].

However, no study has explored whether intervention could revert prediabetes to normal glycemic status as the primary outcome. The previous studies were all aimed at evaluating the efficacy of intervention on preventing diabetes in patients with impaired glucose tolerance (IGT), rather than prediabetes. We undertook the present study to evaluate whether lifestyle intervention with or without pioglitazone could revert prediabetic state back to normal glycemia over a 3-year period in patients with prediabetes.

## 2. Materials and Methods

*2.1. Study Design.* As described in our previous publication [18], Beijing Prediabetes Reversion Program (BPRP) is a prospective, multicenter, randomized, double-blinded, and placebo-controlled clinical trial, based on a $2 \times 2$ factorial design. Patients with prediabetes were randomized into four groups: conventional lifestyle intervention plus placebo, conventional lifestyle intervention plus pioglitazone hydrochloride 30 mg daily, intensive lifestyle intervention plus placebo, and intensive lifestyle intervention plus pioglitazone hydrochloride 30 mg daily.

*2.2. Participants.* As published elsewhere [18], individuals who were diagnosed as prediabetes based on previous oral glucose tolerance test (OGTT) were eligible for screening. We recruited male and female patients who were between 25 and 70 years old and had prediabetes (defined as fasting plasma glucose $\geq$ 6.1 mmol/L (110 mg/dL) and <7.0 mmol/L (126 mg/dL), meanwhile 2hPG < 7.8 mmol/L (140 mg/dL) or FPG < 7.0 mmol/L (126 mg/dL), meanwhile, 2hPG $\geq$ 7.8 mmol/L (140 mg/dL) and <11.1 mmol/L (200 mg/dL) during a single oral glucose tolerance) and a body mass index (BMI) between 22 and 35 kg/m$^2$. Informed consent forms were obtained before the individuals could participate in any screening procedures. Eligible participants were then randomized into one of the four arms of the study. The detailed inclusion and exclusion criteria have been published previously [18].

The first participant was screened in March 2007. The enrollment of 1945 participants was completed in March 2011. Participants were followed until they reverted to normal glucose level or developed diabetes, withdrew from the study, were lost to follow-up, or completed the end of the study. There were 4397 individuals who met the screening criteria and were screened. Among these individuals, 1388 participants were identified normal glucose tolerance and 975 participants had diabetes. Finally, 2034 (46.3%) were confirmed to have prediabetes. Among 4397 screened population, the mean $\pm$ SD age and BMI were $52 \pm 15$ years and $26.1 \pm 3.3$ kg/m$^2$, respectively. Following the inclusion and exclusion criteria, 1954 eligible patients were randomized into four groups with equal proportion. The mean age of the participants was $53 \pm 10$ years old, and the median (Q1, Q3) of BMI and HbA1c was 26.0 (23.9, 28.2) kg/m$^2$ and 5.8 (5.6, 6.1)%, respectively. 85% of the participants were IGT, and 15% were IFG.

*2.3. Interventions.* In our previous publication, we have described the intervention in detail [18]. In brief, all the participants were recruited and followed up in the outpatient clinic. The follow-up of the study was three years. There were 19 scheduled visits during the three-year follow-up. To avoid the bias, in all four groups, study visits were scheduled for every 2-3 weeks during the first 13 weeks and every 13 weeks thereafter.

In the intensive lifestyle intervention group, after randomization, educational courses were provided to the participants at each visit and the investigators would prescribe an individualized lifestyle prescription for them at each visit according to their body weight and lifestyle diary with decision support software we made particularly for this study.

The software was built based on the dietary and exercise recommendations from dietary guidelines for Chinese residents and guideline for prevention and treatment of type 2 diabetes. In the intensive lifestyle intervention group, participants were suggested to have at least 150 minutes of moderate intensity (3-6MET) of aerobic exercise every week and were encouraged to have 180-300 minutes per week. Besides the aerobic exercise, participants were also encouraged to have some resistant exercise every week.

In the dietary instruction, the aim was to achieve negative balance of energy intake. The Harris-Benedict formula was used to calculate the total energy intake [19]. A 300 kcal deficit was provided for overweight population, and a 500 kcal deficit was provided for obese population.

The weight loss goal was 5-10% reduction (or 2–4 kg per week) in body weight from baseline for those who were obese or overweight. At each study visit, the dietary intake component from the lifestyle diary and the body weight was used to generate the automated lifestyle prescription from the software, with the option for manual changes in the prescription if deemed necessary.

In the conventional lifestyle intervention group, participants were provided usual lifestyle education at both baseline and annual visits; they would not receive any individualized consultation. They would only receive general information on healthy lifestyle and would not receive lifestyle evaluation or lifestyle-related prescription. To avoid

the contamination between groups, all four groups were designed with similar visit schedule.

Among participants who received pioglitazone, the dose of the medication (30 mg/day) would not change throughout the follow-up period. The active drug and matched placebo were manufactured by Beijing Taiyang Pharmaceutical Company.

At annual visit, participants who were identified to have reverted to normal glucose state were requested to stop the medication and were invited for an OGTT two weeks after the last visit. This procedure was followed for those participants who remained prediabetic during the course of 3 years of follow-up. Those who were identified to have developed diabetes after 2 weeks of washout period were advised to seek standard care for diabetes. At the end of follow-up, all participants were advised to follow standard lifestyle modification and those who still had elevated glucose level were transferred to outpatient clinic.

*2.4. Objectives.* This study was used to examine whether intensive lifestyle intervention and/or pioglitazone 30 mg once daily would increase the reversion rate of patients with prediabetes to normal glycemia, compared to conventional lifestyle intervention only.

*2.5. Primary and Secondary Outcomes.* As mentioned in our previous publication [18], the primary aim of the study was to evaluate the proportions of participants who reverted to normal glucose state during follow-up. The normal glucose level were was defined by OGTT glucose level with fasting plasma glucose less than 6.1 mmol/L and 2-hour postchallenge glucose less than 7.8 mmol/L. The secondary outcomes of the study included the following: (1) incidence of type 2 diabetes; (2) time to achieving normal glucose level; (3) change in HbA1c; (4) change in body weight and waist circumference; (5) changes in blood pressure, LDL-cholesterol, and HDL-cholesterol and triglyceride; (6) changes in adiponectin, hsCRP, and insulin and C-peptide at fasting and postchallenge; (7) change in urine albumin-creatinine ratio and serum creatinine; (8) composite of the incidence of at least one of the events, heart failure, nonfatal myocardial infarction, nonfatal stroke, or all-cause mortality; and (9) quality of life.

*2.6. Sample Size.* As described in our previous publication [18], we calculated that the sample size of this study would be 2000 participants (500 in each group). With this sample size, it could provide a 90% power with 5% type 1 error to detect a 10% relative increase in the rate of primary outcomes among participants assigned to intensive lifestyle intervention compared with conventional lifestyle intervention group under the following assumptions:

(1) 35.3% for the conventional lifestyle plus placebo, 44.3% for the conventional lifestyle plus pioglitazone, 45.3% for the intensive lifestyle plus placebo, 54.3% for the intensive lifestyle plus pioglitazone would be reverted to normal glucose

(2) Participants would be recruited in half a year

(3) Up to 30% of the participants might be lost of follow up during the study period

*2.7. Randomization.* We have mentioned in our previous publication [18] that randomization was undertaken by an independent statistician using a computer-generated random sequence and was performed as block randomization. The allocation ratio in four groups was 1 : 1 : 1 : 1. Each study site would receive sealed envelopes for randomization of the participants. Both the participants and healthcare providers were blinded by the medication, while they were open to the lifestyle intervention.

*2.8. Statistical Methods.* Basic statistics are presented by number (%), mean (SD), or median (Q1, Q3). The primary and secondary outcomes of the study were evaluated following the intention to treat approach, with additional supporting analyses based on the per protocol population.

To evaluate the proportion of people reverting to normoglycemic state and the proportion of people developing T2DM during the three years of follow-up in the intensive lifestyle intervention plus placebo, conventional lifestyle intervention plus pioglitazone, and intensive lifestyle intervention plus pioglitazone groups, compared to conventional lifestyle intervention plus placebo group, the discrete-time survival regression model with development of diabetes being the competing risk while evaluating the risk of reverting to normoglycemia. The hazard ratios and 95% CI were estimated. As an additional analysis, the logistic regression was also used, and the odds ratio (95% CI) was estimated with the significance level based on Bonferroni corrections. Changes in anthropometric, clinical and laboratory based secondary outcome measures at 1, 2, and 3 years of the study were estimates and presented as mean change (95% CI).

## 3. Results

*3.1. Participants' Flow.* In our study, 4397 individuals who met the screening criteria were screened. Among these individuals, 2034 (46.3%) were finally identified to have prediabetes. Following the inclusion and exclusion criteria, 1945 eligible patients were ultimately randomized into four groups (Figure 1). The baseline characteristics of the participants are described in Table 1. At the end of the first year, 366 participants were lost in follow-up. At the end of the second and third years, 232 and 92 participants were lost in follow-up, respectively. The overall dropout rate was 35% during three years of follow-up. With the software built for the lifestyle intervention prescription, we also evaluated the compliance of the lifestyle intervention in two intensive arms. The percentage of good compliance with diet was 18%, 20%, and 19% for the first, second, and third years, while the percentage of good compliance with exercise was 65%, 67%, and 66% for the first, second, and third years (supplement Figure 1).

*3.2. Primary Outcome.* The proportions of individuals reverting to normoglycemia and diabetes at 1, 2, and 3 years of follow-up and overall during 3 years of follow-up are

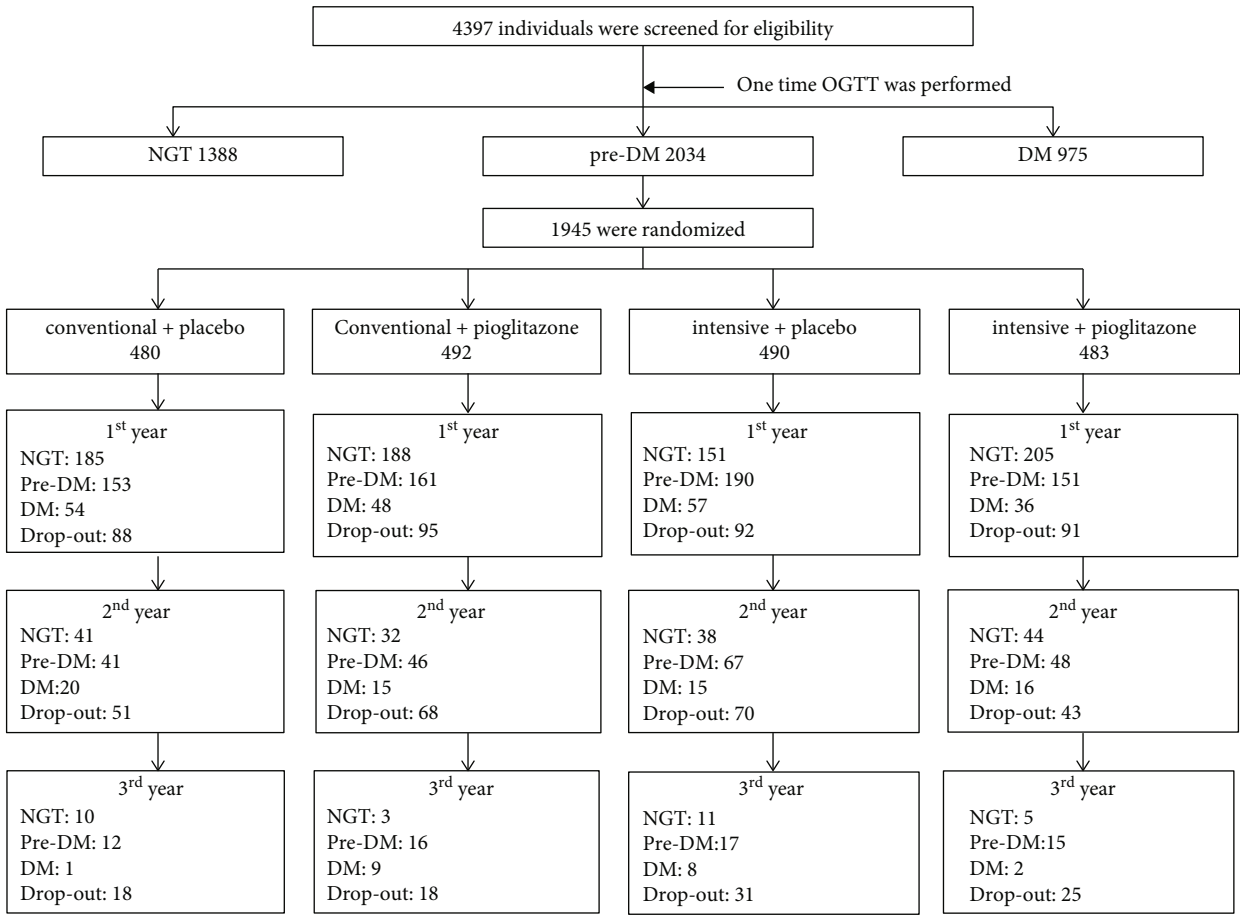


Figure 1: Participant flow.

presented in Table 2. Within 1 year of follow-up, 38.5%, 30.8%, 38.2%, and 42.4% reverted to the normoglycemic state in the conventional lifestyle intervention plus placebo, intensive lifestyle intervention plus placebo, conventional lifestyle intervention plus pioglitazone, and intensive lifestyle intervention plus pioglitazone groups, respectively. Overall, 60.0%, 50.3%, 56.6%, and 65.1% reverted back to normoglycemic state over 3 years of follow-up in these treatment groups, respectively. Compared to the conventional lifestyle intervention plus placebo group, all the other three groups did not show any significant benefit in terms of reverting back to normoglycemic state. We further divided the participants into different age groups and BMI groups. We see that the proportion of participants who reverted to normal glycemia state was highest in the youngest group (age < 40 years) and obese group (BMI $\geq 30\,kg/m^2$) (Figure 2).

*3.3. Secondary Outcomes.* Compared to the conventional lifestyle intervention plus placebo group, individuals in the intensive lifestyle intervention plus pioglitazone group had 45% (95% CI of HR: 0.32, 0.90, $p = 0.024$) reduced risk of developing diabetes, while the likelihood of developing diabetes was similar in the other two study groups. The changes in the secondary outcome measures during 3 years of follow-up from baseline are presented in Table 3. The changes in the fasting and postprandial glucose levels were not different between the treatment groups ($p > 0.05$), while we observed statistically significant reduction in postprandial glucose levels in the conventional lifestyle intervention plus placebo group (95% CI -2.0, -0.12 mmol/L) and intensive lifestyle intervention plus pioglitazone group (95% CI: -0.81, -0.62 mmol/L) ($p < 0.01$ in both groups). A marginal increase in the HbA1c level was observed in all groups (range of 95% CI: 0.05–0.46%), while there was no difference between the groups.

The observed differences in the changes in body weight and waist circumference were not different between the groups. The changes in blood pressure, lipids, and other cardiovascular and renal risk factors were also not different between the treatment groups. A statistically significant reduction in the levels of C-peptide was observed in all groups (range of 95% CI: -0.25, -0.72), while such reduction was not different between the groups.

*3.4. Safety Analysis.* The adverse events, considered relevant by the investigators, are listed in Supplement Table 1. The most common adverse event was edema without any difference among four groups.

Table 1: Baseline characteristics of the participants by study groups.

| | Conventional lifestyle +placebo | Intensive lifestyle +placebo | Conventional lifestyle +pioglitazone | Intensive lifestyle +pioglitazone |
|---|---|---|---|---|
| *N* | 480 | 490 | 492 | 483 |
| Male | 229 (48) | 167 (34) | 221 (45) | 207 (43) |
| Age (year) | 53 (45, 59) | 53 (47, 59) | 53 (45, 59) | 53 (46, 60) |
| <40 years | 68 (14) | 53 (11) | 55 (11) | 61 (13) |
| 40-49 years | 116 (24) | 112 (23) | 124 (25) | 110 (23) |
| 50-59 years | 192 (40) | 213 (43) | 206 (42) | 191 (40) |
| ≥60 years | 104 (22) | 112 (23) | 107 (22) | 121 (25) |
| Ethnicity | | | | |
| Han | 463 (97) | 467 (96) | 471 (97) | 460 (96) |
| Other | 15 (3) | 21 (4) | 16 (3) | 20 (4) |
| Current or ex-smokers | 112 (23) | 90 (18) | 122 (25) | 112 (23) |
| BMI ($kg/m^2$) | 26 (24, 28) | 26 (24, 28) | 26 (24, 28) | 26 (24, 28) |
| Normal: BMI < 25 | 192 (40) | 183 (37) | 193 (39) | 177 (37) |
| Overweight: 25 ≤ BMI < 30 | 224 (47) | 242 (49) | 240 (49) | 253 (52) |
| Obese: BMI ≥ 30 | 63 (13) | 65 (13) | 59 (12) | 53 (11) |
| Waist-hip ratio | 0.89 (0.85, 0.94) | 0.89 (0.84, 0.92) | 0.89 (0.85, 0.93) | 0.89 (0.85, 0.93) |
| Systolic blood pressure (mmHg) | 120 (110, 130) | 120 (110, 130) | 120 (113, 130) | 120 (110, 130) |
| Diastolic blood pressure (mmHg) | 79 (70, 81) | 77 (70, 80) | 78 (70, 82) | 78 (70, 80) |
| Fasting plasma glucose (mmol/L) | 6.0 (5.4, 6.4) | 6.0 (5.5, 6.4) | 6.0 (5.5, 6.4) | 6.0 (5.5, 6.4) |
| 2-hour plasma glucose (mmol/L) | 8.8 (8.1, 9.9) | 9.0 (8.1, 9.9) | 8.9 (8.1, 9.8) | 8.9 (8.0, 9.9) |
| HbA1c (%) | 5.8 (5.6, 6.0) | 5.8 (5.5, 6.1) | 5.8 (5.6, 6.1) | 5.8 (5.6, 6.0) |
| Prediabetes state | | | | |
| IGT | 408 (85) | 424 (87) | 413 (84) | 409 (85) |
| Isolated IFG | 72 (15) | 66 (13) | 79 (16) | 74 (15) |
| Total cholesterol (mmol/L) | 4.8 (4.2, 5.6) | 4.9 (4.2, 5.4) | 4.9 (4.3, 5.5) | 4.9 (4.3, 5.5) |
| LDL-C (mmol/L) | 3.2 (2.6, 3.8) | 3.1 (2.6, 3.7) | 3.2 (2.7, 3.7) | 3.2 (2.7, 3.7) |
| HDL-C (mmol/L) | 1.2 (1.0, 1.4) | 1.2 (1.0, 1.4) | 1.2 (1.0, 1.4) | 1.2 (1.0, 1.4) |
| Triglyceride (mmol/L) | 1.5 (1.1, 2.0) | 1.5 (1.1, 2.0) | 1.5 (1.1, 2.1) | 1.5 (1.1, 2.1) |
| ALT (U/L) | 22 (16, 31) | 20 (15, 28) | 21 (16, 30) | 21 (16, 31) |
| AST (U/L) | 22 (19, 26) | 21 (17, 26) | 22 (18, 27) | 21 (17, 27) |
| Hemoglobin (g/L) | 143 (134, 153) | 141 (132, 151) | 143 (134, 153) | 141 (132, 153) |
| HOMA-IR | 2.4 (1.6, 3.5) | 2.4 (1.7, 3.6) | 2.4 (1.6, 3.4) | 2.5 (1.6, 3.5) |
| HOMA-$\beta$ | 77.3 (51.4, 109.6) | 78.0 (51.5, 119.5) | 78.1 (50.0, 115.8) | 81.9 (52.8, 112.4) |
| Cytokines | | | | |
| CRP ($\mu$mol/L) | 1.1 (0.6, 2.3) | 1.1 (0.7, 2.4) | 1.2 (0.7, 2.4) | 1.3 (0.7, 2.4) |
| SOD | 6.9 (2.0, 10.5) | 6.8 (4.2, 10.0) | 6.7 (4.0, 10.2) | 7.0 (2.2, 11.1) |
| Amylin | 7.6 (6.4, 9.5) | 7.4 (6.4, 9.2) | 7.7 (6.6, 9.5) | 7.6 (6.4, 9.5) |
| IL-6 | 2.3 (1.5, 4.3) | 2.3 (1.5, 4.0) | 2.4 (1.5, 4.2) | 2.3 (1.5, 3.6) |
| Urine albumin/Cr (mg/g) | 6.9 (4.3, 14.5) | 7.5 (4.5, 14.5) | 7.9 (4.6, 15.3) | 7.3 (4.4, 13.9) |
| Daily calorie intake (kcal/day) | 1503 (1222, 1841) | 1548 (1263, 1909) | 1535 (1278, 1929) | 1564 (1233, 1931) |
| Proportion of total calorie intake from carbohydrate (%) | 61 (51, 69) | 59 (49, 66) | 60 (52, 68) | 59 (50, 67) |
| Proportion of total calorie intake from protein (%) | 14 (12, 17) | 14 (12, 17) | 14 (12, 16) | 14 (12, 17) |
| Proportion of total calorie intake from fat (%) | 23 (16, 30) | 24 (19, 31) | 24 (17, 31) | 24 (17, 31) |
| Physical activity | | | | |
| Low | 109 (23) | 116 (25) | 123 (26) | 108 (23) |

Table 1: Continued.

| | Conventional lifestyle +placebo | Intensive lifestyle +placebo | Conventional lifestyle +pioglitazone | Intensive lifestyle +pioglitazone |
|---|---|---|---|---|
| Medium | 244 (53) | 255 (54) | 239 (50) | 246 (53) |
| High | 111 (24) | 99 (21) | 112 (24) | 114 (24) |

Table 2: Proportions of individual developing diabetes and reverting back to normoglycemic state at 1, 2, and 3 years of follow-up, by treatment groups and the odds ratio (95% CI) of the likelihood of reverting to normoglycemic state and developing diabetes during 3 years of follow-up in the three treatment groups, compared to the conventional+placebo group.

| | Conventional lifestyle +placebo | Intensive lifestyle +placebo | Conventional lifestyle +pioglitazone | Intensive lifestyle +pioglitazone |
|---|---|---|---|---|
| Year 1 | | | | |
| *N* | 480 | 490 | 492 | 483 |
| Lost to follow-up | 88 (18.3) | 92 (18.8) | 95 (19.3) | 91 (18.8) |
| DM | 54 (11.3) | 57 (11.6) | 48 (9.8) | 36 (7.5) |
| Normal | 185 (38.5) | 151 (30.8) | 188 (38.2) | 205 (42.4) |
| Year 2 | | | | |
| *N* | 153 | 190 | 161 | 151 |
| Lost to follow-up | 51 (33.3) | 70 (36.8) | 68 (42.2) | 43 (28.5) |
| DM | 20 (13.1) | 15 (7.9) | 15 (9.3) | 16 (10.6) |
| Normal | 41 (26.8) | 38 (20.0) | 32 (19.9) | 44 (29.1) |
| Year 3 | | | | |
| *N* | 41 | 67 | 46 | 48 |
| Lost to follow-up | 18 (43.9) | 31 (46.3) | 18 (39.1) | 25 (52.1) |
| DM | 1 (2.4) | 8 (11.9) | 9 (19.6) | 2 (4.2) |
| Normal | 10 (24.4) | 11 (16.4) | 3 (6.5) | 6 (12.5) |
| Overall | | | | |
| Normal | 236 (60.0) | 200 (50.3) | 224 (56.6) | 255 (65.1) |
| SHR (95% CI) | Ref | 0.61 (0.41, 0.93) | 0.82 (0.55, 1.22) | 1.15 (0.66, 1.57) |
| p | | 0.020 | 1.00 | 0.76 |
| DM | 76 (11.63) | 80 (16.3) | 72 (14.4) | 54 (11.2) |
| SHR (95% CI) | Ref | 1.00 (0.61, 1.57) | 0.83 (0.47, 1.36) | 0.55 (0.32, 0.90) |
| *p* | | 0.86 | 0.26 | 0.024 |

## 4. Discussion

BPRP is the first study to evaluate whether lifestyle intervention and/or pioglitazone could revert prediabetic state back to normoglycemia in Chinese population.

In our study, the reversion rates to normal glucose state were similar among all four groups. This is inconsistent with most of the previous publications. In the most previous prevention study, there always had a benefit from either intensive lifestyle intervention or a certain kind of antidiabetic drugs. Since the primary endpoint was the prevention of diabetes in almost all the studies, there were only a few studies that had reported the reversion rate of normal glucose state. DPP study was one of the most famous diabetes prevention study with lifestyle intervention. After the 3 years' follow-up, the reversion rate in the control group and lifestyle intervention group were around 25 percent and 35 percent, respectively [14], while the overall reversion rate in our study ranged from 50 percent to 65 percent, which was even much higher than that in the intervention group in DPP study. If we take a look at the data in the ACT NOW study, which was also a diabetes prevention study with pioglitazone [12], the conversion to normal glucose tolerance occurred in 48% of the patients in the pioglitazone group and 28% of those in the placebo group. The overall conversion rate in any one of the groups in our study was higher than that reported in the ACT NOW study.

When comparing the incidence of diabetes from our study with other studies, we can see that the incidence of diabetes in the control group in our study is much lower

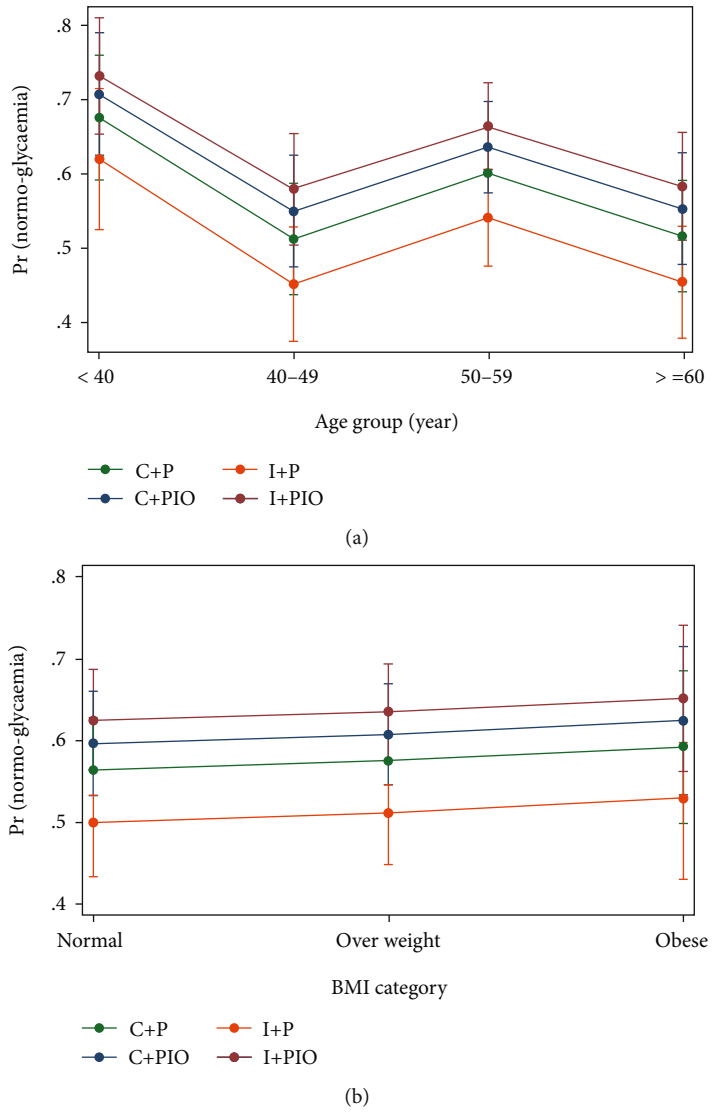


Figure 2: Estimated probability of reaching normoglycemia during three years of follow-up by the treatment group: (a) separately for different age groups at baseline and (b) separately for different BMI categories at baseline.

than that shown in the control group in the previous study. The incidence of diabetes in our study ranged from 11.18 percent to 16.33 percent among all four groups. DPP study was one of the most famous diabetes prevention study. In that study, there were 28.9% in the placebo group and 14.4% in the lifestyle intervention group developed diabetes at three years. If we look at the DPS study, which had a similar follow-up duration with our study, we also found that after the mean 3.2 years follow-up, the cumulative incidence of diabetes was 11 percent in the intervention group, which was similar with our study and 23 percent in the control group, which was much higher than our study [20].

Therefore, we believe that the most possible reason for our negative result is because the conversion rate to normal glucose state in the control group in our study is relatively high. When we designed this study, we designed an exact same follow-up plan for both the conventional lifestyle intervention group and the intensive lifestyle intervention group. This may lead to a minimized difference between the effects on the change of the glucose level from conventional or intensive lifestyle intervention group. Besides this, nowadays, there is also more and more educational information from public media that the participants may receive. So, the participants in our study were totally different with those who were recruited in the Da Qing study twenty years ago.

From the baseline data, we can also see that the BMI of the participants in our study was far lower that shown in many diabetes prevention studies in the Western country. The mean BMI of the participants in our study was only 26 $kg/m^2$, and nearly 40% of the participants had a normal BMI. Therefore, lifestyle intervention may not have as much effect as it does for those who were very obese. However, if we look at the change of the body weight at the end of the study, we can still see that there was a 2.21 kg decrease of the body weight in the conventional lifestyle intervention plus placebo group. This may be another possible explanation for the negative result of the study.

Table 3: Mean change (95% CI) or median change (95% CI of median) * in the levels of secondary study parameters at 3 years of follow-up in the 4 treatment groups. The analysis is based on intention-to-treat analysis.

| | Conventional lifestyle +placebo | Intensive lifestyle +placebo | Conventional lifestyle +pioglitazone | Intensive lifestyle +pioglitazone |
|---|---|---|---|---|
| *N* | 23 | 37 | 28 | 25 |
| FPG change | -0.20 (-0.05, 0.10) | 0.04 (-0.20, 0.28) | 0.08 (-0.20, 0.35) | 0.05 (-0.24, 0.34) |
| *N* | 23 | 37 | 28 | 25 |
| PPG change | -1.05 (-2.0, -0.12) | -0.05 (-0.78, 0.69) | 0.83 (-0.22, 1.67) | -0.92 (-0.81, -0.62) |
| *N* | 27 | 43 | 32 | 30 |
| HbA1c change | 0.30 (0.14, 0.46) | 0.21 (0.09, 0.34) | 0.20 (0.05, 0.34) | 0.20 (0.05, 0.35) |
| *N* | 24 | 39 | 28 | 24 |
| Weight change | -2.21 (-3.86, -0.56) | -1.06 (-2.40, 0.22) | -0.37 (-0.21, 0.94) | -1.35 (-3.00, 0.29) |
| *N* | 24 | 39 | 27 | 24 |
| Waist change | -3.74 (-6.14, -1.33) | -0.95 (-2.83, 0.94) | -1.05 (-3.31, 1.21) | -2.45 (-4.56, -0.05) |
| *N* | 24 | 39 | 28 | 24 |
| SBP change | -2 (-8, -3) | -3 (-7, 1) | 0.3 (-5, 5) | -3 (-9, 2) |
| *N* | 27 | 43 | 32 | 30 |
| LDL change | 0.29 (-0.01, 0.60) | 0.12 (-0.12, 0.36) | 0.11 (-0.17, 0.38) | 0.19 (-0.10, 0.47) |
| *N* | 27 | 43 | 32 | 30 |
| HDL change | 0.12 (0.04, 0.21) | 0.17 (0.09, 0.24) | 0.09 (-0.01, 0.17) | 0.24 (0.16, 0.33) |
| *N* | 27 | 43 | 32 | 30 |
| Triglyceride change* | -0.12 (-0.41, 0.13) | -0.17 (-0.42, 0.20) | -0.03 (-0.28, 0.14) | -0.40 (-0.62, -0.13) |
| *N* | 27 | 43 | 32 | 30 |
| hsCRP change* | -0.10 (-0.34, 0.40) | -0.20 (-0.53, -0.02) | -0.08 (-0.40, 0.27) | -0.10 (-0.40, 0.19) |
| *N* | 33 | 53 | 36 | 39 |
| C-peptide change* | -0.50 (-0.67, -0.31) | -0.47 (-0.55, -0.37) | -0.31 (-0.72, -0.25) | -0.65 (-0.78, -0.31) |
| *N* | 27 | 43 | 31 | 30 |
| Urine ACR change* | -2.11 (-12.27, -0.99) | -1.74 (-3.12, 0.32) | -0.07 (-3.74, 4.70) | -4.00 (-7.56, -1.24) |

There are some limitations of our study. First of all, compliance to the diet intervention in the intensive lifestyle intervention group was relatively poor. However, we had a mix of secondary- and tertiary-level hospitals, with likely differences in the levels of engagement and experience among study nurses and clinicians. Secondly, the dropout rate was 5% higher than assumed, although still maintaining the statistical power to 85%. The intensity of the lifestyle intervention and the resource for the investigator were much less compared to the previous studies. This may lead to the higher dropout rate and nonideal compliance. However, these limitations are unlikely to adversely affect the robustness of the study results. Our study should be considered a pragmatic clinical trial, reflecting the population level scenario in terms of adherence to medication and other lifestyle interventions. Therefore, the findings of this study may provide more evidence-based information to support our daily practice. Lastly, the participants in our study were all from Beijing area and almost all of them were of Han ethnicity. Therefore, from our study, it would be difficult to evaluate how the regional habit and ethnical habit might affect the glucose outcome among patients with prediabetes.

Recently, a paper named "Dubious Diagnosis" was published in Science [21], arguing that a war on 'prediabetes' has created millions of new patients and a tempting opportunity for pharmaceutical companies and questioned the resource allocation principle for the effective management of prediabetes. Our study answered some of important global questions in relation to management of prediabetes. Clearly, while a significant number of people revert back to normoglycemia from prediabetes with or without active intervention (s), there is also a large high-risk prediabetes population to a focus on.

## 5. Conclusion

BPRP was the first study to determine if lifestyle modification and/or pioglitazone could revert prediabetic state to normoglycemia in Chinese population. There was not any significant increase of the reversion rate from both intervention strategies. However, this makes us reconsider the prevention policy nowadays in China. We may need some more feasible method to manage prediabetes in the developing country such as China in this era of information explosion.

## Abbreviations

ACR: Albumin/creatinine
ADD: Antidiabetes drug

BMI: Body mass index
BPRP: Beijing Prediabetes Reversion Program
CRP: C-reactive protein
CVD: Cardiovascular disease
DPP: Diabetes Prevention Program
DPS: Finnish Diabetes Prevention Study
HbA1c: Hemoglobin A1c
HDL-C: HDL-cholesterol
HOMA-beta: Homeostatic model assessment for beta cell function
HOMA-IR: Homeostatic model assessment for insulin resistance
IFG: Impaired fasting glucose
IGT: Impaired glucose tolerance
IL-6: Interleukin-6
LDL-C: LDL-cholesterol
OGTT: Oral glucose tolerance test
PG: Plasma glucose
SOD: Superoxide dismutase
TC: Total cholesterol
TG: Triglyceride
TZD: Thiazolidinedione.

## Data Availability

The data used to support the findings of this study may be accessed on request through the BPRP study group by contacting at the corresponding author of this manuscript.

## Additional Points

*Study Sites and Principle Investigator of Each Site*. Beijing Tongren Hospital: Jingkui Yang. Beijing Hospital: Lixin Guo. The Military General Hospital of Beijing PLA: Xiaofeng Lv. Peking University First Hospital: Xiaohui Guo. Peking Union Medical Colledge Hospital: Hongding Xiang. Fuxing Hospital: Xiaoming Zhuang. Beijing No.6 Hospital: Shangnong Wang. The Second Artillery General Hospital of Chinese People's Liberation Army: Quanmin Li. Beijing Zhanlanlu Hospital: Shuling Chang. Peking University Third Hospital: Tianpei Hong. Chinese PLA General Hospital: Juming Lu. The 304th Hospital: Shinan Yin. The 309th Hospital of Chinese People's Liberation Army: Yan Zhang. Navy General Hospital: Qiyu Guo. Air Force General Hospital: Xiaohong Guan. Beijing Haidian Hospital: Wei Huang. China-Japan Friendship Hospital: Guangwei Li. The 306th Hospital of PLA: Zhangrong Xu. Beijing Chaoyang Hospital: Yuan Xu. Beijing Chuiyangliu Hospital: Cuiping Liu. China Meitan General Hospital: Hongmei Li. Civil Aviation General Hospital: Dingqiong Peng. Xuanwu Hospital: Li Wang. Beijing Tiantan Hospital: Lirong Zhong. Aerospace 731 Hospital: Yuming Liu. Beijing Electric Power Hospital: Dongmei Ni. The Luhe Teaching Hospital of the Capital Medical University: Dong Zhao. People's Hospital of Beijing Daxing District: Changchun Xue. Beijing Pinggu Hospital: Yufeng Li. Daxing Hospital of Tranditional Chinese Medicine: Li Ma. Beijing Chaoyang Hospital (Jingxi Campus): Shan Gao. Beijing Hepingli Hospital: Yaping Liu. Peking University Shougang Hospital: Xiaoping Lu. Nanyuan Hospital: Kaijie Yang. Beijiao Hospital: Z. Jing. Nanfaxin Hospital: Rongmin Zhang.

## Ethical Approval

Approval of protocol and consent forms by the local institutional review board was obtained at Peking University Health Science Center.

## Consent

Informed consent forms were obtained before the individuals could participate in any screening procedures.

## Disclosure

This funding source had no role in the design of this study and would not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

## Conflicts of Interest

Sanjoy K Paul has acted as a consultant and speaker for Sanofi, AstraZeneca, Novartis, GI Dynamics and Amylin Pharmaceuticals LLC. He has received grants in support for investigator and investigator-initiated clinical studies from Merck, Novo Nordisk, Hospira, AstraZeneca, Amylin Pharmaceuticals, and Pfizer. All the other authors declare that there is no conflict of interest regarding the publication of this paper.

## Authors' Contributions

Linong Ji, Hongyuan Wang, Yingying Luo, Xianghai Zhou, Cuiqing Chang, and Wei Chen were responsible for the conceptualization and design of the trial. Yingying Luo, Xianghai Zhou, Xiaohui Guo, and Jinkui Yang were involved in data acquisition. Sanjoy K Paul performed the analysis and interpretation of the data. Y.Y.Luo, Sanjoy K Paul, Xianghai Zhou, and L.N.Ji drafted the manuscript. Cuiqing Chang, Wei Chen, Xiaohui Guo, Jinkui Yang, and Hongyuan Wang revised the manuscript for critical intellectual content. All authors approved the final the final version of the manuscript. Yingying Luo, Sanjoy K Paul, Hongyuan Wang and Linong Ji had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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## Supplementary Materials

*Supplementary 1*. Supplement Figure 1: compliance of the lifestyle intervention in intensive lifestyle intervention groups.

*Supplementary 2*. Supplement Table 1: adverse events which were considered maybe relevant to treatment by investigators among four groups.

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*Research Article*

# Influence of Fasting Plasma Glucose Level on Admission of COVID-19 Patients: A Retrospective Study

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*Background*. The coronavirus disease 2019 (COVID-19) is a serious global health threat and has spread dramatically worldwide. Prolonged viral shedding is associated with a more severe disease course and inflammatory reaction. Blood glucose levels were significantly associated with an increased hazard ratio (HR) for poor outcomes in COVID-19 patients. *Objective*. Previous studies focused primarily on the relationship between blood glucose and mortality or severe outcomes, but there were few research studies on the relationship between fasting plasma glucose (FPG) and duration of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA positive status. To explore the relationship between FPG levels and prolonged duration of SARS-CoV-2 viral positivity, the clinical data of COVID-19 patients were analyzed. *Method*. In this retrospective study, 99 cases of COVID-19 patients in Beijing Ditan Hospital were recruited, and their clinical and laboratory findings at admission were collected and analyzed. Furthermore, the risk factors for prolonged duration of SARS-CoV-2 RNA shedding were identified, and the relationship between FPG levels and the prolonged presence of SARS-CoV-2 RNA was evaluated. *Result*. We found that elevated FPG levels were correlated with longer duration of SARS-CoV-2 RNA positivity, classification of COVID-19, imaging changes of chest CT, inflammation-related biomarkers, and $CD8^+$ T cell number in COVID-19 patients. In a logistic regression model, after adjusting for gender and age, COVID-19 patients with elevated FPG were more likely to had longer duration of SARS-CoV-2 RNA positivity than those with normal FPG levels (OR 3.053 [95% CI 1.343, 6.936]). *Conclusion*. Higher FPG levels (≥6.1 mmol/l) at admission was an independent predictor for prolonged SARS-CoV-2 shedding, regardless of a known history of diabetes. It suggests that intensive monitoring and control of blood glucose are important for all COVID-19 patients.

## 1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, which was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, is a novel and serious global health threat and has dramatically spread worldwide [1]. COVID-19 is transmitted primarily through respiratory droplet and direct contact. At the time of this article's drafting, 188,616,093 confirmed cases and 4,065,804 deaths have been reported worldwide with new confirmed cases and deaths occurring per day [2].

Diabetes mellitus (DM) impacted outcomes of COVID-19 patients. A retrospective, single-center study in Iran showed that COVID-19 patients with DM had more comorbidities such as hypertension and complications than those without diabetes. Treatment failure and death were significantly higher in COVID-19 patients with diabetes compared to those without diabetes [3]. Serum levels of inflammation-related biomarkers such as IL-6, C-reactive protein, serum ferritin and coagulation index, and D-dimer were significantly higher in COVID-19 patients with DM compared with those without DM [4].

Fasting plasma glucose (FPG) level ≥ 7.0 mmol/l is one of the important criteria for the diagnosis of DM [5]. Regardless of a known history of DM, higher FPG levels significantly predicted mortality of COVID-19 ($P < 0.05$).

Among nondiabetic COVID-19 patients, higher FPG remained a significant predictor of mortality [6, 7]. COVID-19 patients with an admission glucose level of >11 mmol/L were more likely to require intensive care unit (ICU), to develop acute respiratory distress syndrome (ARDS) and acute cardiac injury, and they had a higher death rate than patients with an admission glucose level of ≤11 mmol/L. In the multivariable analysis, COVID-19 patients with admission glucose level of >11 mmol/L had an increased risk of death and in-hospital complications, respectively [8].

Combined with some research results mentioned above, both persistent positivity of SARS-CoV-2 RNA and FPG affected outcomes of COVID-19 patients. Currently, there lacks research on the relationship between FPG and persistent positivity of SARS-CoV-2 RNA. We retrospectively analyzed the clinical data of COVID-19 patients in our hospital and further evaluated the relationship between FPG level and duration of SARS-CoV-2 RNA viral shedding/clearance. We tried to find the relevant factors affecting virus clearance and the possible factors that may influence the continuous positive status of SARS-CoV-2 RNA. These results will offer valuable information for the early control of COVID-19 in the real world and help reduce the social burden.

## 2. Materials and Methods

*2.1. Study Design and Participants.* All COVID-19 patients consecutively admitted to the hospital between June 1, 2020, and July 31, 2020, were collected. The diagnosis and clinical classification (mild, moderate, severe, and critical) of COVID-19 patients were carried out by two independent doctors based on the Guideline of Novel Coronavirus Pneumonia (8 th revised Edition) issued by the Chinese National Health Commission [6], mainly according to the criteria as follows: (1) patients with epidemiological history of novel coronavirus pneumonia (NCP), in contact with novel coronavirus infected people within 14 days prior to the onset of the disease; (2) any 2 of the clinical manifestations such as (i) fever and/or respiratory symptoms; (ii) the aforementioned imaging characteristics of NCP; (3) normal or decreased white blood cell count (WBC), and lymphocyte count in the early stage of onset; (4) positive real-time reverse transcription polymerase chain reaction (RT-PCR) results or highly homologous of viral gene sequence to known new coronaviruses [9].

Exclusion criterions included (1) age < 18 years, (2) pregnant women, (3) duplicated cases, (4) nonavailable or incomplete demographic or clinical data, (5) malignant tumor status, and (6) no FPG data available at admission (for example: FPG detected before admission or 24 h after admission, or routine blood glucose tests not being detected for each COVID-19 patient) (Figure 1).

All subjects were divided into 3 groups according to the FPG value at admission: group 1 (patients with FPG < 6.1 mmol/l), group 2 (patients with FPG 6.1 – 6.9 mmol/l), and group 3 (patients with FPG > 6.9 mmol/l).

The definitions and descriptions of some outcomes are as follows:

(1) *Prolonged Viral RNA Shedding of SARS-CoV-2*. The negative conversion time (NCT) of SARS-CoV-2 RNA or the duration of SARS-CoV-2 RNA shedding was greater than the mean duration in the study

(2) *Repositive or Recurrence of SARS-CoV-2*. SARS-CoV-2 nucleic acid was redetected in discharged patients.

*2.2. Data Collection.* FPG levels were measured at admission. For the test of FPG levels, blood samples were collected after an overnight fast lasting at least 8 h within 24 h after admission. The normal reference range of FPG in Beijing Ditan Hospital of Beijing is 3.9-6.1 mmol/L. To confirm SARS-CoV-2 infection, the real-time RT-PCR assay was used for detecting upper respiratory specimens (nasopharyngeal and oropharyngeal swabs), with or without a lower respiratory specimen (sputum). Nasopharyngeal swabs were collected on average every 3 to 7 days (serial time points).

We also obtained clinical, other laboratory, radiological, treatment, and outcome data from patients' electronic medical records for hospitalized patients. Past medical histories were obtained from hospital databases or through self-reporting, including type 2 diabetes mellitus (T2DM), hypertension, chronic lung disease, chronic heart disease, chronic liver disease, chronic kidney disease, cerebrovascular disease, and carcinoma, which were diagnosed according to standard criteria. The common complications that developed after hospitalization included acute respiratory distress syndrome (ARDS), acute cardiac injury, acute kidney injury, acute liver injury, cerebrovascular accident, coagulopathy, and secondary infection.

*2.3. Statistical Analysis.* All the patients completed the specimens collection and lung computed tomography (CT) scan within 48 hours after admission to ensure their diagnosis and classification of COVID-19. Continuous data were expressed as mean ± standard deviation (SD) or median (interquartile ranger, IQR). Categorical data were expressed as counts and proportions. The Kruskal-Wallis $H$ test or Mann–Whitney $U$ test was used to compare the differences between groups. Categorical variables were compared using the Chi-square ($\chi^2$) test or Fisher's exact test (if more than 20% of the cells had an expected count < 5), if appropriate. The data was analyzed through Spearman's bivariate correlations to evaluate the covariation between the duration of SARS-CoV-2RNA positive and FPG level or other indicators. Significant risk factors identified on univariate analyses were further analyzed by the multivariable logistic regression analysis to identify the independent risk factors associated with the prolonged duration of SARS-CoV-2 shedding, with adjustment for age, gender, and other potential confounding factors. Data were analyzed using SPSS 26.0 (IBM, Chicago, IL). For all the statistical analyses, $P < 0.05$ was considered significant.

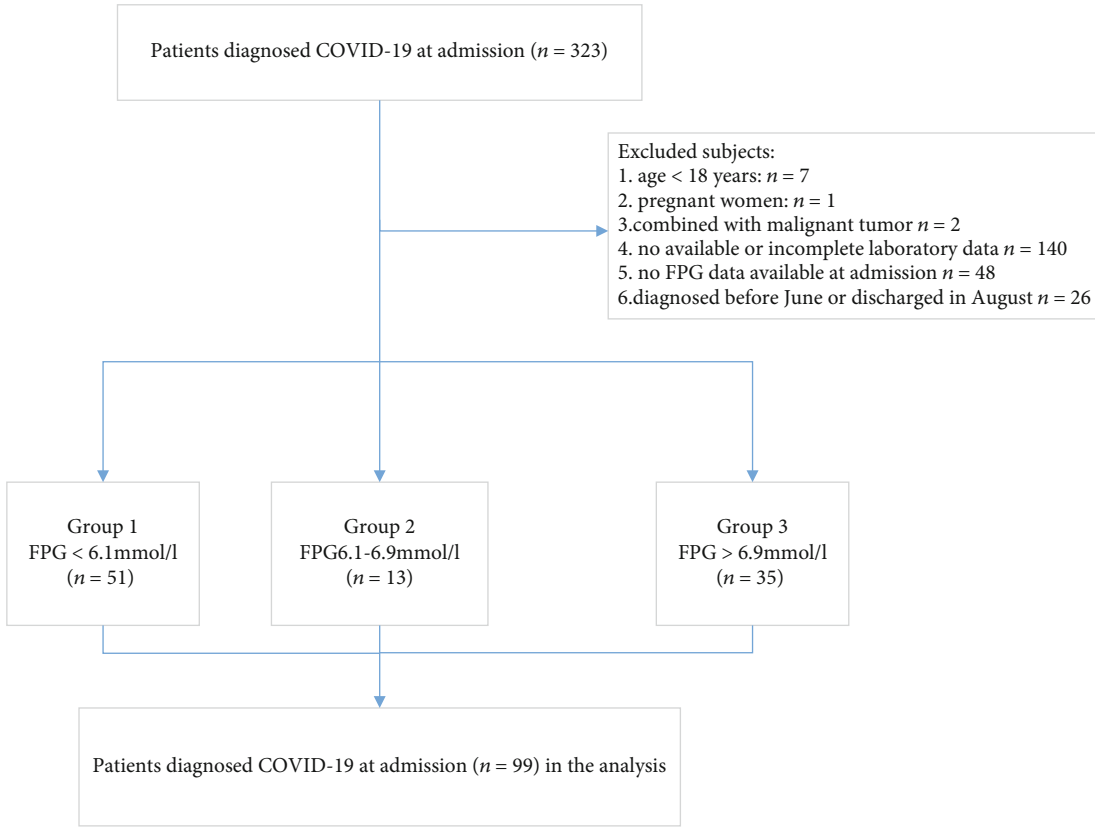


Figure 1: Flow diagram of patient selection.

## 3. Results

*3.1. Characteristics of COVID-19 Patients at Admission.* Among 323 diagnosed COVID-19 patients from June to July 2020, some patients were excluded for age < 18 years ($n = 7$), pregnant women ($n = 1$), combined with malignant tumor ($n = 2$), no available or incomplete laboratory data ($n = 140$), no FPG data available at admission ($n = 48$), and patients diagnosed before June or discharged in August ($n = 26$) were excluded. Finally, 99 cases were included in the study (Figure 1).

The mean age of the patients was $45.84 \pm 12.87$ years, and 54 patients (54.5%) were male. Forty-eight patients (48.5%) had FPG equal or higher than 6.1 mmol/L, and 51 patients (51.5%) had FPG < 6.1 mmol/L. Older patients were more prone to have elevated FPG ($P < 0.05$).

Among these patients, the most common symptom at onset of illness was fever [63 (63.6%)] (Table 1). Less common symptoms were cough (17 patients [17.2%]) and shortness of breath (11 patients [11.1%]). The proportions of sputum production ($n = 8$), muscle soreness ($n = 6$), and headache ($n = 3$) were 8.1%, 6.1%, and 3.0%. Of these patients, 15 patients (15.1%) had a history of diabetes. Other than diabetes, hypertension was the most common comorbidity (20patients [20.2%]) followed by chronic liver disease [6 patients (6.1%)]. The proportions of chronic lung disease, hyperlipidemia, and hyperuricemia were 5.1% (5 patients), 5.1% (5 patients), and 4% (4 patients), respectively.

The mean duration of SARS-CoV-2 RNA detection was $26.01 \pm 6.71$ days (Table 1). The shortest duration was 14 days, and the longest duration was 42 days. Those patients with higher FPG levels were also with a longer duration of viral persistence. There was a significant difference in the prolongation rate between the elevated FPG group and the normal FPG group ($\chi^2 = 7.292$, $P = 0.007$).

The proportions of SARS-CoV-2 RNA repositive in the three groups were not significantly different ($P > 0.05$) (Table 1). Recurrence of SARS-CoV-2 was correlated with past history of hypertension ($r = 0.214$, $P = 0.033$) (data not shown).

After stratification, the FPG levels in mild cases, moderate cases, and severe or critical cases were different ($P < 0.05$). Compared to mild cases, FPG levels were higher in moderate cases and severe or critical cases ($P < 0.05$) (Table 1).

Lung condition was assessed by chest CT scan. CT findings of lung involvement were significantly different among 3 groups ($\chi^2 = 12.139$, $P = 0.009$). Indicating patients were also with a higher FPG level accompanied by more severe CT chest findings ($r = 0.296$, $P = 0.003$).

We evaluated the proportion of major complications such as acute ARDS, liver injury, and secondary infection. The results showed that the percentages of patients with secondary infection were different in the 3 groups ($\chi^2 = 10.486$, $P = 0.003$). Compared with patients FPG < 6.1 mmol/l at admission, fever was not significantly related to longer viral

Table 1: Characteristics of COVID-19 patients at admission.

| Characteristic | All ($n = 99$) | Group 1 ($n = 51$) | Group 2 ($n = 13$) | Group 3 ($n = 35$) | $P$ value |
|---|---|---|---|---|---|
| Age (years) | 45.84 ± 12.87 | 41.1 ± 11.97 | 48.69 ± 12.34 | 51.68 ± 11.87 | <0.001 |
| Gender $n$ (%) | | | | | 0.106 |
| Male | 54 (54.5) | 32 (32.3) | 4 (4.0) | 18 (18.2) | |
| Female | 45 (45.5) | 19 (19.2) | 9 (9.1) | 17 (17.2) | |
| DSRP days | 26.01 ± 6.71 | 24.37 ± 6.20 | 27.85 ± 5.83 | 27.71 ± 7.27 | 0.042 |
| PDVPS $n$ (%) | | | | | 0.026 |
| Yes | 46 (46.5) | 17 (17.2) | 8 (8.1) | 21 (21.2) | |
| No | 53 (53.5) | 34 (34.3) | 5 (5.1) | 14 (14.1) | |
| DCC $n$ (%) | | | | | 0.002 |
| Mild | 19 (19.2) | 17 (17.2) | 0 (0) | 2 (2.0) | |
| Moderate | 72 (72.7) | 32 (32.3) | 12 (12.1) | 28 (28.3) | |
| Severe/critical | 8 (8.1) | 2 (2.0) | 1 (1.0) | 5 (5.1) | |
| RPV $n$ (%) | | | | | 0.440 |
| Yes | 21 (21.2) | 12 (12.1) | 1 (1.0) | 8 (8.1) | |
| No | 78 (78.8) | 39 (39.4) | 12 (12.1) | 27 (27.3) | |
| Temperature $n$ (%) | | | | | 0.321 |
| <37.3 °C | 36 (36.4) | 22 (22.2) | 5 (5.1) | 9 (9.1) | |
| 37.3-38°C | 23 (23.2) | 14 (14.1) | 2 (2.0) | 7 (7.1) | |
| 38.1-39°C | 26 (26.3) | 11 (11.1) | 4 (4.0) | 11 (11.1) | |
| 39.1-41°C | 14 (14.1) | 4 (4.0) | 2 (2.0) | 8 (8.1) | |
| Complications $n$ (%) | | | | | 0.873 |
| Acute liver injury | 35 (35.4) | 16 (16.2) | 5 (5.1) | 14 (14.1) | 0.691 |
| Secondary infection | 8 (8.1) | 0 (0) | 2 (2.0) | 6 (6.1) | 0.003 |
| ARDS | 7 (7.1) | 1 (1.0) | 1 (1.0) | 5 (5.1) | 0.089 |
| T2DM $n$ (%) | 15 (15.2) | 0 (0) | 2 (2.0) | 13 (13.1) | <0.001 |
| Chest CT $n$ (%) | | | | | 0.009 |
| No sign of pneumonia | 6 (6.1) | 6 (6.1) | 0 (0) | 0 (0) | |
| Unilateral pneumonia | 21 (21.2) | 13 (13.1) | 5 (5.1) | 3 (3.0) | |
| Bilateral pneumonia | 72 (72.7) | 32 (32.3) | 8 (8.1) | 32 (32.3) | |

Note: group 1: FPG < 6.1 mmol/l; group 2: FPG 6.1 – 6.9 mmol/l; group 3: FPG > 6.9 mmol/l; Abbreviation: ∗RPV: repositive of virus, repositive of SARS-CoV-2 RNA; DSRP: duration of SARS-CoV-2 RNA positive; PDVPS: prolonged duration of SARS-CoV-2 RNA positive status; DCC: different classification of COVID-19. ∗$P$ values were calculated by Kruskal-Wallis $H$ test, Mann–Whitney $U$ test, $\chi^2$ test, or Fisher's exact test, as appropriate.

RNA conversion time or the proportion of SARS-CoV-2 RNA repositive ($P > 0.05$) (Table 1).

*3.2. Laboratory Findings.* The analysis of laboratory parameters at admission showed that the levels of serum amyloid A (SAA), hemoglobin (HB), C-reactive protein(CRP), $CD8^+$ T cell number, and erythrocyte sedimentation rate (ESR) were different among 3 groups (all $P < 0.05$) (Table 2). D-dimer was correlated with fever ($P < 0.01$). In addition, D-dimer was positively correlated with SAA ($r = 0.253$, $P = 0.011$), CRP ($r = 0.238$, $P = 0.017$), and ESR ($r = 0.297$, $P = 0.003$) (data not shown). While significantly negative correlations were showed between D-dimer and LYM ($r = -0.231$, $P = 0.021$), B cell ($r = -0.200$, $P = 0.047$), T cell ($r = -0.204$, $P = 0.043$), and $CD8^+$T cell ($r = -0.221$, $P = 0.028$) (data not shown).

*3.3. The Association between SARS-CoV-2 Related Indicators and Other Parameters at Admission.* Spearman's correlation analysis showed that PDVPS was positively correlated with levels of FPG ($r = 0.327$, $P = 0.001$), different degrees of elevated FPG ($r = 0.257$, $P = 0.010$), FPG ≥ ULN ($r = 0.271$, $P = 0.007$), D-dimer ($r = 0.241$, $P = 0.016$), and gender ($r = 0.207$, $P = 0.040$). And creatinine was negatively correlated with PDVPS ($r = -0.208$, $P = 0.038$).

Furthermore, levels of FPG ($r = 0.281$, $P = 0.005$), different degrees of elevated FPG ($r = 0.245$, $P = 0.015$), and FPG ≥ ULN ($r = 0.257$, $P = 0.010$) were positively correlated with DSRP. In addition to elevated FPG related indicators, D-dimer ($r = 0.215$, $P = 0.033$), creatinine ($r = -0.210$, $P = 0.037$), and gender ($r = 0.208$, $P = 0.039$) were also correlated with DSRP (Table 3).

T2DM was related to severity of COVID-19 ($r = 0.256$, $P = 0.011$), fever ($r = 0.212$, $P = 0.035$), secondary infection ($r = 0.288$, $P = 0.004$), and imaging changes of chest CT ($r = 0.257$, $P = 0.010$) (data not shown). While T2DM was not significantly related to prolonged viral RNA conversion time or the recurrence proportion of SARS-CoV-2 RNA ($P > 0.05$) (Table 3).

Table 2: Laboratory examination of COVID-19 patients at admission according to different FPG levels.

| | All ($n = 99$) (%) | Group 1 ($n = 51$) | Group 2 ($n = 13$) | Group 3 ($n = 35$) | $P$ value |
|---|---|---|---|---|---|
| FPG (mmol/l) | 5.96 (4.89, 7.35) | 4.95 (4.51, 5.29) | 6.34 (6.27, 6.56) | 8.03 (7.24, 9.65) | <0.001 |
| SAA (mg/l) | 21.8 (3.2, 75.95) | 11.4 (1.4, 38.7) | 44.1 (2.4, 137.8) | 49 (16.9, 141.15) | 0.003 |
| LAC (mmol/l) | 2.98 (2.44, 3.65) | 2.92 (2.27, 3.6) | 2.78 (2.25, 3.6) | 3.35 (2.52, 4.03) | 0.261 |
| BUN (mmol/l) | 4.54 ± 1.20 | 4.54 ± 1.07 | 4.16 ± 1.36 | 4.70 ± 1.33 | 0.387 |
| Crea ($\mu$mol/l) | 65.6 (56, 78.2) | 66.9 (59.3, 78.9) | 60.5 (52.4, 66.95) | 62.6 (53.4, 79.6) | 0.172 |
| URCA ($\mu$mol/l) | 317 (256, 382) | 343 (267, 417) | 286 (220.5, 379) | 303 (245, 358) | 0.155 |
| WBC ($\times 10^9$/l) | 5.12 ± 1.68 | 5.08 ± 1.63 | 5.30 ± 1.34 | 5.11 ± 1.89 | 0.916 |
| LYM ($\times 10^9$/l) | 1.49 (1.1, 1.9) | 1.63 (1.23, 2.09) | 1.19 (1.09, 1.88) | 1.37 (1, 1.82) | 0.120 |
| HB (g/l) | 144 (130, 153) | 148 (134, 160) | 128 (119.5, 138) | 143 (131, 149) | 0.001 |
| PLT ($\times 10^9$/l) | 193 (160, 235) | 196 (171, 223) | 212 (161.5, 251.5) | 170 (144, 248) | 0.337 |
| ALT (U/L) | 19.1 (13.6, 33.3) | 19.9 (13.6, 32.7) | 14.4 (11.5, 24.25) | 19.6 (15.6, 35) | 0.191 |
| AST (U/L) | 21.3 (17.1, 27.7) | 19.5 (16.4, 27) | 20.8 (17.7, 24.25) | 22.7 (17.9, 30.3) | 0.353 |
| CRP (mg/l) | 3.50 (0.8, 18.5) | 1.7 (0.4, 6) | 5 (1, 23.55) | 9.1 (1.1, 35.5) | 0.004 |
| B cell number (cells/$\mu$l) | 171.00 (129, 259) | 168 (142, 280) | 148 (106.5, 233) | 186 (122, 247) | 0.485 |
| NK cell number (cells/$\mu$l) | 217 (139, 313) | 235 (150, 327) | 228 (130.5, 311) | 188 (139, 281) | 0.379 |
| T cell number (cells/$\mu$l) | 1004 (655, 1399) | 1088 (846, 1497) | 822 (540, 1220) | 908 (612, 1314) | 0.050 |
| $CD4^+$T cell number (cells/$\mu$l) | 583 (382, 825) | 626 (493, 825) | 395 (354, 899) | 513 (373, 769) | 0.159 |
| $CD8^+$T cell number (cells/$\mu$l) | 348 (220, 469) | 396 (288, 483) | 221 (146.5, 434) | 290 (217, 420) | 0.034 |
| D-dimer (mg/l) | 0.27 (0.14, 0.45) | 0.25 (0.13, 0.42) | 0.29 (0.20, 0.53) | 0.3 (0.13, 0.45) | 0.614 |
| ESR (mm/h) | 15 (7, 24) | 10 (5, 19) | 15 (12, 24.5) | 20 (13, 40) | 0.001 |

Note: group 1: FPG < 6.1 mmol/l; group 2: FPG 6.1 – 6.9 mmol/l; group 3: FPG > 6.9 mmol/l; abbreviation: ∗ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; Crea: creatinine; UA: uric acid; LAC: lactic acid; SAA: serum amyloid A; ESR: erythrocyte sedimentation rate; WBC: white blood cell count; LYM: lymphocyte; NK cell: natural killer cell; Hb: hemoglobin; PLT: platelet; CRP: C-reactive protein. Data are mean, median (IQR), $n$ (%), or $n/N$ (%). ∗$P$ values were calculated by Kruskal-Wallis $H$ test, Mann–Whitney $U$ test, $\chi^2$ test, or Fisher's exact test, as appropriate.

Table 3: Spearman's correlation between PDVPS/DSRP and other parameters.

| Parameters | PDVPS | | DSRP | |
|---|---|---|---|---|
| | Correlation | $P$ value | Correlation | $P$ value |
| RPV | -0.038 | 0.712 | 0.034 | 0.740 |
| DCC | 0.159 | 0.117 | 0.021 | 0.840 |
| Degree of fever | 0.158 | 0.118 | 0.087 | 0.392 |
| Gender | 0.207 | 0.040 | 0.208 | 0.039 |
| T2DM history | 0.171 | 0.090 | 0.164 | 0.105 |
| FPG grades | 0.257 | 0.010 | 0.245 | 0.015 |
| FPG ≥ ULN | 0.271 | 0.007 | 0.257 | 0.010 |
| FPG mmol/l | 0.327 | 0.001 | 0.281 | 0.005 |
| Crea $\mu$mol/l | -0.208 | 0.038 | -0.210 | 0.037 |
| $CD8^+$ T cell number cells/$\mu$l | -0.239 | 0.017 | -0.191 | 0.058 |
| D-dimer | 0.241 | 0.016 | 0.215 | 0.033 |

Abbreviation: ∗PDVPS: prolonged duration of SARS-CoV-2 RNA positive status; ULN: upper limit of normal; RPV: repositive of virus, repositive of SARS-CoV-2 RNA; DCC: different classification of COVID-19; FPG grades: different groups of FPG levels, including <6.1 mmol/l, 6.1-6.9 mmol/l, and >6.9 mmol/l; Crea: creatinine.

The univariate logistic regression analysis suggested that creatinine (OR 0.972 [95% CI 0.946, 0.999]), gender (OR 2.326 [95% CI 1.036, 5.226]), FPG levels (OR 1.219 [95% CI 1.009, 1.474]), FPG ≥ 6.1 mmol/l (OR 3.053 [95% CI 1.343, 6.936]), and FPG grades (OR 1.756 [95% CI 1.124, 2.744]) were significantly associated with higher likelihood of PDVPS (Table 4). After adjusting for the gender, age, and diabetes history, the multivariable logistic regression analysis further suggested that FPG ≥ ULN (OR 3.053 [95% CI 1.343, 6.936]) was an independent predictor for PDVPS (Table 4). And the risk model was constructed as following: $\text{Logit}(P) = -0.693 + 1.116 \times (\text{FPG} \geq \text{ULN})$.

In addition, FPG was positively correlated with classification of COVID-19 ($r = 0.393$, $P < 0.001$), past DM history ($r = 0.498$, $P < 0.001$), SAA ($r = 0.432$, $P < 0.001$), CRP ($r = 0.378$, $P < 0.001$), ESR ($r = 0.440$, $P < 0.001$), and

Table 4: Bivariate logistic regression of the association between clinical parameters and PDVPS.

| Parameters | PDVPS OR (95% CI) | *P* value |
|---|---|---|
| FPG mmol/l | 1.219 (1.009, 1.474) | 0.041 |
| FPG ≥ ULN | 3.053 (1.343, 6.936) | 0.008 |
| FPG grades | 1.756 (1.124, 2.744) | 0.013 |
| Gender | | |
| Male | | |
| Female | 2.326 (1.036, 5.226) | 0.041 |
| Crea $\mu$mol/l | 0.972 (0.946, 0.999) | 0.041 |

Abbreviation: ∗PDVPS: prolonged duration of SARS-CoV-2 RNA positive status; ULN: upper limit of normal; FPG grades: different groups of FPG levels, including <6.1 mmol/l, 6.1-6.9 mmol/l, and >6.9 mmol/l; Crea: creatinine.

imaging changes of chest CT ($r = 0.318$, $P = 0.001$), while negatively correlated with $CD8^+$ T cell number ($r = -0.272$, $P = 0.006$) (Table 5).

Similar to FPG levels, it showed consistent correlation between elevated FPG and DCC ($r = 0.375$, $P < 0.001$), past DM history ($r = 0.436$, $P < 0.001$), SAA ($r = 0.332$, $P < 0.001$), CRP ($r = 0.326$, $P = 0.001$), ESR ($r = 0.366$, $P < 0.001$), imaging changes of chest CT ($r = 0.251$, $P = 0.012$), and $CD8^+$ T cell number ($r = -0.252$, $P = 0.012$) (Table 5).

## 4. Discussion

There have been few studies on the correlations between clinical features and the duration of SARS-CoV-2 RNA shedding. In our study, we evaluated the relationship between FPG level and PDVPS or DSRP, and identified factors that may affect the virus clearance and the prognosis of COVID-19.

We retrospectively analyzed the clinical data of COVID-19 patients in our hospital and further discussed the relationship between FPG levels and duration of SARS-CoV-2 RNA viral shedding or virus clearance. In our study, the mean duration of SARS-CoV-2 RNA viral shedding was $26.01 \pm 6.71$ days. In our cohort, patients with the longest viral shedding duration were 42 days. The range of duration SARS-CoV-2 RNA viral shedding was consistent with other studies, which ranged between 11 and 31 days [10].

The mean age of overall patients was $45.84 \pm 12.87$ years in our study. In this retrospective analysis, we found that DM patients with senior age and severe inflammatory characteristics of lung had increased incidences of comorbidities compared with those patients without diabetes. DM history was related to the severity of COVID-19 patients, fever, secondary infection, and imaging changes of chest CT (all $P < 0.05$). However, there was no significant relationship between preexisting diabetes or hypertension and the classification of COVID-19. Consistent with the study from Bennasrallah et al., diabetes and hypertension could not act as predisposing factors for late SARS-CoV-2 viral clearance [11]. In addition, T2DM was not significantly related to prolonged viral RNA conversion time or the proportion of RPV ($P > 0.05$). The possible reason was that although they had been diagnosed with diabetes before, their blood sugar levels were well controlled, which may be beneficial to themselves.

The Spearman's bivariate correlations showed that women were more likely to have long-term duration of SARS-CoV-2RNA positive. The univariate logistic regression analysis suggested gender was associated with a higher risk of PDVPS. Zhou et al. demonstrated that female sex was an independent predictor for prolonged SARS-CoV-2 RNA shedding [12]. While Xu et al. demonstrated that male sex was an independent predictor for prolonged SARS-CoV-2 RNA shedding [13]. Males and females might differ in immune reactivity [14]. However, these inconsistent results of the effect of gender on the duration of SARS-CoV-2 RNA shedding need to be further investigated in the follow-up research. Fu et al. found that D-dimer was related to the severity of COVID-19 [15]. However, we found that D-dimer was related to fever in this study. We also found that D-dimer was positively correlated with PDVPS and DSRP ($P < 0.05$). Moreover, D-dimer was positively related to inflammation-related biomarkers such as SAA, CRP, and ESR (all $P < 0.05$). Furthermore, D-dimer was negatively correlated to LYM, B cell, T cell, and $CD8^+$T cell (all $P < 0.05$ ). It suggested the potential mechanisms were that D-dimer affected inflammation and immune responses.

Creatinine level was negatively related to PDVPS ($P < 0.05$). The univariate logistic regression analysis showed that creatinine was also associated with an increased risk of PDVPS. Low serum creatinine (SCr) could reflect to low skeletal muscle mass or sarcopenia and poor nutritional status [16, 17]. The condition of malnutrition could result in weaker cellular mediated immunity and impaired surfactant production [18, 19]. Therefore, patients with low serum creatinine may increase the risk of the inflammatory reaction in lung tissues of COVID-19 patients, which is related to PDVPS.

Although there was a correlation between FPG level and diabetes history in the study population, higher level of FPG was positively related to prolonged duration of SARS-CoV-2 clearance. The univariate logistic regression analysis suggested that FPG levels or FPG≥ULN were significantly associated with higher risk of PDVPS. The multivariate logistic regression analysis further indicated that FPG≥ULN was an independent predictor for PDVPS. There are some mechanisms indicated that higher FPG might play a role in the viral clearance of COVID-19 patients. Since immunity is the first line of defense against SARS-CoV-2, it appears that the disturbed immunity in patients is due to hyperglycemia. Hyperglycemia inhibits neutrophil chemotaxis, decreases phagocytosis by neutrophils, macrophages, and monocytes, and impairs innate cell-mediated immunity [20]. In the present study, the levels of T cell number and $CD8^+$ T cell number were decreased. In addition, $CD8^+$ T cell number was negatively correlated with FPG levels ($P < 0.05$). Moreover, serum levels of inflammation-related biomarkers such as SAA, CRP, and ESR were significantly elevated among COVID-19 patients with higher FPG ($P < 0.05$). Furthermore, after controlling for possible confounders, elevated FPG levels were related to the seriousness of COVID-19,

Table 5: Spearman's correlation between FPG and other parameters.

| Parameters | FPG | | FPG ≥ ULN | |
|---|---|---|---|---|
| | Correlation | *P* value | Correlation (95% CI) | *P* value |
| DCC | 0.393 | <0.001 | 0.375 | <0.001 |
| Degree of fever | 0.252 | 0.012 | 0.224 | 0.026 |
| Chest CT | 0.318 | 0.001 | 0.251 | 0.012 |
| $CD8^+$ T cell number cell/$\mu$l | -0.272 | 0.006 | -0.252 | 0.012 |
| ESR mm/h | 0.440 | <0.001 | 0.366 | <0.001 |
| CRP mg/l | 0.378 | <0.001 | 0.326 | 0.001 |
| SAA mg/l | 0.432 | <0.001 | 0.332 | 0.001 |

Abbreviation: ∗ULN: upper limit of normal; DCC: different classification of COVID-19; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; SAA: serum amyloid A.

accompanied by more severe CT chest findings and inflammation. And the probable mechanisms are that elevated FPG levels increase susceptibility to inflammation due to impaired T cell response, neutrophil function, and humoral immunity [21].

Recently, Zhao et al. found COVID-19 patients with hypertension had higher risk of recurrent detection of viral RNA by RT-PCR [22]. In the current study, we found that hypertension was associated with repositive of SARS-CoV-2 RNA ($P < 0.05$). The underlying mechanisms might be that hypertension can increase the expression of angiotensin converting enzyme-2 (ACE2). As is known to us, human cells that express ACE2 and transmembrane serine protease 2 (TMPRSS2) receptors act as portal of entry via direct interaction of the human body and immune system. The primary site of infection in COVID-19 is the upper and lower respiratory tract. There, SARS-CoV-2 infects goblet secretory cells of the nasal mucosa and alveolar type II pneumocytes by binding to membrane-bound ACE2, and further strengthening virus entry [23].

There were some limitations of our study. First, the interpretation of our results might be limited by the sample size. Second, owing to the retrospective design of the study, the lack of data did not allow us to analyze the mean in-hospital FPG. Third, because the number of critical cases was relatively small, the research on FPG among severe or critical cases may be limited in this study.

## 5. Conclusion

We found that higher FPG was an independent predictor of prolonged duration of SARS-CoV-2 RNA shedding/clearance in the present study. Our findings indicate that screening FPG level is an effective and simple method to evaluate the prognosis of patients with COVID-19, and intervention should be taken in time when patients with FPG ≥ 6.1 mmol/l regardless of a history of diabetes.

## Data Availability

The clinical data used to support the findings of this study are included within the article.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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*Review Article*

# Potential Role of Natural Plant Medicine *Cyclocarya paliurus* in the Treatment of Type 2 Diabetes Mellitus

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Type 2 diabetes mellitus (T2DM) is a common chronic metabolic disease that has become increasingly prevalent worldwide. It poses a serious threat to human health and places a considerable burden on global social medical work. To meet the increasing demand for T2DM treatment, research on hypoglycemic drugs is rapidly developing. *Cyclocarya paliurus* (Batal.) Iljinskaja is a medicinal plant that grows in China. The leaves of *C. paliurus* contain polysaccharides, triterpenoids, and other chemical components, which have numerous health benefits. Therefore, the use of this plant has attracted extensive attention in the medical community. Over the past few decades, contemporary pharmacological studies on *C. paliurus* extracts have revealed that it has abundant biological activities. Multiple in vitro and in vivo experiments have shown that *C. paliurus* extracts are safe and can play a therapeutic role in T2DM through anti-inflammatory and antioxidation activities, and intestinal flora regulation. Its efficacy is closely related to many factors, such as extraction, separation, purification, and modification. Based on summarizing the existing extraction methods, this article further reviews the potential mechanism of *C. paliurus* extracts in T2DM treatment, and we aimed to provide a reference for future research on natural plant medicine for the prevention and treatment of T2DM and its related complications.

## 1. Introduction

The prevalence of diabetes has been increasing globally. According to the International Diabetes Federation, there were 463 million diabetic patients in 2019, and this number is expected to reach 578.4 million by 2030. Among the diabetic patients, about 90-95% is T2DM [1]. The shortage of medical resources, the difference in levels of technology, and the gap between the rich and the poor have brought challenges to disease control. Existing research on the mechanism to fully clarify the etiology of the disease is insufficient. Available evidence shows that insulin resistance (IR) and $\beta$-cell dysfunction are two major pathological characteristics of the disease [2]. As a result, patients often have elevated blood glucose levels due to insufficient insulin secretion or utilization. If blood glucose cannot be controlled in a timely and effective manner, it will lead to a series of life-threatening complications, such as renal failure, heart disease, amputation, and blindness [3].

In the past decades, the potential of traditional Chinese herbal medicines in diabetes treatment has gradually been recognized and accepted by the medical community. In contemporary pharmacological research, an increasing number of natural plants have been explored and applied for their medicinal value. The Juglandaceae plant *C. paliurus* is also known as a sweet tea tree because of its unique sweetness. This plant is primarily distributed in the subtropical plateau of southern China. According to the literature, the leaves, seeds, and bark of *C. paliurus* can be used medicinally. At present, *C. paliurus* leaves are generally accepted as health care products or medicines by local and medical communities [4], and dozens of compounds have been extracted from the plant. Previous research has confirmed that the plant has numerous biological activities, such as hypoglycemic,

antihypertensive, lipid-lowering, anticancer, antioxidant, antibacterial, hepatoprotective, and colon health [5–8]. Through further investigation of the extracts, researchers proved that the biological activity of plants was affected by geographical location, molecular content, and molecular structure [9, 10]. Furthermore, these compounds were affected by the method of extraction.

As a natural plant medicine, *C. paliurus* has a clear and sweet taste and can be easily consumed by the majority of patients. Moreover, this plant is relatively safe. There are no reports of hepatorenal toxicity or obvious side effects in cases where this plant is used in large doses [11–14]. The medicinal value of *C. paliurus* presents considerable potential in various diseases. Therefore, *C. paliurus* may also have great potential in the field of medicine.

Although there is substantial evidence that *C. paliurus* can alleviate diabetes in several ways, no study has systematically summarized the role of this natural plant in diabetes treatment. This paper reviews the literature on the extraction methods and therapeutic mechanisms of *C. paliurus*, and we aimed to provide new ideas and directions for the application of *C. paliurus* in T2DM and its related complications in future research.

## 2. Extraction of Effective Components from *C. paliurus*

To obtain the effective components of *C. paliurus*, various extraction methods should be adopted. These methods mainly include crude extraction, optimized extraction, separation, and purification. In addition, it has been confirmed that the structure of compounds can be further ameliorated by modification, and biological activities can be improved [15].

*2.1. Extraction and Optimized Extraction.* Existing studies have supported the use of *C. paliurus* leaves to explore its medicinal value. To date, the extraction of *C. paliurus* predominantly includes water extraction, ethanol extraction, ultrasonic-assisted extraction, and the hypoglycemic mechanism of polysaccharide microwave-assisted extraction. Among them, hot water and ethanol are the main methods used for preliminary crude extraction to obtain the effective components [16]. Nevertheless, there are certain limitations to these traditional approaches, such as time consumption, high-temperature requirements, and low extraction efficiencies. To optimize the extraction outcome, researchers have used ultrasonic and microwave-assisted methods to obtain more effective extracts. It has been confirmed that the *C. paliurus* polysaccharides after ultrasonic treatment have better scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals, indicating that ultrasonic treatment can promote the antioxidant activity of *C. paliurus* polysaccharides [17]. Another study reported that polysaccharides extracted by ultrasonic-assisted extraction have the advantages of being highly efficient, less solvent, and less time consuming and has the function of scavenging free radicals and inhibiting lipid peroxidation to a certain extent [8]. Xie et al. found that the microwave-assisted extraction method also has clear advantages in the extraction of *C. paliurus* polysaccharides, featuring high extraction efficiency and reduced time consumption [18]. The compounds obtained were also different under the influence of different extraction methods. A study showed that water extract is rich in polysaccharides, while ethanol extract is rich in triterpenoids and flavonoids [9].

*2.2. Separation and Purification.* Crude extracts generally contain a variety of impurities, such as low-molecular-weight compounds and proteins; therefore, they are separated and purified after preliminary extraction. The ethanol precipitation method has the advantage of simple steps; however, this method is not conducive to purification and consumes a large amount of organic solvents [19]. Ultrafiltration is a common separation method that has the advantages of high efficiency, low cost, and environmental friendliness, and after crude extraction and separation, certain low-molecular-weight compounds can be removed; however, extracts still require further purification by ultrapure water dialysis [20], Sevage method [21], decolorization, and chromatography [22].

*2.3. Modification.* Elevated levels of chronic oxidative stress markers appear in the early stages of IR or T2DM [23]. In addition, a variety of inflammatory chemokines can be used as predictive markers of T2DM [24]. Modern studies have confirmed that although traditional Chinese medicine (TCM) has anti-inflammatory and antioxidant effects, its pharmacological effects can be significantly enhanced through structural modification [25, 26]. The modification of compounds is considered to improve their utilization value. At present, the modification of *C. paliurus* mainly includes acetylation, sulfation, phosphorylation, and carboxymethylation. Liu et al. demonstrated that acetylated *C. paliurus* polysaccharides have better immunomodulatory activity [27]. Sulfated polysaccharides exhibit outstanding anti-inflammatory and antioxidative stress properties. Han et al. elucidated that sulfated *C. paliurus* polysaccharides enhanced the immunomodulatory activity of dendritic cells through the TLR2/4-mitogen-activated protein kinase (MAPK)/nuclear factor kappa-B (NF-$\kappa$B) signaling pathway. Moreover, after sulfation, phosphorylation, and carboxymethylation modification, the antioxidant capacity of polysaccharides was also improved [28, 29]. Appropriate modification plays a vital role in the improvement of biological activities; however, according to the existing literature search, the modification of *C. paliurus* extract mainly focused on polysaccharides. Therefore, it is necessary to explore more effective modification methods to better exert their biological activities.

Bioactive compounds with medicinal value have been successfully obtained using various extraction methods. According to existing research, common compounds primarily include polysaccharides, triterpenoids, flavonoids, phenolic acids, and saponins. Meanwhile, a small number of carbohydrates, proteins, mineral elements, sterols, amino acids, organic acids, and unsaturated fatty acids are present in this plant. [4, 30–32]. These active substances are the material basis of drug efficacy.

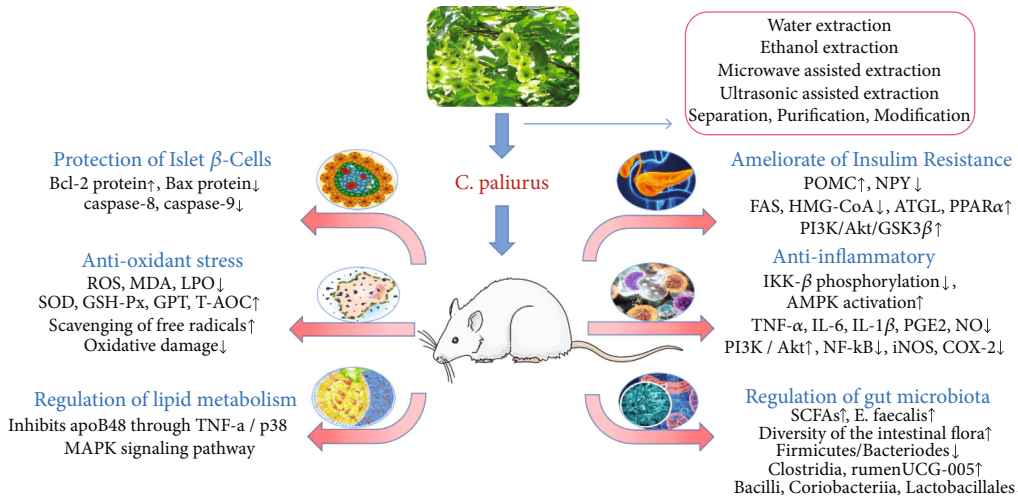


Figure 1

## 3. The Therapeutic Effect of *C. paliurus* on T2DM

Following centuries of application, *C. paliurus* has been recognized to exhibit the benefits of regulating glucose and lipid metabolism. In vitro and in vivo studies have shown that water and ethanol extracts can reduce blood glucose and lipid levels in T2DM patients. Among these compounds, *C. paliurus* flavonoids have a potential hypoglycemic effect, triterpenoids have a more obvious lipid-lowering effect [9], and quercetin and kaempferol are the main factors that inhibit $\alpha$-glycosidase activity. This inhibition ability is even stronger than that of acarbose [33]. In addition, they can reduce serum total cholesterol (TC) and triglycerides (TG), ameliorate liver fat levels, inhibit pancreatic lipase activity, reduce body weight, and improve liver steatosis [34].

## 4. Summary on Potential Mechanism of *C. paliurus* on T2DM (Figure 1)

*4.1. Improvement of IR.* The majority of T2DM patients experience progression from IR to impaired islet $\beta$-cell function [35]. Therefore, alleviating IR and protecting islet $\beta$-cell function are key points of T2DM treatment. Lipid metabolism disorders and chronic inflammation caused by obesity are thought to be closely related to the occurrence of IR [36]. In addition, mitochondrial dysfunction, gut microbiota dysbiosis, and remodeling of the adipose extracellular matrix also play a bridging role between obesity and IR [37]. Xu et al. found that *C. paliurus* aqueous extract can inhibit energy intake in animal experiments, which may be due to hypothalamic insulin signaling pathway regulation and controlling proopiomelanocortin (POMC) and neuropeptide Y expression in order to inhibit excessive food intake [11]. In T2DM, the levels of hepatic TG in overweight and obese patients are directly related to the severity of IR in the liver and skeletal muscles [38, 39]. Recently, the effects of *C. paliurus* extracts on IR have attracted increasing attention. It has been shown that *C. paliurus* extracts can reduce IR and regulate the activity of key enzymes such as CCAAT/enhancer binding protein alpha and peroxisome proliferator-activated receptor $\gamma$ in lipid metabolism [13]. In addition, in high-fat diet- (HFD-) induced mice and palmitic acid-induced HepG2 cells, it was found that *C. paliurus* triterpenoids (CPT) upregulated the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/glycogen synthase-3$\beta$ (GSK3$\beta$) signaling pathway to alleviate IR [34]. The hepatolipid-lowering activity of *C. paliurus* extract is also reflected in mammalian target of rapamycin (mTOR)/70 kDa ribosomal protein S6 kinase (p70S6K) pathway regulation, lipophagy activation, and promotion of lipid decomposition in HepG2 cells [40]. In addition, CPT acts on the ng to improve insulin sensitivity of adipocytes and increase glucose intake, which may have potential as insulin sensitizers [41, 42]. Extensive research shows that, in T2DM, low chronic inflammation and IR are the two interdependent key processes; inflammation can interfere with insulin signaling; therefore, IR is closely related [43]. Persistent hyperglycemia can affect the inflammatory mechanism of the liver, leading to lipid accumulation and aggravated IR [44]. Jiang et al. found that *C. paliurus* extracts regulate adipokine expression and improve IR by inhibiting inflammation in mouse models [45]. These results provide support for the role of *C. paliurus* in improving insulin sensitivity and alleviating IR. This plant appears to be effective for T2DM treatment due to its effect on weight loss and IR alleviation.

*4.2. Anti-Inflammatory and Antioxidative Stress.* Substantial evidence has shown that oxidative stress and an increase in cytokines are significantly correlated with the occurrence and development of T2DM. Numerous cytokines, including interleukin-6 (IL-6), interleukin-1$\beta$ (IL-1$\beta$), high-sensitivity C-reactive protein, and tumor necrosis factor-$\alpha$ (TNF-$\alpha$), can act as central mediators of the inflammatory response and have been confirmed to be positively correlated with the risk of T2DM [46, 47]. Therefore, active intervention against proinflammatory cytokines is considered beneficial for T2DM treatment. Certain studies have shown that the hypoglycemic effect of triterpenoids may be mediated by the activation of adenosine 5′-monophosphate- (AMP-)

activated protein kinase (AMPK), thereby inhibiting adipose tissue inflammation. Zhu et al. found that the chloroform extract of *C. paliurus* and its two triterpenoids can reduce inhibitor kappa kinase $\beta$ (IKK-$\beta$) phosphorylation induced by inflammatory injury, and its anti-inflammatory effect may be related to AMPK activation [42]. The compound polysaccharide and dammarane triterpene saponin from *C. paliurus* has also been proven to be conducive to proinflammatory cytokine inhibition and inflammatory pathway regulation, and research has demonstrated that the expression and release of nitrate oxide, TNF-$\alpha$, and prostaglandin E2 (PGE2) were significantly inhibited in lipopolysaccharide- (LPS-) mediated inflammatory stimulation of RAW 264.7 [48–50]. Jiang et al. confirmed in vivo and in vitro that *C. paliurus* triterpenoids improve diabetes-induced liver inflammation through the Rho/Rho-associated coiled-coil-containing protein kinase (ROCK)/NF-$\kappa$B signaling pathway, and the expression of Rho kinase and NF-$\kappa$B in the liver was significantly reduced [51]. Transcriptome analysis showed that *C. paliurus* aqueous extract alleviated inflammation by inhibiting cytochrome P450 and enhancing fatty acid metabolism in diabetic rat livers, as evidenced by decreased TNF-$\alpha$ and IL-6 levels [52]. These results suggest that *C. paliurus* extracts might be beneficial for T2DM treatment through the regulation of inflammation.

It is widely acknowledged that oxidative stress is another major hallmark of T2DM. The most common markers of oxidative stress are malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH). To a certain extent, the increase in reactive oxygen species (ROS) production can cause or aggravate diabetes by damaging $\beta$-cells, reducing insulin secretion, affecting glucose transport pathways, and negatively interfering with the balance of oxidant and antioxidant levels [53, 54]. Several clinical studies have shown that oxidative stress is directly related to IR, which can induce pancreatic $\beta$-cell apoptosis and cause abnormal glucose and lipid metabolism. Currently, exogenous antioxidants have gradually attracted attention for inhibiting apoptosis, enhancing autophagy, and improving oxidative stress [53]. Multiple studies have confirmed that the main polysaccharide compounds in *C. paliurus* have antioxidant activities. After using RNA-seq technology to study the polysaccharide synthesis pathway at different stages of leaf development and to detect the polysaccharide content and antioxidant activity, Liu et al. found that the hydroxyl scavenging activity was the highest and the antioxidant capacity was the strongest in the first stage [55]. The health beverage composed of *C. paliurus* polysaccharides and *Momordica* saponin has been proven in the worm model to ameliorate oxidative damage by reducing the levels of ROS, MDA, and nonesterified fatty acids [56]. Lin et al. demonstrated that *C. paliurus* polysaccharides effectively enhanced the stress resistance of *Caenorhabditis elegans* through longevity-promoting factor 1 and heat shock transcription factor 1, which may include the scavenging of free radicals and the alleviation of oxidative damage [57]. Another study showed that *C. paliurus* polysaccharides play an important role in scavenging free radicals and have the function of autoxidation of 1,2,3-pyrogallol, which has a significant effect on inhibiting lipid peroxidation [8]. For carbon tetrachloride- (CCl4-) induced oxidative stress in the livers and kidneys of mice, *C. paliurus* polysaccharides can reduce the induction of recombinant cytochrome P450 2E1 (CYP2E1) expression in the liver by CCl4 and then have a protective effect on the liver, which is mainly manifested by the reduction of ROS and MDA levels and the recovery of SOD and GSH peroxidase (GSH-PX) activities in the liver and kidneys [58]. In addition to polysaccharides, triterpenoids have also been proven to improve oxidative stress in vitro, exerting an antioxidant effect in HepG2 hepatic steatosis cells induced by free fatty acid (FFA) [59]. Compared with other *C. paliurus* compounds, polysaccharides exhibit good antioxidant activity, which has attracted attention in the medical field [60]; however, there is still a lack of extensive and in-depth mechanistic research.

*4.3. Regulation of Gut Microbiota.* The intestinal tract contains a microbiota composed of a large number of microorganisms, which directly affects the health of the host. As shown with a previous in-depth study, the intestinal microbiota plays an increasingly prominent role in the pathogenesis of T2DM [61]. At present, it is believed that regulation of the gut microbiome has a positive impact on blood glucose homeostasis and the prognosis of T2DM [62]. Clinically, certain active components of TCM or active ingredients isolated from plants have been shown to have potential regulatory effects on the microbiota [63, 64]. In vivo experiments on *C. paliurus* showed that the main effects of its compounds represented by polysaccharides and flavonoids were reflected in increasing short-chain fatty acid (SCFA) content, enriching intestinal and fecal microbiota diversity, and regulating the relative balance of dominant bacterial phyla and genera in the intestinal tract [17, 20]. Other studies have shown that the intervention of *C. paliurus* compounds represented by polysaccharides and flavonoids can be beneficial for the species diversity ($\alpha$ diversity and $\beta$ diversity) of intestinal microbiota [65]. In terms of species composition of the microbiota, *C. paliurus* administration changed the composition of intestinal microbiota in mice/rats at multiple levels, for example, reducing the ratio of Firmicutes/Bacteroides, increasing the relative abundance of *Clostridia*, *rumenUCG-005*, and reducing the relative abundance of *Bacilli*, *Coriobacteriia*, *Lactobacillales*, *Faecalibacterium*, and *Mitsuokella*; simultaneously, the relative abundance of *Ruminococcaceae* and *Veillonellaceae* decreased in the community structure [20, 66, 67]. *Enterococcus faecalis* is a beneficial intestinal bacteria [68], and an acidic environment (low pH) can damage the membrane of *E. faecalis* to some extent [69, 70]. Transcriptome analysis showed that *C. paliurus* flavonoids could enhance the acid resistance of *E. faecalis* by downregulating the major facilitator superfamily transporter gene and other pathways, alleviating the negative effects caused by low pH, thus showing a positive impact on the production of intestinal probiotics [71]. Furthermore, *C. paliurus* polysaccharides can also help activate G-protein-coupled receptors, promoting intestinal L cells to secrete intestinal hormones GLP-1 and peptide tyrosinetyrosine (PYY), thus contributing to T2DM treatment [72]. *C. paliurus*-related compounds actively regulate the intestinal microbiota of animal models, and its therapeutic value on intestinal microbiota has been gradually clarified; however, there is still a lack of clear

verification of specific pathways and targets of action, and the mechanism requires further investigation.

*4.4. Protection of Islet β-Cells.* As another important factor in the development of disease, the quantity and quality of islet β-cells directly affect the progression of T2DM patients. Therefore, the protection of islet β-cell function can largely assist in T2DM treatment. Based on transcriptome and biochemical analysis, it was previously found that *C. paliurus* formula extract reduced proinflammatory cytokines and islet damage, inhibited β-cell apoptosis, ensured normal insulin secretion, and reduced blood glucose in rat models [73]. Certain studies have reported that *C. paliurus* and its related extracts can effectively reduce the expression of proapoptotic factors caspase-8 and caspase-9 in the pancreatic tissue of streptozotocin- (STZ-) induced mice or rats, reduce the ratio of Bax/Bcl-2, effectively avoid islet β-cells apoptosis, and alleviate pancreatic injury, which may be related to the regulation of *C. paliurus* on the MAPK and Akt pathways [66, 74].

*4.5. Regulation of Lipid Metabolism.* Dyslipidemia is a major risk factor for T2DM. T2DM patients are often accompanied by dyslipidemia, which aggravates the risk of macrovascular complications [75]. As the initiating, inducing, and aggravating factors of T2DM, lipid metabolism disorders play a crucial role in the course of the disease. Abnormal glucose and lipid metabolism caused by hyperlipidemia and hyperglycemia aggravate the progression of metabolic diseases. A cross-sectional study of 4807 Chinese adults showed that 67.1% of T2DM patients had dyslipidemia [76]. Statins are one of the most widely used lipid-lowering drugs, which can effectively reduce blood lipid levels and reduce the occurrence of cardiovascular events [77]. Nevertheless, a significant number of patients may develop intolerance after receiving statins [78].

Numerous studies have found that certain elements in plants, such as *C. paliurus* leaves and related extracts, have significant effects in dyslipidemia treatment. Research shows that *C. paliurus* polysaccharides play a certain role in lipid-lowering in HFD-induced hyperlipidemic rat models, which is mainly reflected in the downregulation of fatty acid synthase (FAS) and hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA) and the upregulation of the expression of triglyceride lipase (ATGL) and peroxisome proliferator-activated receptor α (PPARα) [79], while reducing the deoxyribonucleic acid (DNA) methylation level of leptin and microsomal triacylglycerol transfer protein (MTTP) and downregulating the mRNA content of leptin and MTTP to ameliorate lipid metabolism disorders in rats [80]. Moreover, *C. paliurus* polysaccharides can reduce the whole-genome DNA methylation level of mouse liver induced by high-fat emulsion by regulating AMPK, adipocytokines, fatty acid metabolism, and other signaling pathways and play a lipid-lowering role in mouse models [81].

Using high-fat *C. elegans* as an animal model, the results showed that the polysaccharide-enriched extract from *C. paliurus* reduced the size and number of lipid droplets in *C. elegans* and reduced the accumulation of lipids through the monounsaturated fatty acid (MUFA) biosynthesis pathway, and the mediator 15 (MDT-15)/selenium binding protein-1(SBP-1) and nuclear hormone receptor NHR-49 (NHR-49)/MDT-15 signaling pathways [82]. In hyperlipidemic mice, *C. paliurus* chloroform extract improved the activity and gene expression of cholesterol 7α-hydroxylase (CYP7A1), inhibited 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) reductase, promoted the transformation of cholesterol to bile acid, and exerted a lipid-lowering effect [83].

Recent studies have shown that *C. paliurus* extract can prevent intestinal absorption of dietary fat by inhibiting the secretion of apoB48, thus effectively preventing hyperlipidemia and obesity [12]. In addition to polysaccharides, triterpenoids also play an important role in improving lipid metabolism disorders and are expected to be effective in regulating T2DM complicated with dyslipidemia. Wu et al. found that the triterpene acid-enriched components and ethanol extract of *C. paliurus* inhibited the excessive production and secretion of apoB48 in hyperlipidemic rats through the TNF-α/p38 MAPK signaling pathway [84, 85]. Wu et al. demonstrated that the triterpenoids of *C. paliurus* can also significantly reduce the oversecretion of apoB48 in Caco-2 cells [86]. Based on this, we hypothesized that *C. paliurus* has a therapeutic effect on T2DM complicated with dyslipidemia. However, the molecular mechanism has not been well established and requires further exploration and summary.

## 5. Application of *C. paliurus* in the Complication of T2DM

Previous studies have shown that *C. paliurus* plays a significant role in reducing blood glucose, alleviating IR, and regulating lipid metabolism. In vivo and in vitro experiments also showed that the plant exerts good effects in the treatment of diabetes-related complications. Ample evidence indicates that approximately 40% of T2DM is associated with diabetic nephropathy (DN) to varying degrees, and DN has become one of the main causes of end-stage renal disease (ESRD). The presence of DN significantly increases the risk of cardiovascular disease-related mortality, which has placed a considerable burden on the social health system [87]. The main features of DN include increased urinary albumin excretion, decreased glomerular filtration rate, persistent hyperglycemia, and persistent renal function decline. In terms of DN treatment, the benefits of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers have been recognized clinically and widely used to alleviate DN. Bergamo Nephrology Diabetes Complication Trial research shows that ACEIs have a protective effect on the kidney and can effectively prevent the production of microalbuminuria [88]. A meta-analysis showed that, compared with single-use, a low-dose combination of the two drugs can compensate for their respective shortcomings; therefore, it is more prominent in reducing urinary total proteinuria and urinary albumin excretion rate [89]. However, certain researchers have opposing views, they affirm the effectiveness of the combination of these drugs in reducing urinary protein; however, they emphasize that the

potential risk of hyperkalemia cannot be ignored. In addition to hyperkalemia, patients also have a significantly increased risk of decreased renal function or renal failure [90]. The advantages of TCM in treating DN have become increasingly prominent [91]. A population-based cohort study included 125490 patients with DN, and the results showed that the incidence and mortality of ESRD in DN patients actively treated with TCM were lower than those without TCM treatment [92]. Recently, *C. paliurus* has been reported to be a promising therapy for DN. Studies have demonstrated that the extract of *C. paliurus* can improve the extensive thickening of the glomerular capillary basement membrane in HFD-STZ-induced diabetic rats and also assist in reducing the levels of blood urea nitrogen, creatinine, and glycated serum protein, which is beneficial for early-stage DN treatment [52, 93]. Xia et al. reported that *C. paliurus* polysaccharides can reduce blood glucose, improve renal function, enhance antioxidant capacity, and downregulate the expression of advanced glycosylation end products and transforming growth factor-$\beta$1, thus playing a protective role in DN rats [94]. Furthermore, autophagy-induced oxidative stress, inflammation, and apoptosis aggravate renal injury [95], and the *C. paliurus* triterpenic acid fraction regulates autophagy through the AMPK-mTOR pathway and reduces high glucose-induced HK-2 cell apoptosis [96]. Meanwhile, *C. paliurus* aqueous extract can inhibit oxidative stress and aldose reductase activity and effectively improve renal function and urinary protein excretion in DN rats, alleviating renal damage in diabetic rats [14].

Increasing evidence suggests that diabetic patients are at a higher risk of heart disease and heart failure. Currently, diabetic cardiomyopathy (DCM) is generally recognized as a cardiac structural and functional disorder in diabetic patients, excluding hypertension, coronary artery disease, or severe valvular heart disease [97].

As one of the most common pathogenic factors of diabetes, inflammation has been confirmed to be associated with DCM. NF-$\kappa$B is an important nuclear transcription factor in cells and is involved in the regulation of inflammation and myocardial cell injury. Simultaneously, NF-$\kappa$B activation can lead to the release of pro-inflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$, and IL-6, resulting in myocardial injury, dilated cardiomyopathy, and other myocarditis-related conditions [98]. Furthermore, cardiomyocyte apoptosis may also be involved in the occurrence and progression of DCM [99]. To date, there is no specific medicine for targeted treatment of the disease. In clinical practice, individualized interventions are adopted based on controlling blood glucose levels. Following intragastric administration of *C. paliurus* ethanol extract to db/db mice, Wang et al. found that the levels of myocardial injury markers (cardiac troponin I and creatine kinase MB), oxidative stress markers (MDA and SOD), and proinflammatory cytokines (TNF-$\alpha$, IL-1ß, and IL-6) were effectively alleviated. Further mechanistic exploration revealed that *C. paliurus* extract reduced the expression of NF-$\kappa$B and myocarditis by activating the PI3K/Akt signaling pathway, improving antiapoptotic Bcl-2, and reducing prapoptotic cle-caspase-3, cle-caspase-9, and Bax, to reduce myocardial inflammation and injury in mouse models. Therefore, it is speculated that it may effectively reduce pathological damage and fibrosis of the heart tissue in diabetic patients [100].

These results indicate that *C. paliurus* is a good target for the treatment of DN and DCM. However, the application of other diabetic complications requires further improvement, and the specific components of the corresponding bioactivities in the extract require further exploration.

Current research on the prevention of diabetes complications by *C. paliurus* extract is still in its initial stages. Xiao et al. found that after *C. paliurus* intervention, in addition to the effective improvement of biochemical indexes such as liver and kidney function, pathological changes such as glomerular basement membrane thickening, hepatic steatosis, and myocardial hypertrophy in model mice have also been effectively alleviated. *C. paliurus* has remarkable potential in the treatment of diabetes mellitus and various related complications, such as inflammation, oxidative stress modulators, and apoptosis inhibitors, which are worth exploring in depth.

## 6. Conclusion and Perspectives

As a natural plant, *C. paliurus* mainly grows in southern China and plays an important supporting role in numerous metabolic diseases, such as diabetes, obesity, and hypertension. The extracted plant components have been shown to have abundant biological activities in vivo and in vitro. Table 1 summarizes the potential effects of major compounds and extracts from *C. paliurus* on T2DM and related complications.

This study focused on the application of *C. paliurus* in T2DM and its related complications, with particular focus on the acquisition of effective components and extracts. The extraction method of the active components directly affects biological activities. Researchers should select the extraction method according to the purpose, requirements, and conditions of the study and further optimize the extraction process. The development of more efficient separation and purification technologies will effectively prevent the loss of effective plant components and bring benefits for the next step of pharmacological value research. Through a review of the existing literature, it was found that *C. paliurus* has the characteristics of multiple targets and pathways in T2DM treatment. It was found that the compounds represented by polysaccharides and triterpenes and the extracts represented by water extraction and ethanol extraction had unique advantages.

For instance, *C. paliurus* polysaccharides have a significant therapeutic effect on T2DM and can effectively inhibit $\beta$-cell apoptosis, regulate intestinal flora, and exert anti-inflammatory and antioxidant effects. *C. paliurus* triterpenoids play a significant role in alleviating IR and regulating lipid metabolism and can be used as a promising effective component in T2DM complicated with lipid metabolism disorder or obesity. Moreover, existing research has shown that *C. paliurus* polysaccharides and triterpenoids have significant effects on the regulation of inflammation and

Table 1: Potential role of the major compounds and extracts of *C. paliurus* in T2DM and its complications.

| Chemical compound/ extract | Method of extraction | Model for the experiments | Effect | Potential mechanism | References |
|---|---|---|---|---|---|
| Polysaccharide | Water extraction | Male Wistar rats | Regulation of gut microbiota | UCG-005, SCFAs↑ IL-1$\beta$, IL-6, TNF-$\alpha$↓ Leptin↓, adiponectin, GLP-1↑ Bcl-2↑, Bax↓ | [66] |
| | Ethanol extraction | Sprague-Dawley rats | Regulation of gut microbiota | SCFA-producing bacteria↑ mRNA in GPR41, GPR43, GPR109a↑ SCFA-GLP1/PYY associated sensory mediators↑ | [72] |
| | Ethanol extraction | Sprague-Dawley rats | Hypolipidemic | FAS, HMG-CoA↓, ATGL, PPAR↑ | [79] |
| | Ethanol extraction | Sprague-Dawley rats | Hypolipidemic | DNA methylation of leptin and MTTP↓, mRNA contents of leptin and MTTP↓ | [80] |
| | Hot water extraction | RAW264.7 cell | Anti-inflammatory | Accounted for synergistic effect on the release of NO and TNF-$\alpha$, accounted for antagonistic effect on the release of IL-1$\beta$ and PGE2. | [50] |
| | Hot water method assisted by ultrasonic | Female ICR mice | Hypolipidemic | The expression of ATGL and PPAR$\alpha$ gene in liver↓, high expression level of fatty acid synthesis gene induced by HFD↓, SOD, GSH-PX, GPT, T-AOC↑, LPO, MDA↓ | [13] |
| | Water extraction | Kunming mice | Regulation of gut microbiota | SCFAs↑, diversity of the intestinal flora↑, specific metabolic functions of the gut microbiota↑ | [20] |
| | Water extraction | C. elegans | Hypolipidemic | Size distribution of lipid droplets↓, number of lipid droplets↓, through MUFA biosynthetic pathways, MDT-15/sbp-1 and NHR-49/ MDT-15 signaling pathway | [82] |
| | Ethanol extraction | Kunming mice | Antioxidant | Induction of CYP2E1 expression in liver by CCl4↓, ROS↓, SOD, GSH-Px↑, MDA↓ | [58] |
| | Water extraction | C. elegans | Antioxidant | ROS, MDA, NEFA, GSSG↓, SOD, CAT, GSH-Px, GSH↑ | [57] |
| | Water extraction | Kunming mice | Anti-inflammatory | NO, iNOS, COX-2, TNF-$\alpha$ and IL-1$\beta$↓, SCFAs↑, TLR4 protein expression↓, ERK, JNK p38↓ | [7] |
| Triterpenoid | Ethanol extraction | C57BL/6J mice, HepG2 cells | Ameliorate of insulin resistance | Upregulation of PI3K/Akt/GSK3$\beta$pathway | [34] |
| | Ethanol extraction | db/db mice, HepG2 and LO2 cells | Anti-inflammatory | IL-6, IL-1$\beta$, TNF-$\alpha$↓, hepatic expression of Rho-kinase and NF-$\kappa$B↓ | [51] |
| | Ethanol extraction | Mouse C2C12 myoblasts and 3T3-L1 preadipocytes | Improve glucose uptake | Activate AMPK-p38 pathway, Insulin sensitivity of adipocytes↑ | [41] |
| | Ethanol extraction | Male Sprague-Dawley rats | Attenuates kidney injury | AMPK phosphorylation↑, mTOR phosphorylation↓ | [96] |
| | Ethanol extraction | Male Sprague-Dawley rats | Hypolipidemic | Inhibition of intestinal apoB48 production through TNF-$\alpha$/p38 MAPK signaling pathway, MDA↓, GSH-P, SOD, CAT↑ | [85] |
| | Ethanol extraction | 3T3-L1 adipocytes | Promote glucose uptake in 3T3-L1 adipocytes | AMPK activation↑, IKK$\beta$ phosphorylation in adipocytes↓, restored insulin-mediated phosphorylation of IRS-1 tyrosine and Akt | [42] |

Table 1: Continued.

| Chemical compound/ extract | Method of extraction | Model for the experiments | Effect | Potential mechanism | References |
|---|---|---|---|---|---|
| Flavonoid | Ethanol extraction | FFA-induced HepG2 steatosis cells | Antioxidant | SOD↑, MAD↓ | [59] |
| | Ethanol extraction | RAW 264.7 cells | Anti-inflammatory | NO, TNF, PGE2, IL-6↓, COX-2, iNOS, NF/p65↓ | [48] |
| | Ethanol extraction | RAW264.7 cell | $\alpha$-Glucosidase inhibitory and anti-inflammatory | mRNA expression of iNOS, COX-2, NF-$\kappa$B, IL-6, IL-1$\beta$, and TNF-$\alpha$↓, protein expression of iNOS, NF-$\kappa$B/p65 and COX-2↓ | [49] |
| | Hot water extraction | Male C57BL/6 J mice | Regulation of gut microbiota | Faecal microbiota diversity↑, Bacteroidetes↑, Firmicutes, Proteobacteria↓ | [65] |
| | Ethanol extraction | RAW264.7 cell | Inhibit XOD activity, inhibition of NO production in LPS induced RAW264.7 cells | Not mentioned | [32] |
| | Ethanol/ chloroform extraction | KM male mice | Hypolipidemic | CYP7A1↑, HMG-CoA reductase↓ | [83] |
| Extract | Water extraction | Male Wistar albino rats | Alleviates diabetic nephropathy | Reduce oxidative stress, suppress the activation of the polyol pathway through aldose reductase inhibition | [14] |
| | Ethanol extraction | Kunming mice | Hypolipidemic | TNF-$\alpha$, mRNA↓, p38 phosphorylation↓, inhibit MAPK signaling along the TNF-$\alpha$/ p38MAPK pathways | [84] |
| | Ethanol/ water extraction | SD male rats | Antioxidant | MDA↓, SOD, GSH-Px↑ | [93] |
| | Hot water extraction | Male C57/BL6J mice | Inhibit $\beta$ cell apoptosis | Caspase-8, caspase-9, cleaved caspase-3↓, Bax/Bcl-2↓, p38, ERK and JNK phosphorylation↓, Akt phosphorylation↑ | [74] |
| | Ethanol extraction | HepG2 cells | Attenuates hepatic lipid deposition | p-mTOR↓, Beclin↑, p-p70S6K↓, p62↓ | [40] |
| | Water extraction | SHR/cp rats | Improving insulin signaling in the hypothalamus | m-PI3Kp85↑, p-Akt↑, p-FoxO1↑, POMC↑, NPC↓ | [11] |
| | Ethanol extraction | db/db mice | Protect against diabetic cardiomyopathy | TNF-$\alpha$, IL-1$\beta$, IL-6↓, Bcl-2↑, cle-caspase-3, cle-caspase-9, Bax↓ | [100] |
| | Hot water extraction | Male Sprague-Dawley rats | Ameliorate diabetes | SOD↑, MDA↓, Ins1, Ins2↑, $\beta$-cell mass↑, Ddit4, Fgf21↑, DNA replication↑, cytochrome P450↓ | [52] |

oxidative stress. As for the regulation of gut microbiota as a way of antidiabetes, *C. paliurus* flavonoids and polysaccharides are more prominent. However, certain studies only proposed the bioactivities of the components obtained by different extraction methods and did not further detect the exact components, and further research on the main chemical components and mechanisms of biological activities may become a focus in the next stage.

Existing studies have shown that the natural product *C. paliurus* can have a good curative effect while being safe compared with the limitations of adverse reactions, drug resistance, toxicity, and side effects of commonly used modern drugs. At present, there are no reports of toxicity or liver injury. Surprisingly, many animal experiments have confirmed that *C. paliurus* extracts have a hypoglycemic effect similar to that of metformin. In addition, studies have shown that the inhibitory activity of *C. paliurus* extracts on glucosidase is even stronger than acarbose. The authors of this paper speculate that it may become the main component of drugs or functional foods to supplement the shortcomings of traditional therapies. Notably, all existing studies on *C. paliurus* are based on animals or cells and lack randomized controlled trials. Due to the differences among different species, although much evidence shows that *C. paliurus* has considerable potential in rodent research, its effectiveness in humans remains to be further established.

*C. paliurus* is widely used as a health drink, and its medicinal value requires further promotion and application. There are no reports on the dose-response relationship of *C. paliurus* as a therapeutic drug. Further exploration of *C.*

*paliurus* is needed to fill these gaps, and the evaluation of *C. paliurus* as a means of intervention still requires further research.

## Data Availability

All data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Dr. Han Wang and Dr. Cheng Tang contributed equally to this work as co-first authors. Dr. Linhua Zhao and Dr. Xiaolin Tong are cocorrespondence authors.

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*Research Article*

# Exosomes Secreted by Umbilical Cord Blood-Derived Mesenchymal Stem Cell Attenuate Diabetes in Mice

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Mesenchymal stem cell (MSC) therapy is an innovative approach in diabetes due to its capacity to modulate tissue microenvironment and regeneration of glucose-responsive insulin-producing cells. In this study, we investigated the role of MSC-derived exosomes in pancreatic regeneration and insulin secretion in mice with streptozotocin-induced diabetes. Mesenchymal stem cells (MSCs) were isolated and characterized from umbilical cord blood (UCB). Exosomes were isolated and characterized from these MSCs. Diabetes was induced in male C57Bl/6 mice by streptozotocin (STZ; 40 mg/kg body weight, i.p.) for five consecutive days. The diabetic mice were administered (i.v.) with MSC ($1 \times 10^5$ umbilical cord blood MSC cells/mice/day), their derived exosomes (the MSC-Exo group that received exosomes derived from $1 \times 10^5$ MSC cells/mice/day), or the same volume of PBS. Before administration, the potency of MSCs and their exosomes was evaluated in *vitro* by T cell activation experiments. After day 7 of the treatments, blood samples and pancreatic tissues were collected. Histochemistry was performed to check cellular architecture and $\beta$ cell regeneration. In body weight, blood glucose level, and insulin level, cell proliferation assay was done to confirm regeneration of cells after MSC and MSC-Exo treatments. Hyperglycemia was also attenuated in these mice with a concomitant increase in insulin production and an improved histological structure compared to mice in the PBS-treated group. We found increased expression of genes associated with tissue regeneration pathways, including *Reg2*, *Reg3*, and *Amy2b* in the pancreatic tissue of mice treated with MSC or MSC-Exo relative to PBS-treated mice. MicroRNA profiling of MSC-derived exosomes showed the presence of miRs that may facilitate pancreatic regeneration by regulating the Extl3-Reg-cyclinD1 pathway. These results demonstrate a potential therapeutic role of umbilical cord blood MSC-derived exosomes in attenuating insulin deficiency by activating pancreatic islets' regenerative abilities.

## 1. Introduction

Diabetes is a major growing health problem that has been estimated to afflict well over 300 million people globally. The two most common types of diabetes are type 1 (T1DM) and type 2 diabetes mellitus (T2DM). Together, they account for a large and growing patient population with pancreatic $\beta$ cell deficiency. In the case of T1DM, $\beta$ cell deficiency is associated with autoimmune destruction of $\beta$ cells, while in the case of T2DM, it is associated with $\beta$ cell dysfunction [1, 2]. Nevertheless, substantial $\beta$ cell loss results in permanent endocrine deficiency and irreversible diabetes due to their limited regenerative capacity in adults [3]. Thus, any treatment modality to restore $\beta$ cell mass would be beneficial for all forms of diabetes [4, 5]. Mesenchymal stem cells (MSCs) present a promising approach for restoring $\beta$ cell mass in diabetes [6, 7]. Immune modulatory properties of MSCs have been attributed to their therapeutic effects in T1DM [8, 9]. Bone marrow-derived MSCs have been found to inhibit T cell proinflammatory response to islet antigen

GAD65, in patients with T1DM [10, 11]. Moreover, these MSCs were shown to affect the antigen-presenting function of dendritic cells (DC) by promoting an immature DC phenotype [12]. The immunomodulatory effects of autologous bone marrow MSCs in patients with recent onset T1DM showed preservation of C peptide levels in response to a mixed meal tolerance test (MMTT) [13]. Similar immunomodulatory potential of MSCs derived from other sources, such as umbilical cord blood (UCB), will have a profound impact in developing therapeutic modalities for diabetes due to their availability and noninvasive nature of collection [14–16]. The mechanisms of action of MSCs have been attributed to their secretome rather than to their transdifferentiation [17]. However, MSC administration for therapeutic purposes may expose individuals to the side effects of cell-based therapy and immune rejection [18]. This has geared a great scientific interest in exploring alternative approaches inspired by the paracrine hypothesis in regenerative medicine, namely, using MSC-derived extracellular vesicles, such as exosomes, rather than the cells themselves. Exosomes have been shown to play a key role in cell-to-cell communication. Ample of data suggests that exosomes may transfer beneficial outputs from cells to neighboring diseased or injured cells through the delivery of biomolecules with therapeutic potential. The protective effect of UCB-derived MSC-secreted exosomes has been studied in myocardial repair and autoimmune diseases [14, 16]. However, the potential of UCB-MSCs and their derived exosomes in attenuating endocrine deficiencies caused due to nonautoimmune pancreatic $\beta$ cell loss needs to be tested. In this study, we aimed to understand the therapeutic effect of exosomes secreted by UCB-derived MSC on pancreatic repair/regeneration in diabetic mice, where streptozotocin-induced pancreatic beta cell destruction leads to diabetes.

## 2. Materials and Methods

*2.1. Isolation and Characterization of Human UCB-Derived MSCs.* MSCs were isolated from human UCB as previously described [17]. The protocol has been approved by Institutional Committee for Stem Cell Research (IC-SCR; Code: 2019-03-IMP-08). MSCs propagated to the $3^{rd}$-$8^{th}$ passages were characterized using accepted MSC-positive markers anti-CD73, CD90, and CD105 (BioLegend, San Diego, CA, USA), negative hematopoietic markers anti-CD45 and CD34 (BioLegend, San Diego, CA, USA), HLA-DR (BD Biosciences, San Jose, CA, USA) by confocal microscopy (FluoView F10i confocal microscope, Olympus), and flow cytometry (Becton Dickinson, New Jersey, USA). Negative gates were set relative to isotype controls. No chromosomal aberrations were observed at high passage (data not shown). *In vitro* differentiation was performed using StemPro™ Adipogenesis Differentiation Kit (A1007001, Thermo Scientific, Pittsburg, PA Scientific, USA), StemPro™ Osteogenesis Differentiation Kit (A1033201, Thermo Scientific, Pittsburg, PA Scientific, USA), and StemPro™ Chondrogenesis Differentiation Kit (A1007101, Thermo Scientific, Pittsburg, PA Scientific, USA) [19, 20].

*2.2. Isolation and Characterization of Exosomes from Human UCB-Derived MSCs (MSC-Exo).* Exosomes were enriched by differential centrifugation as described previously [21]. Briefly, samples were centrifuged at 17,000$g$ for 10 min at 4°C followed by ultracentrifugation (Beckman Coulter LE80K, CA, 362, USA) at 126,000$g$ for 2 h at 4°C. The exosomal pellet was resuspended in an isolation buffer (PBS). Isolated exosomes were characterized for size using dynamic light scattering (DLS) analysis. The sample temperature was allowed to equilibrate for 10 min before each measurement and was shaken for 20 min at 37°C to dissolve any aggregation, followed by DLS measurements at 20°C using a Zetasizer Nano (Malvern Instruments Ltd., UK). The light scattering was recorded for 200 s with 10 replicate measurements. DLS signal intensity was calculated by the Dispersion Technology Software v.5.10 (Malvern Instruments Ltd., UK). The mean hydrodynamic diameter of exosomes was calculated by fitting a Gaussian function. The peak maximum of the Gaussian function was used to estimate exosome size. Gaussian fitting, mean value, and standard deviation were calculated and compared using Origin Pro 9.0.0 (Origin Lab Corp, USA). Shape characterization was done by scanning electron microscopy (SEM). MSC-Exo suspensions were mixed 1 : 1 with 4% paraformaldehyde and were then applied to 200-mesh nickel grids. They were dried and coated with gold particles, and images were taken by scanning electron microscope (JSM 6490).

T cell activation analysis was performed by using mouse spleen (source of T cell) and followed by $CD3^+$ T cell enrichment column (cat MTCC-25, R&D systems, USA) as per the manufacturer's instruction. Proliferation analysis was performed on (i) unactivated T cells, (ii) activated T cells, (iii) activated T cells treated with MSCs, and (iv) activated T cells treated with MSC-Exo. For this, $1 \times 10^5$ T cells were seeded in 10% FBS (exosome free) RPMI media in 96-well plates. Cells were activated by using 50 $\mu$l of 5 $\mu$g/ml anti-CD3 antibodies (or 2C11) (BioLegend, San Diego, CA, USA) for 4 days at 37°C. Similarly, in the treated group, these activated T cells were cocultured with MSCs ($5 \times 10^4$ cells/well) and MSC-derived exosome (collected from $5 \times 10^4$ MSCs cells for 24 h). After 4 days, nonadherent T cells were collected and live T cell counting was performed by using trypan blue and haemocytometer.

*2.3. Microarray Analysis for MicroRNA Profile.* For microarray analysis, RNA was isolated from MSC and MSC-Exo by miRNAeasy kit (Qiagen India Pvt. Ltd.) as per the manufacturer's protocol. The quantity was checked by NanoDrop ND-2000 (Thermo Fisher Scientific, Pittsburgh, PA). Samples with A260/280 more than 1.8 were subjected to microarray analysis by GeneChip™ miRNA 4.0 Array according to the manufacturer's instruction (Thermo Fisher Scientific). Transcriptome Analysis Console program (version 4.0.0.25, Applied Biosystems) conducted a statistical analysis of the output files (.CEL). Conditions used were background correction, quantile, normalization, description, and $\log_2$ value conversion using the RMA+DABG algorithm. Data represented as heat map and volcano plot.

*2.4. In Vivo Diabetic Mouse Model Development.* 8-week-old male C57Bl/6 mice (body weight~20 g) were used throughout the study. The protocol has been approved by Institutional Animal Ethics Committee (Ref. no: P-03/20/2017). Mice were procured from CSIR-Indian Institute of Toxicology Research, Lucknow, and were housed at the animal care facility of Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow. All mice were housed under the specific pathogen-free conditions in individually ventilated cages with 12 h light and dark cycle. All mice received drinking water and diet *ad libitum*. To induce T1DM, mice were injected intraperitoneal with multiple-dose injections of streptozotocin (STZ) 40 mg/kg body weight, freshly dissolved in 0.1 mmol/l sodium citrate (pH 4.5) for 5 consecutive days as previously described [22]. Blood samples for glucose levels monitoring were collected daily (from day 1 to 10) from tail vein using a glucometer (Elite Diabetes Care System). Animals were considered diabetic when their blood glucose levels were ≥200 mg/dl. The control nondiabetic mouse group received a corresponding volume of 0.1 mmol/l of sodium citrate (pH 4.5) (Figure 1). Insulin was measured on pancreatic tissue using mouse insulin ELISA kit (Thermo Scientific, Pittsburg, PA Scientific, USA) on day 10 and day 17 in the control and diabetic mice treated with PBS, MSCs, or MSC-Exo ($n = 6$/group).

*2.5. Proliferation Assays in Diabetic Mice Administered with MSCs and MSC-Exo.* After 10 days of STZ treatment, mice were randomly divided into three groups: (i) vehicle group that received i.v. PBS injections from day 11 to day 17 ($n = 6$), (ii) MSC group that received i.v. injection of $1 \times 10^5$ MSC cells/mice from day 11 to day 17 ($n = 6$), (iii) Exo group that received i.v. injection of MSC-Exo derived from $1 \times 10^5$ MSCs/mice from day 11 to day 17 ($n = 6$). To investigate the homeostatic turnover of pancreatic cells in all three groups, two doses of BrdU (at 2 h and 16 h) were administered at day 18 (three mice for each group, BrdU at 50 $\mu$g/kg in saline, i.p.) (Sigma-Aldrich, St. Louis, Missouri, USA).

*2.6. Histology.* Mice were euthanized on day 18 by cervical dislocation. For excision of the pancreas, mice were perfused with 1x PBS. Pancreases were carefully cleaned to remove excess fat tissue, fixed in 4% buffered paraformaldehyde (PFA) and then embedded in paraffin wax for histological processing. Paraffin-embedded tissues were sectioned (5 $\mu$m thickness) using a microtome (Thermo Scientific, Pittsburg, PA Scientific, USA). The sections were then processed for histological and/or immunohistochemical analysis.

For histopathogical analysis, five random fields were selected on three different sections from each group and the number of cells were counted as healthy and damaged on the basis of cellular morphology.

*2.7. Proliferation Assay.* Proliferating cells in pancreas sections were detected by indirect immunoperoxidase test as described previously [23]. Briefly, the sections (5 $\mu$m thickness) were deparaffinized using xylene and rehydrated by descending gradings of ethanol. The sections were washed in 1x PBS and were further processed for heat-mediated antigen retrieval by using antigen-unmasking solution (Vector Labs, USA). The sections were washed with 1x PBS followed by blocking with 3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, Missouri, USA). The sections were then probed with anti-BrdU antibody (B2531, Sigma-Aldrich, St. Louis, Missouri, USA) overnight at 4°C. In the next morning, the sections were washed with 1x PBS and incubated with HRP-tagged anti-mouse secondary antibody for 1 h at 37°C. The sections were then washed for three times with 1x PBS, and color was developed by 3,3′-diaminobenzidine (DAB) chromogen solution (106038, GeNei™) at 37°C for 10 min. Sections were counterstained with haematoxylin, dehydrated in ethanol gradings, and mounted in DPX. Images were captured on an Olympus IX73 microscope and analysed by ImageJ software.

*2.8. Western Blotting.* Exosomes were lysed in RIPA buffer with a freshly added protease inhibitor cocktail (Roche Diagnostics, Germany). Protein concentrations were determined by BCA (Pierce, BCA Protein Assay Kit, Thermo Scientific, Pittsburg, PA). Equal amount of proteins was denatured and resolved on denaturing SDS-PAGE gel (12%) and transferred to PVDF membrane. To prevent any protein loss due to stripping and for the ease of interpretation, membrane was cut into the lane after transfer of gel. The membrane was probed overnight with mouse anti-TSG101 (ab125011, Abcam, Cambridge, MA, USA), anti-CD63 (ab59479, Abcam), and anti-CD81 (ab109201, Abcam, Cambridge, MA, USA) antibody followed by HRP-conjugated secondary antibody. The membrane was visualized by an enhanced chemiluminescence detection system (Clarity™ western ECL substrate). Images were acquired on a ChemiDoc imaging system (Universal Hood III, Bio-Rad, California, USA).

*2.9. Next-Generation Sequencing for Transcriptome Profile.* RNA was extracted from diabetic mouse pancreas treated with PBS, MSCs, and MSC-Exo for sequencing and qPCR experiments. The RNA quality was tested using 1% agarose gel electrophoresis, quantities were measured by Qubit system (Invitrogen), and absorbance ratios (A260/280 and A260/230) were tested using NanoDrop ND-2000 (Thermo Fisher Scientific, Pittsburgh, PA, USA). Samples with A260/280 and A260/230 ratios more than 2.0 were used for sequencing using a MinION sequencer according to the manufacturer's instructions (Oxford Nanopore Technologies). In brief, the RNA samples (50 ng per sample) were reverse transcribed with a cDNA-PCR sequencing kit (SQK-PCS109); then, the samples were barcoded with a PCR barcoding kit (SQK-PBK004). Samples were primed with a flow cell priming kit (FLP001) and loaded on MinION flow cells, according to the manufacturer's instruction (Oxford Nanopore Technologies). MinKNOW was used to convert the generated Fast_5 files into FastQ format. Fold expressions were summarised in a heat map using HeatMapper software (http://www.heatmapper.ca/). Pathway analysis was performed using Reactome (https://reactome.org/), KEGG (http://www.genome.jp/kegg/), and Panther (http://pantherdb.org/).

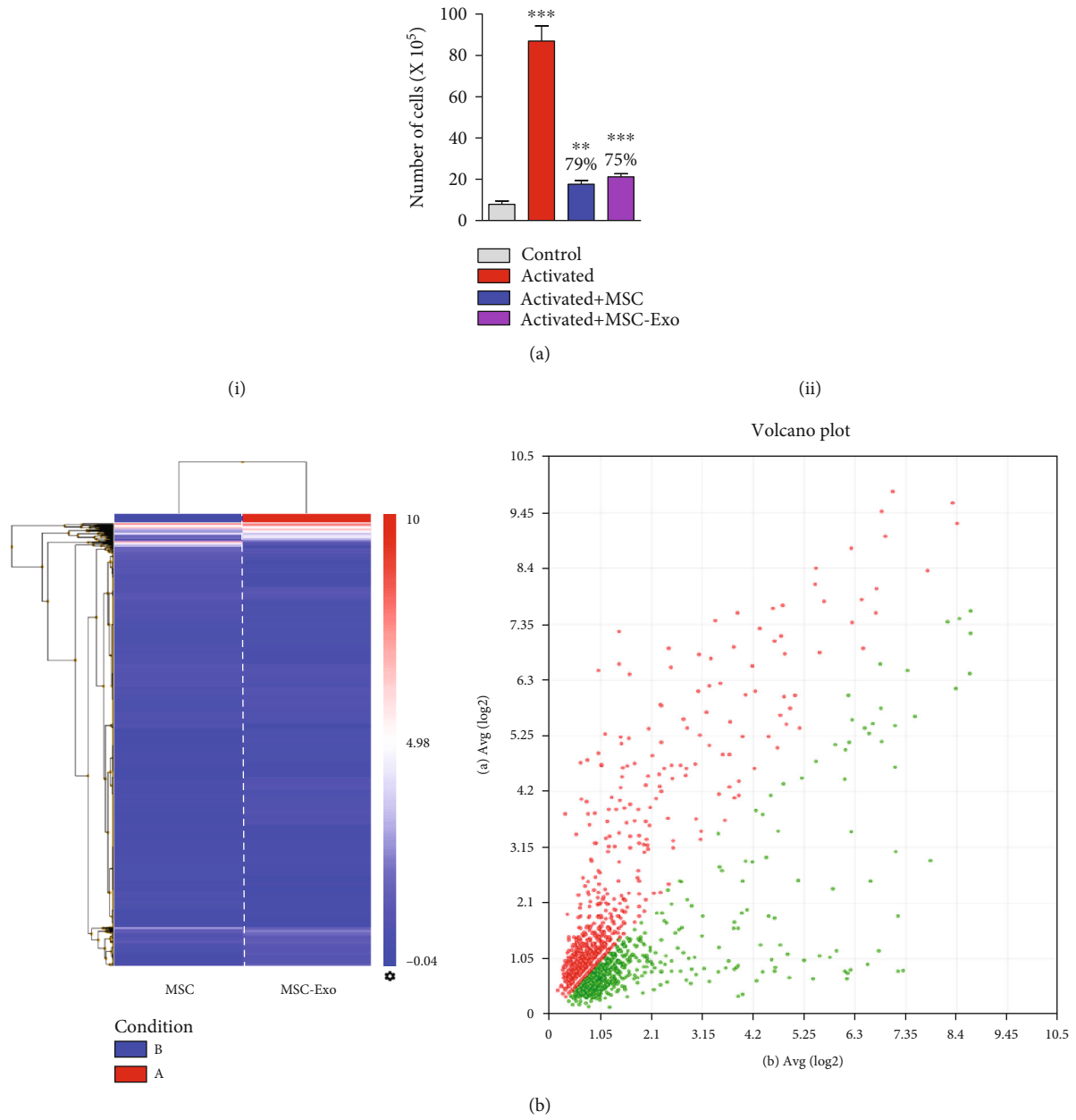



FIGURE 1: Human UCB-MSC and their derived exosomes showed similar microRNA profile and function. (a) Histogram representing T cell activation or proliferation *in vitro* attenuated after treatment with MSCs and MSC-Exo. Data are shown as the mean ± SD. The significance of differences between mean values was estimated by Student's *t*-test (unpaired, two-tailed). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control. (b) (i) Heat map and (ii) volcano plot representing the comparative miRNA profile of MSCs and MSC-Exo.

*2.10. Real-Time PCR.* cDNA synthesis was performed with 1 $\mu$g of total RNA template per sample (the PBS, MSC, and MSC-Exo groups) by cDNA RT kit (Thermo Scientific, Pittsburgh, PA, USA). The protocol was used according to the manufacturer's instructions. Real-time PCR was performed using an ABI 7500 Sequence Detection System (Applied Biosystems, California, USA) in the presence of SYBR Green Master Mix (Takara Bio, Shiga, Japan). Standard PCR conditions were used as prescribed in the reagent protocol.

The primer sequences used were as follows: *Reg3* forward primer-5′-GAATATACCCTCCGCACGCA-3′ and *Reg3* reverse primer-5′-TCTTTTGGCAGGCCAGTTCT-3′, *Reg2* forward primer-5′-AATCAACTGCCCAGAGGGTG-3′ and *Reg2* reverse primer-5′-GCCACAAAGTTGCTCTCAGC-3′, *Amy2b* forward primer-5′-TGGGAGGACTGCTATTGTCC-3′ and *Amy2b* reverse primer-5′-CATTGTTGCACCTTGTCACC-3′, *TLR4* forward primer-5′-ATGCATGGATCAGAAACTCAGCAA-3′ and *TLR4* reverse primer-5′-AAACTTCCTGGGGAAAAACTCTGG-3′, and 18S forward primer-5′-GGCCCTGTAATTGGAATGAGTC-3′ and 18S reverse primer-5′-CCAAGATCCAACTACGAGCTT-3′

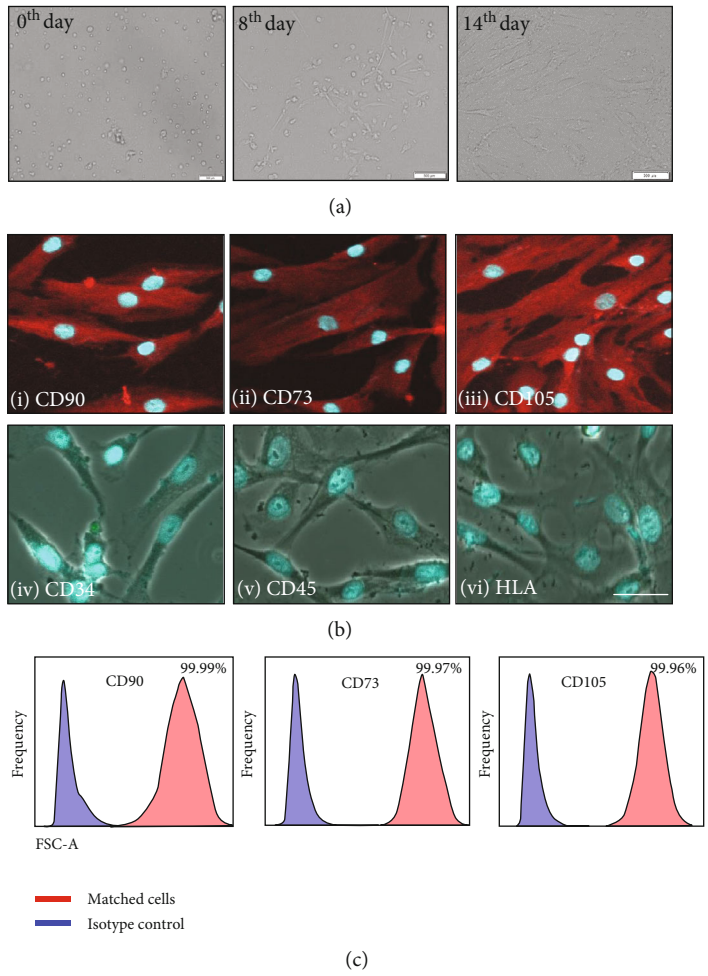



Figure 2: Characterization of human UCB-derived mesenchymal stem cells (MSCs). (a) Typical fibroblastic morphology of MSCs (i) at day of seeding, (ii) at day 8, and (iii) at day 14 of seeding. Scale bar = 500 $\mu$M. (b) Confocal microscopy images showing expression of (i) CD90, (ii) CD73, and (iii) CD105 in isolated MSCs and lack of expression of (iv) CD34, (v) CD45, and (vi) HLA-DR in isolated MSCs. Scale bar = 100 $\mu$M. (c) Flow cytometry showed expression of typical MSC surface markers CD90, CD73, and CD105. FSC-A: forward scattering area.

. Gene expression was calculated relative to the endogenous control sample (18S). Fold change was calculated relative to the PBS control group using the $2^{-\Delta\Delta Ct}$ method (where Ct is the threshold cycle).

*2.11. Statistical Analysis.* GraphPad Prism 5 software was used for the statistical analysis. Data are presented as the mean ± standard deviation. The significance of differences between mean values was estimated using two-tailed unpaired Student's $t$-test. The statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. their respective control.

## 3. Results

*3.1. Immunophenotypic Characterization and Differentiation of Human UCB-Derived MSCs.* MSCs isolated from human UCB (hUCB) were identified by their fibroblast spindle-shaped morphology (Figure 2(a)). Expressions of typical MSC markers (Figure 2(b, i–iii)) and absence of staining for hematopoietic markers (Figure 2(b, iv–vi)) were observed using immunofluorescence. The results were confirmed by FACS analysis for the presence of MSC markers CD73 (96%), CD90 (99%), and CD105 (99.9%) (Figure 2(c)). Differentiation assays performed on isolated MSCs exhibited as expected trilineage differentiation potential (Figures 3(a)–3(c)). Adipogenic differentiation was confirmed by the positive Oil Red O staining of neutral lipid vacuoles in the cells on day 14 of culture. Under osteogenic differentiation media, the cells exhibited nodular calcium deposition, which was stained with alizarin red staining on day 21 of culture. Four weeks under chondrogenic conditions, the cells exhibited typical chondrocyte-like lacunae. Alcian Blue staining confirmed the presence of secreted glycosaminoglycans.

*3.2. Human UCB-MSCs (MSC) and Their Derived Exosomes (MSC-Exo) Showed Similar MicroRNA Profile and Function.* Isolated exosomes were characterized by their cup-shaped spheroidal morphology (size: ~132 nm) (Figure 3(d, i and ii))

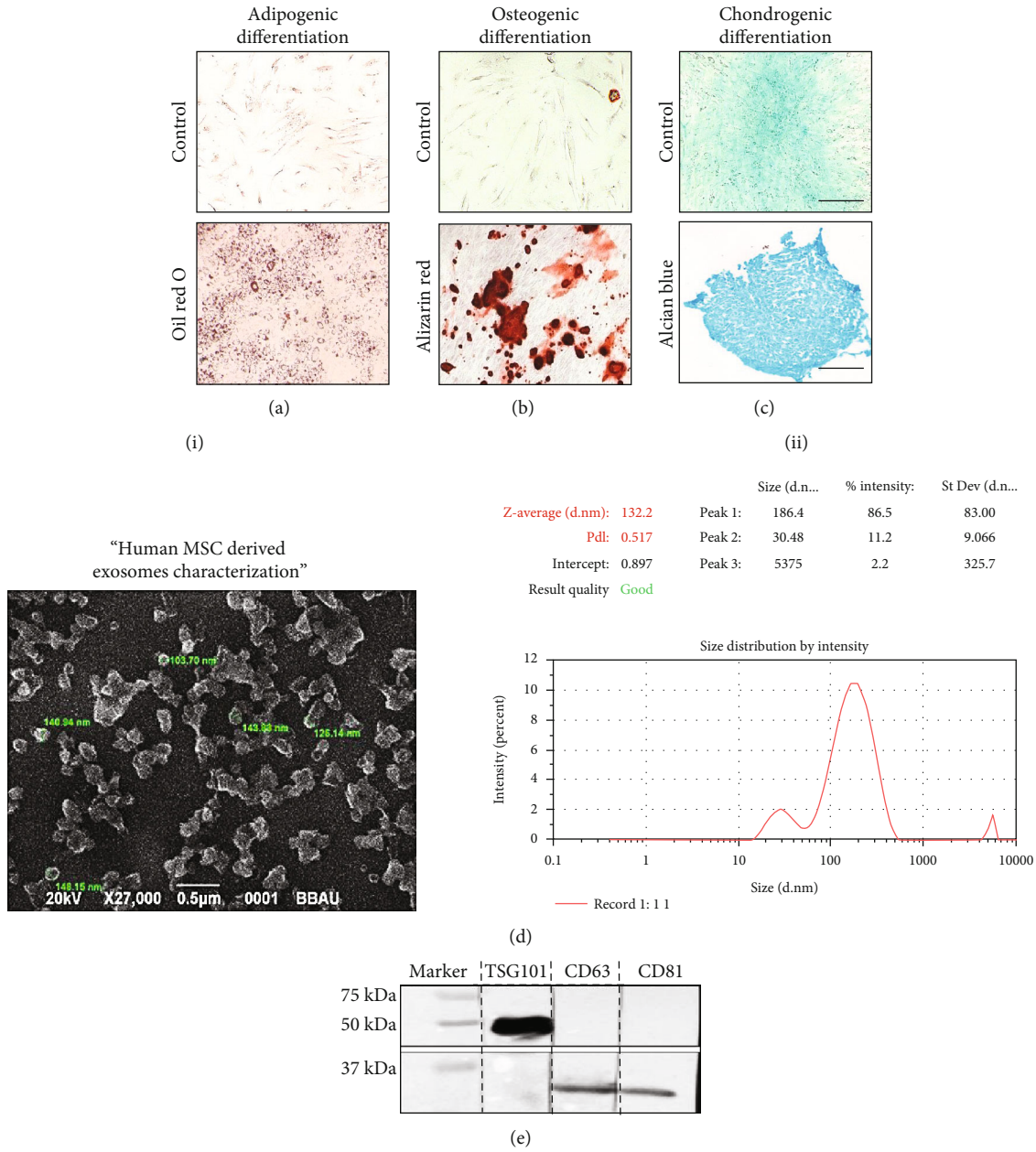



Figure 3: Characterization of MSCs and MSC-Exo. Representative photomicrographs of human umbilical cord blood-derived mesenchymal stem cells differentiated into (a) adipocytes, (b) osteocytes, and (c) chondrocytes and stained with Oil Red, Alizarin Red, and Alcian Blue stain, respectively. Scale bar = 100 $\mu$M. (d) (i) Electron micrograph of an MSC-Exo showing that 92.7% of the exosomes were between 50 and 140 nm (indicated by green letter). Scale bar = 0.5 $\mu$M. (ii) MSC-derived exosome size ranging between 100 and 140 nM by dynamic light scattering analysis. (e) Representative immunoblot showing exosome-specific markers TSG101, CD63, and CD81 in isolated exosomes. Three independent experiments were performed. Full blot is submitted as a supplementary file.

and the presence of exosome-specific markers, i.e., TSG101, CD63, and CD81 proteins (Figure 3(e)). The heat map and volcano plots generated after microarray analysis showed a similar microRNA profile of MSC-Exo and MSCs (Figure 1(b, i and ii)). The expression patterns of miR-let 7a-5p, miR-24-3p, miR-2115-5p, miR-4156, miR-663a, miR-424-5p, miR-30d-5p, miR-450-p, miR-23b-5p, and miR19b-1-3p were similar between MSC-EXO and MSCs.

Suppressive effects of MSCs and their derived exosomes were tested on 2C11 antibody-activated T cells cocultured in the presence/absence of MSCs and MSC-Exo. 2C11 antibody was shown to induce T cell proliferation which was significantly attenuated by MSC-Exo ($p < 0.05$) and MSCs ($p < 0.05$) treatments, compared to control (Figure 1(a)).

*3.3. In Vivo Mouse Model Induction of Diabetes and Pancreatic Injury by STZ Treatment.* The schematic study design is shown in Figure 4. STZ-injected animals had significantly higher blood glucose as compared to vehicle-injected animals' hours of the initial STZ injection

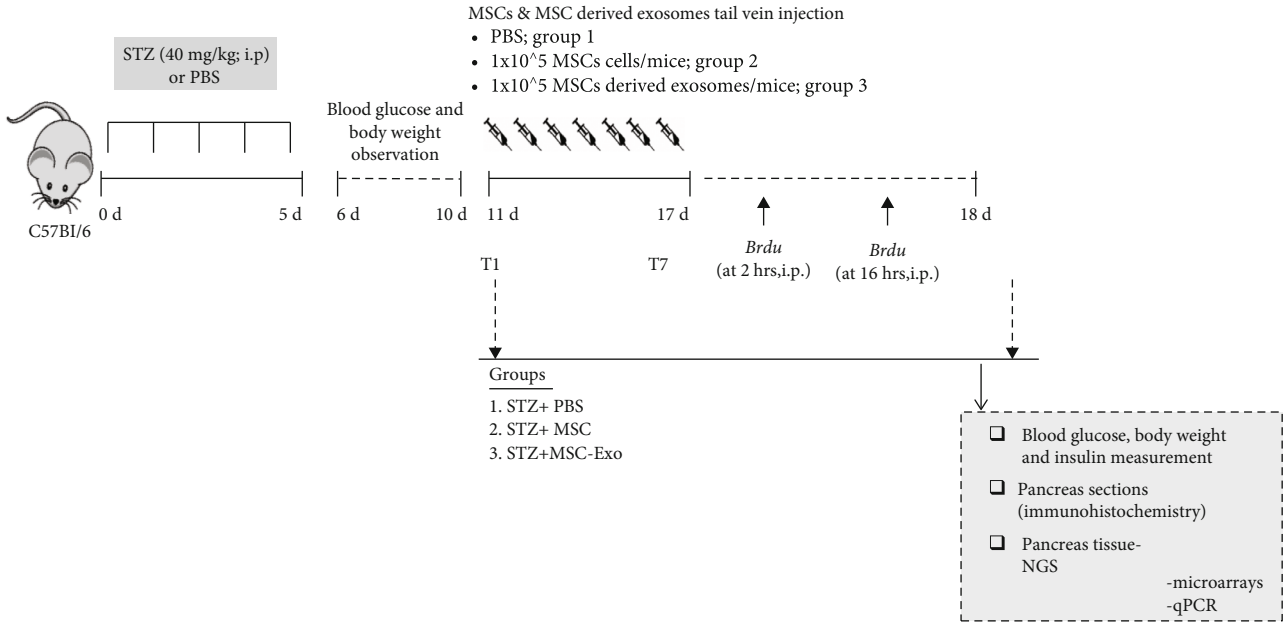



Figure 4: Timeline of the *in vivo* experimental design. Schematic illustration of the experimental plan. (a) In brief, 8-week-old adult male C57BL/6 mice (body weight~20 g) were used throughout the study. To induce diabetes, mice were injected with multiple low-dose injections of streptozotocin intraperitoneal (i.p.) (STZ; 40 mg/kg freshly dissolved in 0.1 mmol/l sodium citrate (pH 4.5)) for 5 consecutive days. Blood samples were collected from tail-vein and were used to monitor glucose levels. Animals were considered diabetic when their blood glucose levels were ≥200 mg/dl. Mice in the nondiabetic control group received a corresponding volume of sodium citrate buffer alone. On day 10, after STZ treatment, mice were randomly divided into three groups: (i) STZ+PBS group that received i.v. injection of PBS ($n = 6$), (ii) STZ +MSC group that received i.v. injection of $1 \times 10^5$ UCB-MSC cells/mice ($n = 6$), and (iii) STZ+MSC-Exo group that received i.v. injection of exosomes derived from $1 \times 10^5$ MSCs/mice from day 11 to day 17 ($n = 6$). To explore the homeostatic turnover of pancreatic cells, two doses of 50 $\mu$g/kg BrdU (at 2 h and 16 h) were administered at day 18 (three mice for each group). At day 18, mice were sacrificed and processed biochemical and molecular analysis.

($p < 0.001$; Figure 5(a)). Body weight decreased in STZ-treated mice from day 5$^{th}$ of STZ treatment; the decrease was significant at days 9 and 10 relative to their baseline (Figure 5(b)). Histopathological analysis showed loss of distinct lobular and cellular boundaries, vacuolar degeneration, fibrosis, and marked decrease in islets of Langerhans size as well as decrease number of cells in islets of Langerhans in the diabetic group compared to the nondiabetic group (Figure 5(c, i)). The number of damaged cells was increased in the diabetic group compared to the control (Figure 5(c, ii)). Pancreatic insulin content as estimated by ELISA showed significantly lower levels in diabetic animals, relative to nondiabetic animals (Figure 5(d)).

*3.4. MSC-Exo Attenuated Hyperglycemia and Pancreatic Injury in STZ-Induced Diabetic Animals.* After 10 days of STZ injection, diabetic animals were randomly assigned into three treatment groups: PBS, MSCs, and MSC-Exo (Figure 4). The treatment was given for 7 consecutive days (T1 to T7). A reduction in blood glucose levels was observed after MSCs or MSC-Exo treatment from day 1 of treatment (T1) onwards; the reduction was found to be significant from day 5 of treatment (T5) compared to their baseline (Figure 6(b)). In PBS-treated diabetic mice (STZ+PBS group), blood glucose further rose from day 1 of treatment (T1) and remained significantly higher compared to MSC/MSC-Exo-treated animals till day 7 (T7) (Figure 6(b)). Body weights did not change significantly during the treatment period (from T1 to T7, Figure 6(c)). Histological analysis showed recovery of the cellular architecture of islets of Langerhans, with the reduction in vacuoles and fibrosis in the MSC-Exo and MSC-treated group as indicated by the increased number of cells (Figure 6(a, ii)) and cellular density in islets of Langerhans compared to the PBS-treated group (Figure 6(a, i)). Although the boundaries of islets of Langerhans were not well defined, they were surrounded by a fine capsule with increased cellular density in diabetic mice treated with MSCs and MSC-Exo (Figure 6(a)).

*3.5. MSC-Exo-Induced Proliferation of Islet Cells and Increased Pancreatic Insulin Production in Diabetic Animals.* Proliferation induction in the pancreatic islets was observed in MSC and MSC-Exo-treated animals compared to the PBS-treated group (Figure 7(a)), as indicated by increased BrdU-positive cells in islets of Langerhans ($p < 0.001$ vs. PBS group; Figure 7(b)). Moreover, the pancreatic insulin content (ELISA assay) estimated at the end of treatment (at T7) was significantly higher in the MSC and MSC-Exo-treated groups relative to the PBS group ($p = 0.057$ vs. PBS group; Figure 7(c)).

*3.6. Regeneration Pathways Were Differentially Regulated in the Pancreas of MSCs and MSC-Exo-Treated Animals.* NGS analysis revealed out 43,000 genes, 667 differentially up

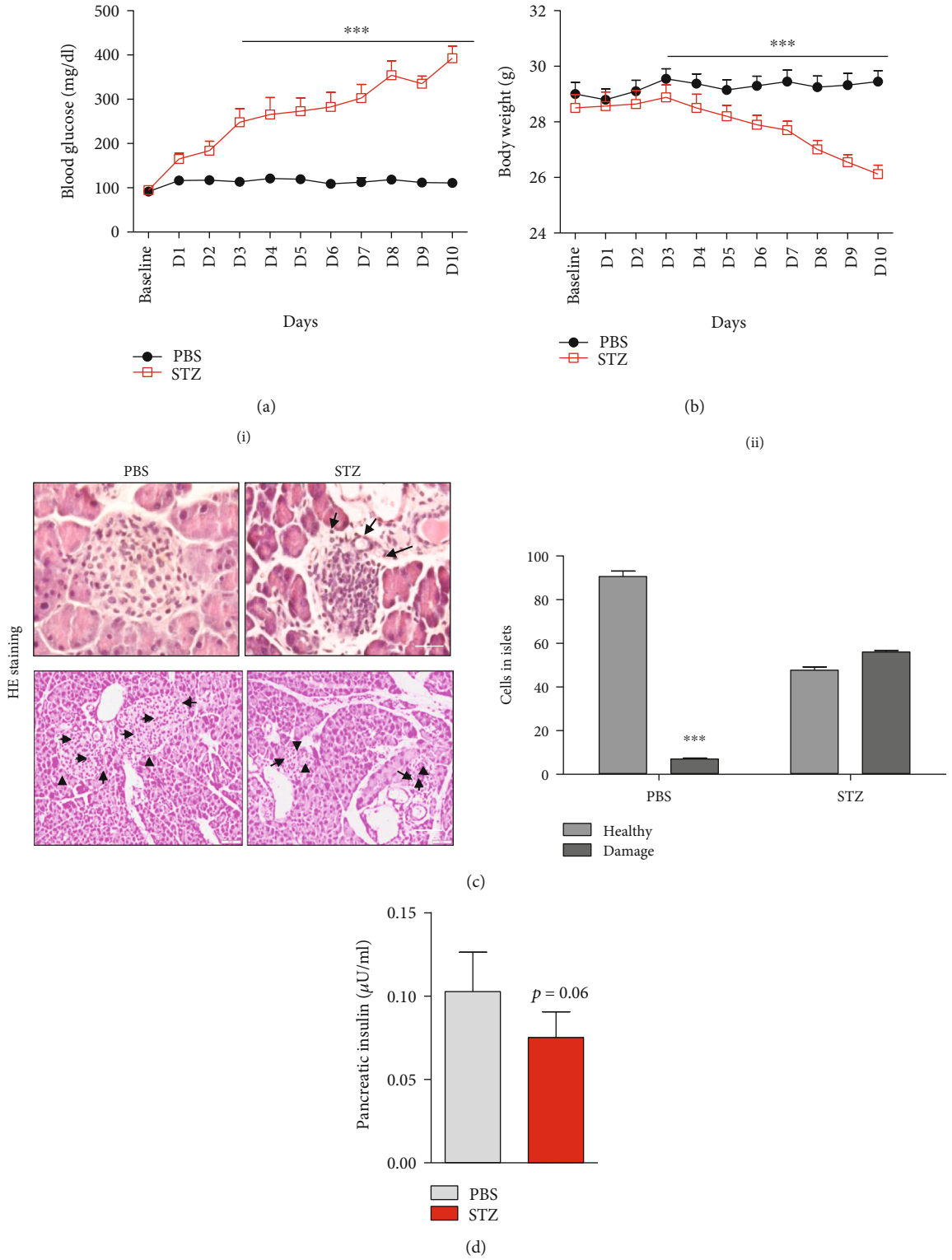



Figure 5: STZ treatment-induced diabetes in mice. (a) Line graph showing blood glucose levels in diabetic mice compared to the control animals over the experiment period ($n = 6$/group). (b) Line graph showing body weight in the diabetic group compared to the control group ($n = 6$/group). (c) (i) Representative images of histological analysis of H&E (scale bar = 20 $\mu$M) in the diabetic and control groups showing the altered pancreatic morphology. The lower panel shows images of H&E staining with multiple islets (low magnification, scale bar = 100 $\mu$M). (c) (ii) Histogram representing the number of healthy and damaged cells in islets counted on three different sections (five random fields) in the diabetic group and control group. (d) Histogram representing insulin levels in pancreatic tissue of the diabetic and control groups. Data are shown as the mean ± SD ($n = 6$/group). The significance of differences between mean values was estimated by nonparametric Student's $t$-test (unpaired, two-tailed). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control animals.

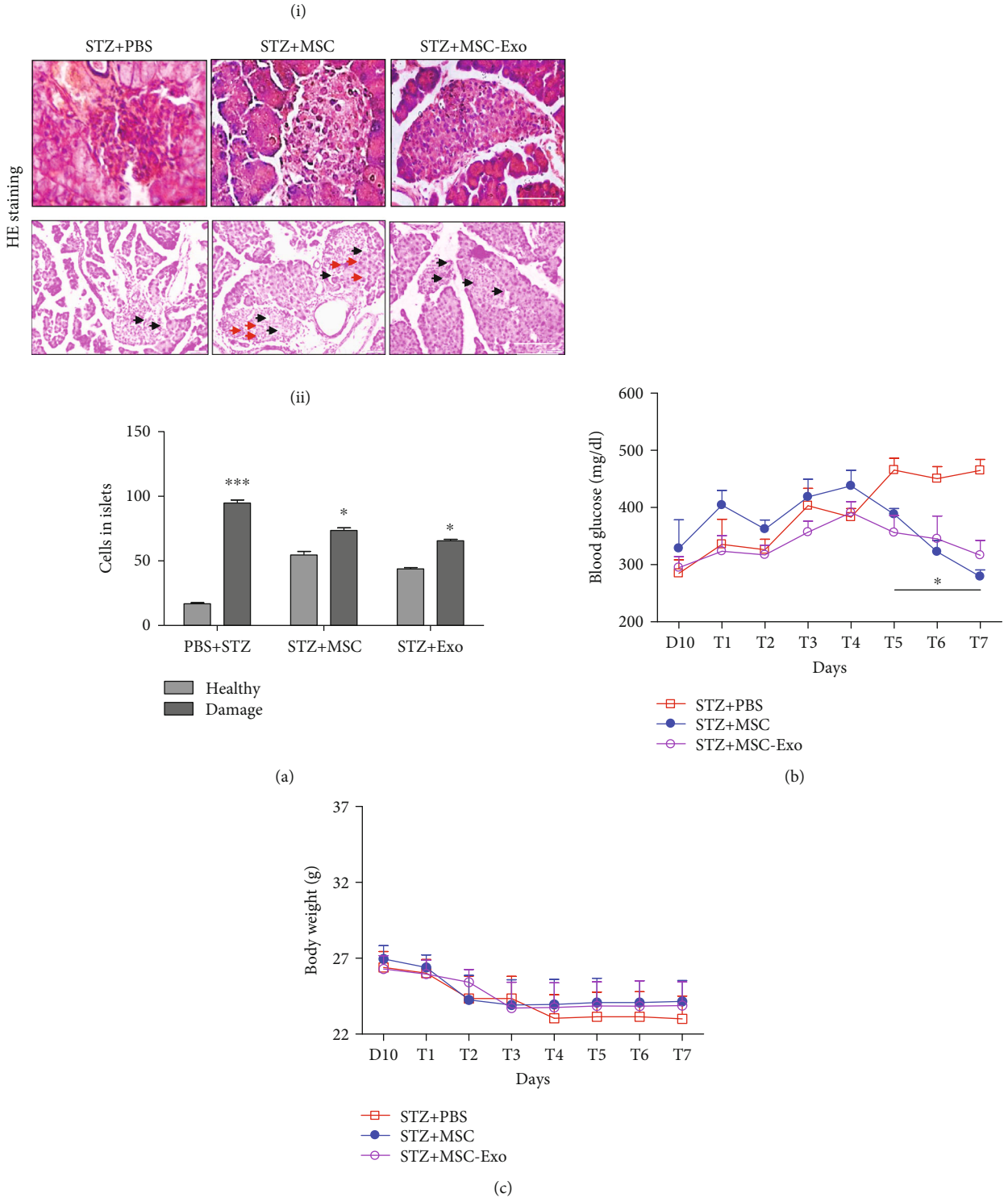



Figure 6: Reduced glucose levels in the MSC and MSC-Exo-treated groups. (a) (i) Representative images of H&E staining (scale bar = 50 $\mu$M ) of pancreatic tissue of the STZ+MSC and STZ+MSC-Exo groups compared to the STZ+PBS group. The lower panel shows images of H&E staining with multiple islets (low magnification, scale bar = 100 $\mu$M). (ii) Histogram representing number of healthy and damaged cells in islets counted on three different sections (five random fields) in the STZ+PBS, STZ+MSC, and STZ+MSC-Exo groups. (b) Line graph showing blood glucose level in diabetic animals treated with the MSC and MSC-Exo groups compared to the PBS-treated group. (c) Line graph showing body weight in diabetic animals treated with the MSC and MSC-Exo groups compared to the PBS-treated group. Data are shown as the mean ± SD ($n = 6$/group). The significance of differences between mean values was estimated by nonparametric Student's $t$-test (unpaired, two-tailed) $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the PBS-treated group.

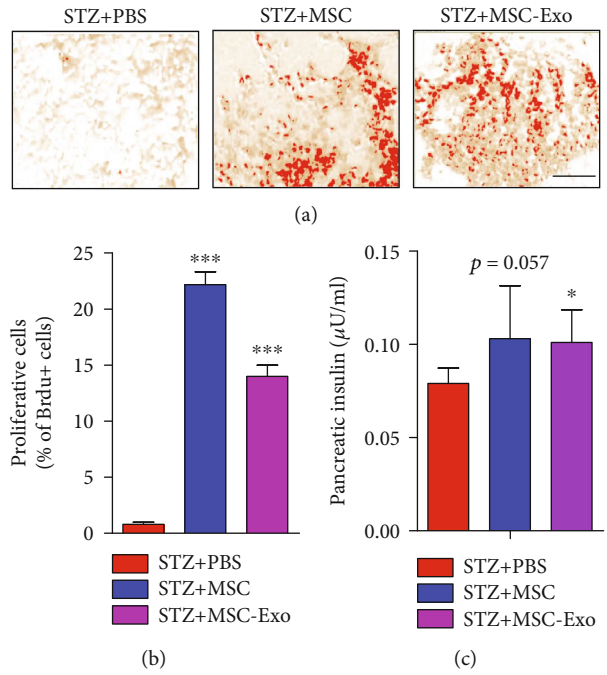
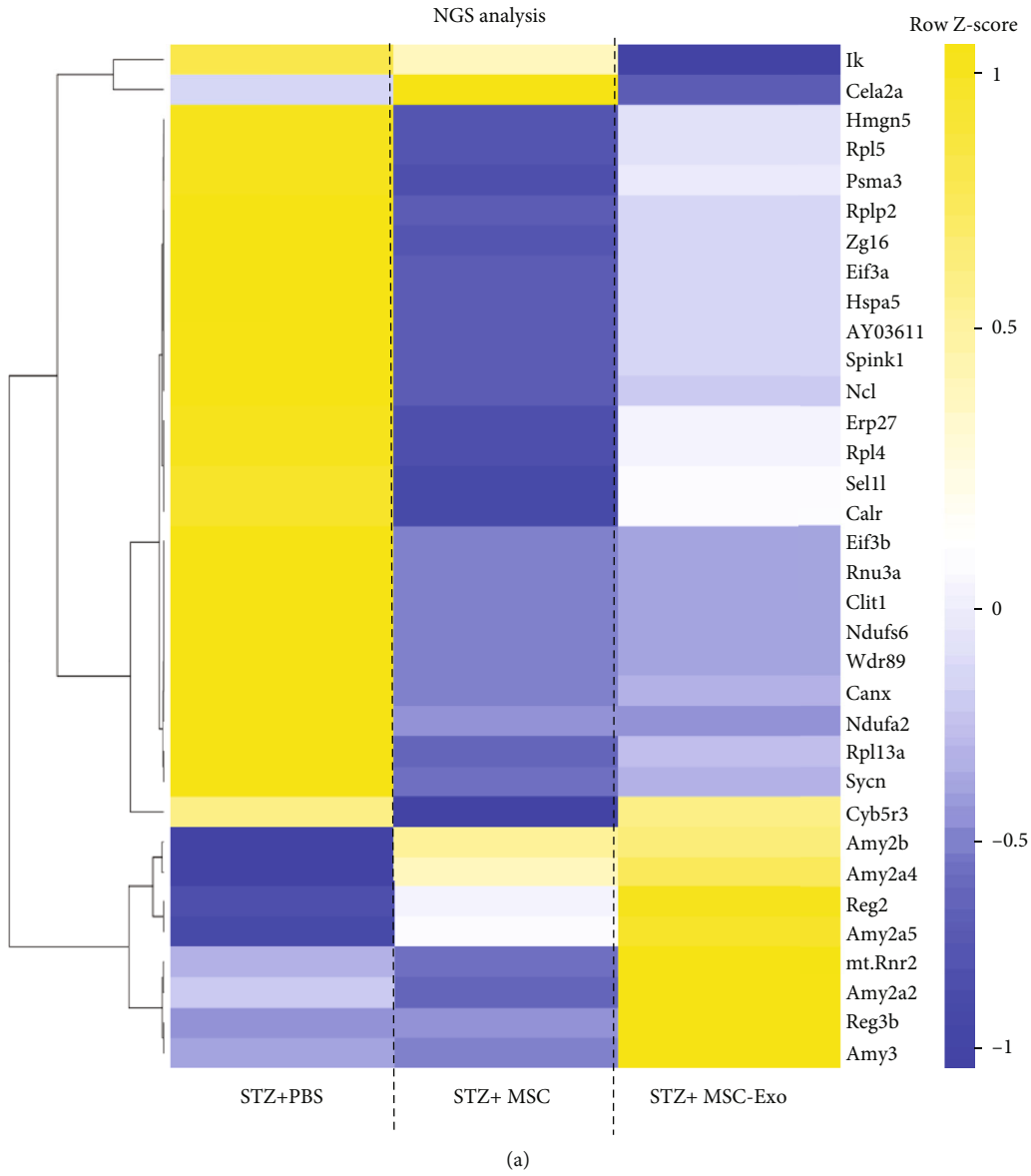



Figure 7: Intravenous post-MSC and Exo treatments increase cell proliferation and insulin level in the MSC and MSC-Exo-treated group. (a) Representative histological images of pancreatic tissue stained with BrdU showing increased BrdU-positive cells in diabetic animals treated with MSC or MSC-Exo in comparison with PBS-treated animals. Scale bar = 100 $\mu$M. (b) Histogram representing the percentage of BrdU-positive cells in MSC and MSC-Exo-treated diabetic animals in comparison with the PBS-treated group. (c) Histogram representing increased insulin level in pancreatic tissue of diabetic animals treated with MSC and MSC-Exo in comparison with PBS-treated animals. Data are shown as the mean ± SD ($n = 6$/group). The significance of differences between mean values was estimated by nonparametric Student's $t$-test (unpaired, two-tailed). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the PBS-treated group.

and downregulated genes (cut off > ±2.0 fold change), in the pancreatic tissue of diabetic animals treated with MSCs and MSC-Exo relative to the PBS group. The expressions of these differentially regulated genes were summarised in a heat map (Figure 8(a)), and pathway analysis of the differentially regulated genes showed involvement of insulin signaling and tissue regeneration pathways. Figure 8(b) shows genes, identified in our analysis, that have been associated with these pathways. A few of the identified genes were further validated using qPCR analysis, including *Reg3* (regenerating islet-derived protein III), *Reg2* (regenerating islet-derived protein II), and *Amy2b* (amylase Alpha 2b) and *TLR4* (Supplementary Table 1).

## 4. Discussion

Restoring $\beta$ cell mass is an important management modality in diabetes [3–5]. Among several strategies for restoring $\beta$ cell mass, MSCs might serve as a promising agent due to their unique properties, including conditional differentiation, tropical support, and capabilities of spontaneous differentiation into connective tissue [24]. Moreover, they possess intrinsic immunosuppressive abilities [25]. MSCs derived from several sources such as bone marrow and dental pulp have been reported to attenuate diabetes and have pancreatic islet regeneration ability [26, 27]. Currently, the therapeutic potential of MSCs was extracted from studies based on the animal models; however, four clinical trials of MSCs in T1DM are known till date, but due to smaller sample size, these results cannot be adequately evaluated and they are unable to demonstrate the significance as well [28]. Along with these, therapeutic use of MSCs may lead to cell-based therapy side effects and immune rejection. Recent studies [29–31] reported the promising role of exosomes in the treatment of T1DM. These studies suggested that MSC-derived exosome could be a better alternative to MSCs due to their fewer side effects [29–31]. However, future studies and clinical trials are required to understand their mechanism of action. Moreover, the potential of MSC and MSC-derived exosomes in attenuating endocrine deficiencies caused by nonautoimmune pancreatic $\beta$ cell loss needs to be tested. With this aim, we isolated and characterized MSCs from h-UCB (a noninvasive source of stem cells) based on their morphology, surface markers, and differentiation ability. After successful isolation of MSCs, we isolated the MSC-derived exosomes. Functional characterization of the MSC and MSC-derived exosomes was confirmed in vitro, where activated T cells were suppressed after treatment with MSC (79%) or MSC-Exo (75%). A similar report also showed the effect of MSC-Exo over MSCs in the rat model of T1DM [31]. Moreover, a therapeutic potential of stem cell-derived exosomal miRNA has been reported. Since the miRNA profiles of exosomes vary with type of stem cells, we compared the regulatory profile of these two treatment strategies (MSC and MSC-Exo) by comparing their miR profile. Using microarray analysis, we observed that hUCB-




(a)

Figure 8: Continued.

| Pathways related to insulin signaling | | Pathways related to regeneration of cells | |
|---|---|---|---|
| Pathways found in our data | Gene | Pathways found in our data | Gene |
| MAPK signaling pathway | 132 | Ribosome | 116 |
| PI3K-AKT signaling pathway | 130 | Oxidative phosphorylation | 106 |
| Ras signaling pathway | 126 | Protein processing in ER | 101 |
| Chemokine signaling pathway | 104 | Cell cycle | 094 |
| Insulin signaling | 067 | Apoptosis | 073 |
| Signaling pathways regulating | 064 | RNA transport | 067 |
| | 062 | Carbon metabolism in cancer | 048 |
| | | Proteasome | 036 |
| | | Basal transcription factors | 030 |

(b)

Figure 8: Differential gene expression in pancreatic tissue of animals treated with MSCs and MSC-Exo. (a) Heat map representing differential gene expression in the pancreatic tissue of diabetic animals treated with MSCs and MSC-Exo in comparison with PBS-treated animals. $p < 0.05$ was considered as the level of significance, and ±2-fold change was the cut off value for heat map generation. (b) The table represents significantly differentially regulated pathways.

derived MSCs and their exosomes share similar miRNA profiles. We noted a huge expression of miRs that were known to be associated with cellular proliferation, cell cycle, inflammation, apoptosis, and metabolism pathways.

After successful isolation and characterization of MSC and MSC-Exo, we assessed their effect on pancreatic regeneration and the associated mechanism of action *in vivo*. STZ-induced diabetes and pancreatic injury were confirmed, respectively, by the induction of hyperglycemia and loss of pancreatic cellular architecture (diminution of islets of Langerhans' size and insulin content) compared to the control mice. Similar to our observation, Sabry et al. [31] reported high inflammation, vacuolar degeneration, congestion, and fibrosis along with the marked decrease in the size of islets of Langerhans. In our study, we have demonstrated regenerative benefits of MSCs derived from human UCB, in STZ-induced diabetic mice. We found that both MSCs and MSC-Exo had similarly attenuated diabetes, as evidenced by decreased blood glucose levels after MSCs and MSC-Exo treatments. The treatments also promoted pancreatic regeneration compared to vehicle-treated diabetic mice. In support of our observations, study in rat pancreas demonstrated marked recovery of pancreatic architecture after treatment with MSC and MSC-Exo [31]. A similar beneficial effect of MSC-Exo, on STZ-induced diabetes, has also been reported in a recent study [32]. The author suggests that exosomes promote insulin sensitivity, increase glucose uptake and metabolism in peripheral tissues, and protect pancreatic islets from damage by inhibiting STZ-induced $\beta$ cell apoptosis. Recently, Mahdipour et al. have shown that exosomes derived from menstrual blood MSCs can regulate $\beta$ cell regeneration through PDX1-dependent mechanism in T1DM rat model [33].

Though the studies have confirmed the beneficial effect of MSC and MSC-Exo on pancreatic regeneration, but the mechanism remained illusive. Here, we performed transcriptome

profiling in diabetic, MSC-, and MSC-Exo-treated pancreas. During transcriptome analysis of the pancreatic tissues, most of the transcripts (~80%) were found to be similarly regulated in MSCs and the MSC-Exo-treated group, relative to the PBS-treated group. However, a few of the transcripts were differentially regulated between MSCs and the MSC-Exo-treated group. For example, *Cyb5r3* (NADH cytochrome b5 reductase (b5R)), *Reg2* (regenerating islet-derived protein II), *Reg3b* (regenerating islet-derived protein 3b), *mtRnr2* (mitochondrial encoded 16S rRNA2), Amy*2a5* (amylase alpha 2A5), *Amy2a2* (amylase alpha 2A2), and *Amy2a3* (amylase alpha 2A3) genes were upregulated in the MSC-Exo group, but not in the MSC-treated group, as compared to the PBS-treated group. Moreover, *Cela2a* (chymotrypsin-like elastase 2A), Amy*2a4* (amylase alpha 2A4), and *Amy2b* (amylase alpha 2b) genes were significantly upregulated in MSC-Exo relative to the MSC-treated group. The genes that regulated in the treated groups compared to the PBS-treated group majorly belong to insulin signaling and tissue regeneration pathways. Within the insulin signaling pathway, the highest number of genes was associated with the MAPK signaling (132 genes) and the PI3K-AKT pathway (130 genes), including Ras, MAPKs, AKT, and IRS, while tissue regeneration pathway includes genes associated with the following processes: oxidative phosphorylation (106 genes), ER protein processing (101 genes), cell cycle (94 genes), ribosomal pathway (116 genes, predominantly Rpl 2-13, Rps 1-30, and Mrps 1-12), and proteasomal pathway (36 genes, Psmb, Psmd, and Psme (proteosomal subunit)).

Furthermore, by using qPCR analysis, we validated the upregulation of *Reg3* (regenerating islet-derived protein III), *Reg2* (regenerating islet-derived protein 2), and *Amy2b* (amylase alpha 2B) in the pancreatic tissue of the MSC and MSC-Exo-treated groups, in comparison to the PBS-treated group. *Reg* gene encodes an endogenous lectin which may be involved in the regeneration and growth of $\beta$ cells [34]. *Reg3* could also stimulate $\beta$ cell regeneration by reducing inflammatory conditions by the Jak-Stat3 signaling pathway [34]. Thus, upregulation of *Reg3* found in the pancreas of MSC-Exo-treated diabetic animals may have a role in the $\beta$ cell regeneration leading to the recovery of the insulin level [35–37]. Furthermore, an indispensable role of Reg protein in the pathophysiology of various human inflammatory diseases, especially $\beta$ cell regeneration in pancreatic inflammation damage models, has been demonstrated [37]. Reg is regulated by multiple genes, including Glp1 [38], PDX1 [33], Extl3 [39, 40], and PARP [41] and in turn affects multiple signaling pathways in a stimulus-dependent manner. Reg3a has been shown to regulate keratinocyte proliferation and differentiation after skin injury via Reg-Extl3-PI3K-Cyclin D1 pathway [42], while Reg3g downregulates the Stat3-Socs signaling which may result in enhanced apoptosis in acute pancreatitis [43]. The pathways analysis in our study revealed that most of the downstream targets of *Reg2* and *Reg3* were altered in the pancreas of MSC-Exo-treated animals. Moreover, several miRNAs regulating these signaling pathways were found in the UCB-MSC-derived exosomes, including miRNA-regulating Extl3 (miR-let-7a-5p, miR-24-3p, and miR-19-b-1-5p) and Cela2a (miR-450-b-5p) expression. Thus, miRNAs in the MSC-derived exosomes may facilitate regeneration by regulating the Extl3-Reg-cyclinD1 pathway in the pancreas.

## 5. Conclusion

In conclusion, we have shown that exosomes derived from human UCB-MSCs significantly reduce blood glucose level, increase insulin production, limit pancreatic tissue damage, and improve disease outcomes in STZ-induced diabetic mice. Furthermore, exosome treatment-induced pancreatic tissue regeneration likely increased the Reg3 signaling pathways. Thus, UCB-MSC-derived exosomes may activate intrinsic regenerative abilities of pancreatic islets and attenuate insulin deficiency caused due to pancreatic cell destruction.

## Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

## Ethical Approval

For stem cells, the protocol has been approved by the Institutional Committee for Stem Cell Research (IC-SCR; Code: 2019-03-IMP-08). For animal models, the protocol has been approved by the Institutional Animal Ethics Committee (Ref. no: P-03/20/2017).

## Disclosure

A part of the manuscript has been presented at the "10th Annual Meeting of the International Society for Extracellular Vesicles (ISEV)-2021, held in July 2021."

## Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

## Authors' Contributions

Rajni Sharma and Manju Kumari share equal authorship.

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## Supplementary Materials

Supplementary Table 1: qPCR analysis for *Reg2*, *Reg3*, *Amy2b*, and *TLR4*. MSC-Exo treatment increases *Reg2*, *Reg3*, and *Amy2b* expressions. Along with this, MSC and

MSC-Exo treatment attenuated STZ-induced increase in the level of *TLR4* mRNA. Data represent as the mean ± SD; $^{*}p < 0.05$ versus the STZ+PBS group, by a two-tailed unpaired *t*-test. *(Supplementary materials)*

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*Review Article*

# Mitochondrial Dysfunction and Diabetic Nephropathy: Nontraditional Therapeutic Opportunities

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Diabetic nephropathy (DN) is a progressive microvascular diabetic complication. Growing evidence shows that persistent mitochondrial dysfunction contributes to the progression of renal diseases, including DN, as it alters mitochondrial homeostasis and, in turn, affects normal kidney function. Pharmacological regulation of mitochondrial networking is a promising therapeutic strategy for preventing and restoring renal function in DN. In this review, we have surveyed recent advances in elucidating the mitochondrial networking and signaling pathways in physiological and pathological contexts. Additionally, we have considered the contributions of nontraditional therapy that ameliorate mitochondrial dysfunction and discussed their molecular mechanism, highlighting the potential value of nontraditional therapies, such as herbal medicine and lifestyle interventions, in therapeutic interventions for DN. The generation of new insights using mitochondrial networking will facilitate further investigations on nontraditional therapies for DN.

## 1. Introduction

Diabetic nephropathy (DN) is a chronic disease that is caused by diabetes and is characterized by microangiopathy and alterations in kidney structure and function. It not only causes end-stage renal disease (ESRD) but also significantly increases the incidence and mortality rate of cardiovascular and cerebrovascular diseases [1]. With the rapid increase in the incidence of diabetes, the number of cases of DN worldwide has increased rapidly. In 2019, the International Diabetes Federation reported that approximately 463 million individuals were diagnosed with diabetes, and its incidence is expected to reach 700 million by 2045. In addition, approximately 30%–40% of these individuals are expected to develop DN [2]. However, current therapies delay rather than prevent the progression of ESRD, necessitating the search for new therapeutic targets to ameliorate the poor prognosis of DN. Current studies suggest that irregularities in key pathways and cellular components promote renal dysfunction and lead to DN. These include enhanced glucose metabolite flux, more glycation end (AGE) products, endoplasmic reticulum stress, mitochondrial dysfunction, abnormally active renin angiotensin system, and oxidative stress [3–6], with mitochondrial dysfunction playing a key role in the occurrence and pathogenesis of DN [7]. Various studies have emphasized the impact of nontraditional treatments, such as herbal medicine, nutrition, exercise, and surgical treatment, on the prevention and delayed progression of DN. Nontraditional therapy is considered a well-proven strategy which robustly improves health in most organisms.

Randomized controlled clinical trials have shown that herbal medicines are efficacious and safe [8, 9]. In terms of experimental research, studies provided evidence for the efficacy of nontraditional therapies from the perspectives of ameliorating mitochondrial dysfunction. This provides a rationale for further exploration of the effect of nontraditional approaches on DN at the molecular level. Mitochondria are important for renal cell survival, as these serve as metabolic energy producers and regulate programmed cell death. The structure and function of mitochondria are regulated by a mitochondrial quality control (MQC) system, which is a series of processes that include mitochondrial biogenesis, mitochondrial proteostasis, mitochondrial dynamics/mitophagy, and mitochondria-mediated cell death. In this review, we have outlined the physiological role of mitochondria in renal function, discussed the role of mitochondrial dysfunction in the occurrence and development of DN, and emphasized on the therapeutic effect of nontraditional treatments, particularly herbal medicine (Table 1) and lifestyle interventions, on DN by targeting mitochondrial networking.

## 2. Critical Mediator of DN: Mitochondrial Dysfunction

The kidney, a highly metabolic organ rich in mitochondria, requires a large amount of ATP for its normal function [30]. The kidney possesses the second highest oxygen consumption and mitochondrial content following the heart [30, 31]. Mitochondrial energetics are altered in DN due to hyperglycemia, which induces changes in the electron transport chain (ETC) which cause an increase in reactive oxygen species (ROS) and a decrease in ATP production. This leads to increased mitochondrial division, decreased PGC1$\alpha$ levels, changes in mitochondrial morphology, increased cell apoptosis, and further aggravation of the condition [32–34] (Figure 1).

*2.1. Mitochondria: The "Energy Station" for the Kidney.* In general, the mechanism of ATP production in kidney cells is determined by the cell type. For example, proximal tubules in the renal cortex are dependent on oxidative phosphorylation for ATP production to fuel active glucose, nutrient, and ion transport [35]. However, glomerular cells such as podocytes and mesangial cells are largely utilized for filtering blood, removal of small molecules (e.g., glucose, urea, salt, and water), and retaining large proteins, including hemoglobin [36]. This passive process does not require direct ATP. Therefore, glomerular cells can perform aerobic and anaerobic respiration to produce ATP for basic cellular processes [37–40]. ATP is produced through the respiratory chain, which includes five multienzyme protein complexes embedded in the inner mitochondrial membrane [19], including complex I: NADH CoQ reductase, complex II: succinate-CoQ reductase, complex III: reduced CoQ-cytochrome c reductase, complex IV: cytochrome c oxidase, and complex V: ATP synthase. One palmitate molecule produces 106 ATP molecules, whereas glucose oxidation yields only 36 ATP molecules [41, 42]. Due to the higher energy requirements of the proximal tubules, they use nonesterified fatty acids, such as palmitate, to maximize the production of ATP through $\beta$-oxidation. In the diabetic state, there is a large amount of substrate in the form of glucose, which provides fuel for the citric acid cycle and produces more NADH and FADH2. However, during the electron transfer process, the generation of a greater reducing force leads to electron leak; these electrons combine with oxygen molecules to produce a large amount of ROS and induce oxidative stress [43, 44].

*2.2. ROS and Mitochondrial Dysfunction in DN: Dangerous Liaisons.* The double membrane structure of mitochondria contains a large number of unsaturated fatty acids which are highly vulnerable to ROS attack. Excessive ROS results in membrane lipid peroxidation as well as triggers the mitochondrial permeability transition pore (mPTP) to abnormally open, which in turn increases its permeability and allows proteins to enter the membrane space. These negatively charged proteins are released into the cytoplasm, causing positive ions in the membrane gap to flow back into the matrix. Subsequently, the ion concentration gradient on both sides of the mitochondrial inner membrane disappears [45], mitochondrial membrane potential decreases, oxidative phosphorylation uncouples, and ATP synthesis is blocked. At the same time, it causes an imbalance of related molecules moving in and out of the mitochondria, leading to the dysfunction of the mitochondrial and cytoplasmic barriers. The greater concentration of positive ions in the mitochondrial matrix than in the cytoplasm aggravates swelling and even ruptures the mitochondria [46]. Since mitochondrial DNA (mtDNA) lacks the protection of introns, histones, and other DNA-related proteins and it is near the electron transport chain where ROS production occurs, it is more susceptible to ROS attack than nuclear DNA. Mutations may occur that lead to mitochondrial dysfunction and contribute to the progression of DN [4, 45, 47]. According to a previous study, mtDNA damage precedes bioenergy dysfunction in DN, indicating that systemic mitochondrial dysfunction and glucose-induced mtDNA changes can lead to DN [48]. In general, ROS and mitochondrial dysfunction are mutually causes and effects, forming a vicious cycle.

*2.3. Imbalance of Mitochondrial Dynamics in DN: A Vicious Cycle.* Mitochondria are highly dynamic organelles that regulate their shape, quantity, distribution, and function through continuous fusion and fission. They form a network-like mode of action in the cell which can be redistributed to meet the energy needs of the cell to the maximum extent as it is important to maintain cell homeostasis [49, 50]. Mitochondrial fusion is mainly involved in the synthesis and repair of mitochondria. When the mitochondria are slightly damaged by harmful stress, such as mtDNA variation and mitochondrial membrane potential decline, the fusion of damaged mitochondria and healthy mitochondria can repair the mutated mtDNA and restore the membrane potential to realize self-repair [51]. Mitochondrial fission also contributes to the maintenance of mitochondrial membrane potential and mtDNA stability. Depolarized

Table 1: Mitochondria-targeted herb medicine in DN.

| Herb medicine | The form of herb medicine | Experimental model | Target | Pathway | Observed effect | Ref. |
|---|---|---|---|---|---|---|
| | | | *Mitochondrial biogenesis* | | | |
| Berberine | Pure chemical | Patients with DN, db/db diabetic mice | PCG-1$\alpha$↑, FAO↑, AMPK↑ | PGC-1$\alpha$ signaling pathway | Restoration of PGC-1$\alpha$ activity and the energy homeostasis | [10] |
| Tangshen formula | Extract | db/db diabetic mice, mTECs | PGC-1$\alpha$↑, LXR↑, ABCA1↑ | PGC-1$\alpha$-LXR-ABCA1 pathway | Improving cholesterol efflux | [11] |
| Salidroside | Pure chemical | db/db diabetic mice | SIRT1↑, PGC-1$\alpha$↑ | SIRT1/PGC-1$\alpha$ axis | Improving mitochondrial biogenesis | [12] |
| Resveratrol | Pure chemical | db/db diabetic mice, HGECs | AdipoR1↑, AdipoR2↑, AMPK↑, SIRT1↑, PGC-1$\alpha$↑, PPAR$\alpha$↑ | AMPK–SIRT1–PGC–1$\alpha$ axis | Ameliorating lipotoxicity, oxidative stress, apoptosis, and endothelial dysfunction | [13] |
| Resveratrol | Pure chemical | db/db diabetic mice | AMPK↑, SIRT1↑, PGC-1$\alpha$↑, PPAR$\alpha$↑ | AMPK–SIRT1–PGC–1$\alpha$ axis | Prevention of lipotoxicity-related apoptosis and oxidative stress | [14] |
| Resveratrol | Pure chemical | STZ-induced diabetic rats, podocytes | SIRT1↑, PGC-1$\alpha$↑, ROS↓ | SIRT1/PGC-1$\alpha$ axis | Inhibition of mitochondrial oxidative stress and apoptosis | [15] |
| Resveratrol | Pure chemical | DN rabbits with AKI, HK-2 cells | SIRT1↑, PGC-1$\alpha$↑, HIF-1$\alpha$↓ | SIRT1–PGC–1$\alpha$–HIF-1$\alpha$ signaling pathways | Reducing renal hypoxia, mitochondrial dysfunction and renal tubular cell apoptosis | [16] |
| Marein | Extract | db/db diabetic mice, HK-2 cells | SGLT2↓, SREBP-1↓, AMPK↑, PGC-1$\alpha$↑ | AMPK/ACC/PGC-1$\alpha$ pathway | Amelioration of fibrosis and inflammation | [17] |
| | | | *Mitochondrial dynamics* | | | |
| Berberine | Pure chemical | db/db diabetic mice, podocytes | DRP1↓, MFF↓, FIS1↓, MID49↓, MID51↓, PGC-1$\alpha$↑ | DRP1 modulator | Inhibiting mitochondrial fission and cell apoptosis | [18] |
| Astragaloside IV | Pure chemical | db/db diabetic mice | Drp1↓, MFF↓, Fis1↓ | Mitochondrial quality control network | Amelioration of renal injury | [19] |
| Polydatin | Pure chemical | KKAy mice, hyperglycemia-induced MPC5 cells | DRP1↓, ROS↓, caspase-3↓, caspase-9↓ | ROS/DRP1/mitochondrial fission/apoptosis pathway | Impairing mitochondria fitness and ameliorating podocyte injury | [20] |
| | | | *Mitophagy* | | | |
| Astragaloside II | Pure chemical | STZ-induced diabetic rats | NRF2↑, Keap1↓, PINK1↑, Parkin↑ | NRF2 and PINK1 pathway | Amelioration of podocyte injury and mitochondrial dysfunction | [21] |
| Huangqi-Danshen decoction | Extract | db/db diabetic mice | DRP-1↓, PINK1↑, Parkin↑ | PINK1/Parkin pathway | Protection kidney injury by inhibiting PINK1/Parkin-mediated mitophagy | [22] |
| | | | *Mitochondria ROS* | | | |
| Nepeta angustifolia C. Y. Wu | Extract | HFD/STZ-induced diabetic rats, mesangial cells | SOD↑, ROS↓, MDA↓ | Mitochondrial-caspase apoptosis pathway | Antioxidative stress, inflammation and inhibiting mesangial cell apoptosis | [23] |
| Resveratrol | Pure chemical | db/db diabetic mice | ROS↓, AMPK↑, SIRT1 | AMPK/SIRT1-independent pathway | Antioxidative stress and enhanced mitochondrial biogenesis | [24] |
| Betulinic acid | Pure chemical | STZ-induced diabetic rats | SOD↑, CAT ↑, MDA↓, AMPK, NF-$\kappa$B↓, NRF2↑ | AMPK/NF-$\kappa$B/NRF2 signaling pathway | Attenuating the oxidative stress and inflammatory condition | [25] |
| Obacunone | Pure chemical | NRK-52E cells | SOD↑, GSK-3$\beta$↓, NRF2↑ | GSK-3$\beta$/Fyn pathway | | [26] |

Table 1: Continued.

| Herb medicine | The form of herb medicine | Experimental model | Target | Pathway | Observed effect | Ref. |
|---|---|---|---|---|---|---|
| | | | | | Inhibiting oxidative stress and mitochondrial dysfunction | |
| Curcumin | Pure chemical | STZ-induced diabetic rats | NRF2↑, FOXO-3a↑, PKC$\beta$II↓, NF-$\kappa$B↓ | PKC $\beta$II/p 66 Shc axis | Antioxidative stress | [27] |
| Notoginsenoside R1 | Pure chemical | db/db diabetic mice, HK-2 cells | ROS↓, NRF2↑, HO-1↑ | NRF2 pathway | Inhibition of apoptosis and renal fibrosis caused by oxidative stress | [28] |
| Oleanolic acid and N-acetylcysteine | Pure chemical | Type 2 diabetic rat model, mesangial cells | ROS↓, NRF2↑, TGF-$\beta$/smad2/3↓, $\alpha$-SMA↓ | NRF2/Keap1 system | Inhibition of oxidative stress and ER stress | [29] |

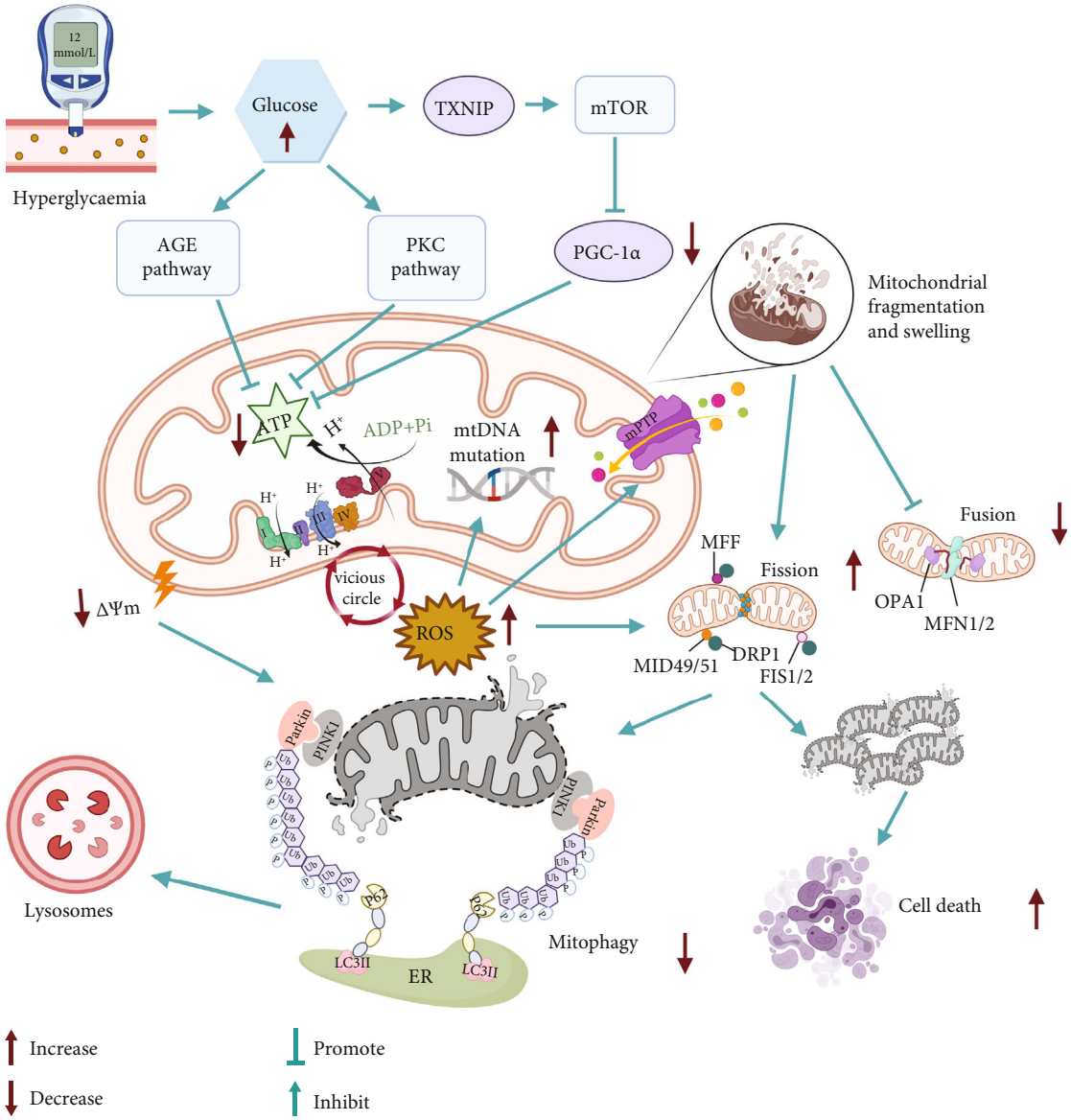


Figure 1: Hyperglycemia serves as the primary factor that influences mitochondrial dysfunction in DN. The increased level of glucose enhances glycolysis, and the subsequent activation of the TXNIP, AGE, and PKC pathways reinforces the decrease in ATP levels. Insufficient ATP levels stimulate the ETC to overwork in response to the energy supply for the kidneys. In turn, excessive ROS production occurs following the overactivation of the ETC, which results in decreased ATP production, mutation of mtDNA, abnormal opening of the mitochondrial permeability transition pore, and ultimately mitochondrial fragmentation and swelling. Decreases in the levels of OPA1, MFN1, and MFN2 may contribute to the decrease in mitochondrial fusion observed in DN. Activation of DRP1 promotes mitochondrial fragmentation and fission. Damaged mitochondria are cleared by mitophagy. However, an excess number of damaged mitochondria that is higher than the rate of mitophagy may result in cell death. Abbreviations: DN: diabetic nephropathy; DRP1: dynamin 1-like protein; PGC-1$\alpha$: PGC1$\alpha$, peroxisome proliferator-activated receptor $\gamma$ coactivator 1$\alpha$; AMPK: 5′-AMP-activated protein kinase; SIRT1: sirtuin-1; PINK1: putative kinase protein 1; Cyt c: cytochrome c; ROS: reactive oxygen species; MFN1 and 2: mitofusin proteins 1 and 2; OPA1: optic atrophy protein 1; MFF: mitofission proteins; FIS1: mitochondrial fission 1; PPAR: peroxisome proliferator-activated receptor; Parkin: E3 ubiquitin-protein ligase parkin; ER: endoplasmic reticulum; TXNIP: thioredoxin-interacting protein; AGE: advanced glycation end; PKC: protein kinase C; ETC: electron transport chain.

mitochondrial membranes and altered mtDNA accumulate during mitochondrial fission and are discarded by autophagy or the ubiquitin-proteasome system in order to maintain normal mitochondrial function [52–54]. Increases in the levels of proteins that facilitate mitochondrial fusion occur early in the disease process in the kidneys of patients with diabetes [33]. These increases may be an early compensatory event for increased ATP demand because increasing mitochondrial fusion induced by high glucose 1 [55] or mitofusins (MFN1 and MFN2) can increase mitochondrial bioenergy function and reduce diabetic kidney damage. Fusion may also prevent renal damage in diabetes by balancing mitochondrial fission and fragmentation, which is generally considered harmful in DN.

When the mitochondrial membrane potential is damaged, the pathway for PTEN-induced putative kinase protein 1 (PINK1) to enter the inner membrane of mitochondria is blocked; therefore, it accumulates in the outer membrane, recruits Parkin to the damaged mitochondria, and phosphorylates it. Activated Parkin can ubiquitinate voltage-dependent anion channel protein 1, MFN1, MFN2, and other substrates embedded in the outer membrane. This leads to further regulation of mitochondrial morphology and dynamic changes in fission and fusion. Subsequently, the ubiquitinated mitochondria, with the assistance of autophagy receptor regulatory proteins, such as P62/SQSTM1 and microtubule-associated protein light chain 3, aggregate into double-layer autophagic vesicles, which are encapsulated to form mitochondrial autophagosomes, and fuse with lysosomes to form mitochondrial autophagic lysosomes that are degraded by hydrolases [56, 57]. Nevertheless, accumulation of autophagosomes containing mitochondria has been found in the kidneys of patients with diabetes [58] and rodent models of DN [58–60]. Although dysfunctional mitochondria can be removed by mitophagy, these can also trigger cell death in the presence of an extremely high number of damaged mitochondria relative to the rate of mitophagy [34]. Programmed cell death may occur in several forms, which include apoptosis, programmed necrosis, and autophagic cell death. Despite these distinct cell death pathways, members of the Bcl-2 family have been implicated in the direct or indirect control of mitochondrial processes [61, 62]. The permeability of the damaged mitochondrial membrane changes, resulting in the disappearance of the membrane potential, the rupture of mitochondria, and the release of intermembrane space cell death proteins (such as Cyt c, Smac/DIABLO, and HtrA2/Omi) into the cytoplasm, ultimately leading to cell death [63–65].

## 3. Maintaining Mitochondrial Homeostasis: The Target of Herbal Medicine in DN

Mitochondrial homeostasis pertains to the balance between mitochondrial fission, fusion, and biogenesis and mitophagy, which maintains mitochondrial energetics. Diseases such as DN can disrupt mitochondrial homeostasis and thus contributes to disease progression. In recent years, most of the studies on the mechanisms of herbal medicine treatment of DN focus on improving mitochondrial homeostasis and function, aiming to restore renal function and slow the progression of DN (Figure 2).

*3.1. Mechanism of Herb Medicine on Mitochondrial Biogenesis in DN.* The complex process of mitochondrial biogenesis involves the generation of new mitochondrial mass and mtDNA replication, which are derived from preexisting mitochondria. This increases ATP production to meet the growing energy demands of cells. Mitochondrial biosynthesis is controlled by various transcriptional coactivating and coinhibitory factors [66, 67]; however, the peroxisome proliferator-activated receptor $\gamma$ coactivator- (PGC-) 1$\alpha$ remains as the predominant upstream transcriptional regulator of mitochondrial biogenesis [68]. In several gain- and loss-of-function experimental studies, the activation of PGC-1 has been demonstrated to upregulate the expression of mitochondrial genes, including nuclear respiratory factor- (NRF-) 1, NRF-2, peroxisome proliferator-activated receptors (PPARs), and estrogen-related receptor alpha [69–71]. PGC-1$\alpha$ binds to PPARs, which act as master regulators of fatty acid oxidation (FAO) and nutrient supply [72]. To note, kidney proximal tubules have high levels of baseline energy consumption, supporting FAO as the preferred energy source in proximal tubules [73]. Defective FAO causes lipid accumulation, apoptosis, and tubule epithelial cell dedifferentiation [74]. Taken together, PGC-1$\alpha$ regulates complex processes of nutrient availability, FAO, and mitochondria biogenesis.

However, reduced PGC-1$\alpha$ expression and consequent dysfunctional mitochondria have been observed in patients with DN and animal models [6, 75–77]. Moreover, cholesterol accumulation in the kidney is a risk factor for DN progression. PGC-1$\alpha$ acts as a master regulator of lipid metabolism by regulating mitochondria [78]. Given the pivotal role of PGC-1$\alpha$ and metabolism in kidney cells, it is important to search for new approaches to restore the activity of PGC-1$\alpha$ in DN. An increasing number of studies have demonstrated that the interventional mechanisms of herbal medicines on DN are associated with this target. Berberine (BBR), an isoquinoline alkaloid present in Chinese herbal medicine (CHM), is widely used for treating DN. In particular, BBR can directly regulate PGC-1$\alpha$ to enhance FAO in DN, which promotes mitochondrial energy homeostasis and energy metabolism in podocytes [10]. Tangshen formula is a CHM that ameliorates kidney injuries in a diabetic model by promoting the PGC-1$\alpha$-LXR-ABCA1 pathway to improve renal cholesterol efflux in db/db mice [11]. Moreover, an active component of the traditional Chinese medicine herb Rhodiola rosea L., salidroside, has been reported to greatly attenuate DN by upregulating mtDNA copy number and ETC protein expression [12].

As PGC-1$\alpha$ is almost ubiquitously expressed, targeting its upstream regulatory sensors such as 5′-AMP-activated protein kinase (AMPK), NAD-dependent protein deacetylase sirtuin-1 (SIRT1) is generally acknowledged as a significant method to restore mitochondrial function. AMPK, an extensively studied upstream regulator of PGC-1$\alpha$, increases the rate of mitochondrial biogenesis by initiating the transcription of the *PPARGC1A* gene and by phosphorylating amino acids threonine-177 and serine-538, which in turn activates PGC-1$\alpha$. In dead, herbal medicine prevents DN via the AMPK-SIRT1-PGC-1$\alpha$ axis that is a hot spot. Resveratrol is a naturally occurring polyphenol that imparts anti-inflammatory, antidiabetic, antioxidative, and neuroprotective effects. Particularly, resveratrol prevents DN via activation of the AMPK-SIRT1-PGC-1$\alpha$ axis, and PPARs were coactivated by PGC-1$\alpha$ in db/db mice [14]. Additional studies revealed resveratrol imparts a protective effect against DN by improving lipotoxicity, oxidative stress, and apoptosis by directly activating AdipoR1 and AdipoR2 that in turn upregulates AMPK and forkhead box protein O (FoxO) expression [13]. Interestingly, Zhang et al. further

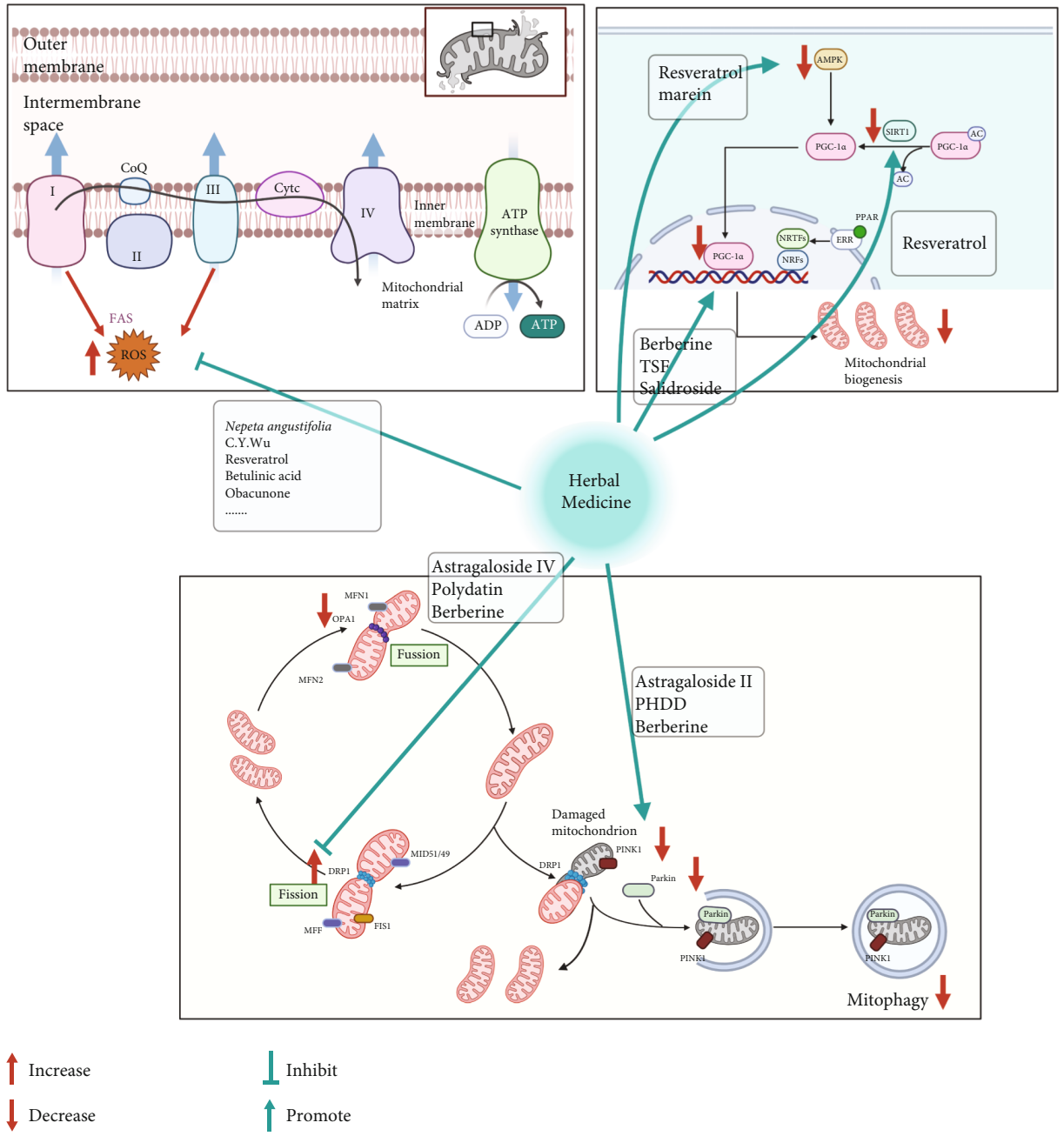


Figure 2: Therapeutic target of herbal medicine on mitochondrial dysfunction in DN. Herbal medicine plays a protective role in inhibiting DRP1-mediated mitochondrial dynamics to improve mitochondrial dysfunction in DN. In addition, herbal medicine enhances mitochondrial biogenesis by inducing the expression of PGC-1$\alpha$ and its upstream regulators (AMPK and SIRT1) and drives PINK1/Parkin-mediated mitophagy. In addition, the renoprotective effects of herbal medicine are associated with antioxidative stress. Abbreviations: DN: diabetic nephropathy; DRP1: dynamin 1-like protein; PGC-1$\alpha$: PGC1$\alpha$, peroxisome proliferator-activated receptor $\gamma$ coactivator 1$\alpha$; AMPK: 5′-AMP-activated protein kinase; SIRT1: sirtuin-1; PINK1: putative kinase protein 1; Cyt c: cytochrome c; ROS: reactive oxygen species; MFN1 and 2: mitofusin proteins 1 and 2; OPA1: optic atrophy protein 1; MFF: mitofission proteins; FIS1: mitochondrial fission 1; PPAR: peroxisome proliferator-activated receptor; Parkin: E3 ubiquitin-protein ligase parkin.

investigate the renoprotection mechanism of resveratrol *in vivo* and *in vitro*, which suggested that SIRT1/PGC-1$\alpha$ was upregulated, accompanied by improved mitochondrial function decreased oxidative stress and apoptosis [15]. In addition to single DN, the protective role of resveratrol has been verified in acute renal injury with DN via activating SIRT1–PGC–1$\alpha$–hypoxia-inducible transcription factor-1$\alpha$ (HIF-1$\alpha$) signaling pathways [16]. Because the role of sodium glucose cotransporter 2 (SGLT2) in excess glucose reabsorption has become a research topic of interest, SGLT2 inhibitors (SGLT2i) could reduce hyperfiltration and inhibit inflammatory and fibrotic responses that are elicited by proximal tubular cells [34, 79]. In addition, SGLT2i that enhances the excretion of urinary glucose triggers AMPK,

a nutrient sensor, which in turn reverses the metabolic disorders associated with DN [80]. Marein is one of the main active components of Coreopsis tinctoria Nutt, which possesses renoprotective activity in DN by directly suppressing SGLT2 expression and then activating the AMPK–acetyl CoA carboxylase (ACC)–PGC-1$\alpha$ pathway to suppress fibrosis and inflammation [17].

*3.2. Mechanism of Herb Medicine on Mitochondrial Dynamics in DN.* Mitochondria are highly dynamic organelles that require correct mitochondrial morphology to maintain maximal ATP production [81]. The major processes of mitophagy, fission, and fusion occur as a response to mitochondrial dynamics as well as to maintain mitochondrial integrity in different metabolic conditions. Fission is essential in the isolation of damaged parts from the rest of the mitochondrial network and is induced by translocating dynamin 1-like protein (DRP1) from the cytosol to the outer membrane of the mitochondria where it binds to its receptors, including mitochondrial fission 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins MID49 and MID51 [82, 83]. Mitochondria fusion involves the recruitment of a series of proteins that include MFN1 and MFN2 that triggers outer membrane fusion, as well as optic atrophy protein 1 (OPA1) that facilitates inner membrane fusion. However, in DN, excessive mitochondrial fission and fusion are associated with key features of renal damage. Mitochondrial dysfunction in podocytes is increasingly recognized as a factor contributing to the pathogenesis of DN [84].

Previous studies have elucidated the correlation between mitochondrial dynamics disorder and DN progression and revealed that DRP1 may be potentially utilized as a therapeutic target in the treatment of DN [85]. Meanwhile, traditional Chinese medicine has definite effects on this point. Besides its function on PGC-1$\alpha$-mediated mitochondrial biogenesis, BBR plays a therapeutic role in positively regulating DRP1-mediated mitochondrial dynamics to protect glomerulus and improve the fragmentation and dysfunction of mitochondria in podocytes [18]. AS-IV is a major and active component of *Astragalus*, which is a traditional Chinese medicinal herb for tonifying. Liu et al. have shown in diabetic db/db mice that AS-IV significantly improves albuminuria and renal pathologic injury. In addition, they found AS-IV decreased the elevation of renal DRP1, Fis1, and MFF expression in db/db mice [19]. More than that, polydatin which is mainly extracted from the roots of Polygonum cuspidatum not only inhibits DRP1 activation and fragmented mitochondria caused by high glucose but also blocked the increase of apoptosis through a DRP1-dependent mechanism [20, 86].

Mitophagy allows the removal of damaged and nonfunctional mitochondria from the network and requires the efficient recognition of targeted mitochondria followed by the engulfment of mitochondria by autophagosomes [87]. As part of a healthy network of mitochondria, mitophagy is regulated by a PINK1-PARKIN pathway for mitochondrial identification and labeling [88]. However, impairment of the mitophagy system aggravated the progression of DN, which was mainly caused by decreases in renal PINK1 and Parkin expression in diabetes following activation of either FOXO1 or NRF2 signal [89, 90]. In recent years, the role and regulation of mitophagy in DN have attracted lots of attention. It has been reported that AS II, another of the active constituents of Astragalus, exerts protective effects on podocyte injury and mitochondrial dysfunction through enhancing mitophagy activation via modulation of NRF2 and PINK1 [21]. Huangqi-Danshen decoction which mainly includes Astragali Radix (Huang-qi) and Salviae Miltiorrhizae Radix et Rhizoma (Dan-shen) significantly alleviated DN, which might be associated with the reversion of the enhanced mitochondrial fission and the inhibition of PINK1/Parkin-mediated mitophagy [22].

## 4. The Modulation of Mitochondrial ROS: The Effect of Herb Medicine in DN

Increased oxidative stress is due to ROS production caused by dysfunctional cellular respiration during hyperglycemia and is the major pathway involved in the pathogenesis of DN [3]. In the early phase of pathogenesis, ROS mainly from mitochondria origin do have a role in the regulation of various metabolic pathways. However, their accumulation that exceeds local antioxidant capacity is a biomarker of mitochondrial dysfunction in DN [91]. Overproduction of ROS can subsequently induce oxidative stress and cause damage to critical cellular components (particularly protein and DNA) and glomerular podocyte, which contributed to inflammation, interstitial fibrosis, and apoptosis [92, 93]. The damaging effect of ROS is thought to be mediated by activation of several pathways such as NF-$\kappa$B, hexosamine, and the formation of AGE products [94]. The transcription of genes that encode antioxidant enzymes, including SOD2, glutathione peroxidase, and catalase, is activated by NRF2, which in turn promotes antioxidant activity and induces negative feedback on NF-$\kappa$B [95, 96]. Therefore, an emerging therapeutic target antioxidant defense mechanism and promoting renoprotection in DN involves the activation of NRF2 with its mediated antioxidant enzymes [97], while traditional Chinese medicine has definite antioxidant effects.

*Nepeta angustifolia* C. Y. Wu, an important medicinal material constituting a variety of traditional Chinese medicine prescription, has significant antioxidant activity [98]. *Nepeta angustifolia* C. Y. Wu inhibits proinflammatory mediators and renal oxidative stress in diabetic rats, as well as improves mitochondrial potential to disrupt mesangial cell apoptosis caused by oxidative stress *in vitro* [23]. AMPK is an energy sensor in metabolic homeostasis [99]. Recent studies have shown that AMPK participates in the attenuation of oxidative stress in DN [100]. The beneficial effects of resveratrol on renal diseases are attributed to its antioxidative properties. Moreover, the study conducted by Kitada et al. [24] indicated that resveratrol can enhance mitochondrial biogenesis and protect against DN through normalisation of Mn-SOD dysfunction via the AMPK/SIRT1-independent pathway. Betulinic acid is extracted from the outer bark of white birch trees and exerts a protective effect

on DN by effectively attenuating oxidative stress and inflammatory conditions via the AMPK/NF-$\kappa$B/NRF2 signaling pathway [25]. Zhou et al. showed that obacunone, a natural bioactive compound isolated from the Rutaceae family, blocks GSK-3$\beta$ signal transduction and subsequently enhances the activity of NRF2 to inhibit oxidative stress and mitochondrial dysfunction in NRK-52E cells [26]. Furthermore, Yahya et al. [27] showed using STZ-induced diabetic rats that curcumin imparts a nephroprotective effect via NRF2 activation, inhibition of NF-$\kappa$B, suppression of NADPH oxidase, and downregulation/inhibition the PKC $\beta$II/p66 Shc axis. The role of herbal medicine in NRF2/AGE signal should also be carefully considered as NRF2/AGE plays a pivotal role in controlling transcriptional regulation of the genes encoding endogenous antioxidant enzymes [97]. Notoginsenoside R1, a novel phytoestrogen isolated from *Panax notoginseng* (Burk.) F. H. Chen, was found to decrease AGE-induced mitochondrial injury and promote NRF2 and HO-1 expression to eliminate oxidative stress and apoptosis in DN [28]. Further, oleanolic acid combined with N-acetylcysteine has therapeutic effects on DN through an antioxidative effect and endoplasmic reticulum stress reduction by the NRF2/Keap1 system [29].

## 5. Lifestyle Interventions

Diabetes is usually accompanied by excessive nutrition and calories, as well as a decrease in physical activity, both of which aggravate nephropathy [91]. In 2020, the consensus statement of the American Association of Clinical Endocrinologists and American College of Endocrinology on the comprehensive management algorithm for type 2 diabetes (T2D) mentioned that lifestyle optimization is essential for all diabetic patients, including healthy eating patterns, weight loss, physical activity, and smoking cessation [101]. We summarize therapeutic strategies about lifestyle intervention, with a focus on mitochondrial biogenesis, to improve the malignant progress of DN.

*5.1. Healthy Eating Patterns and DN.* Healthy eating patterns are important for patients with diabetes and DN to maintain glucose control and inhibit the progression of kidney damage [102]. Particularly in late-stage kidney disease, a low-protein diet (LPD) can maintain the renal function in patients with chronic kidney disease (CKD), including those with DN [103–106]. In terms of the molecular mechanism of LPD against DN, earlier animal studies have revealed that LPD decreases intraglomerular pressure via reduction of afferent arteriole vasoconstriction, which in turn improves glomerular hyperfiltration and hypertension as well as reduces fibrosis of mesangial cells via growth factor-$\beta$ signals. Furthermore, an LPD, particularly a very LPD, can also prevent renal tubular cell injury, apoptosis, inflammation/oxidative stress, and fibrosis within the tubule-interstitial region by reducing the accumulation damaged mitochondria, which is triggered by the reduction in the activity of mammalian target of rapamycin complex 1 and the restoration of autophagy [107]. However, due to the insufficiency of clear results from present clinical trials, the renal protective effect of LPD against DN is controversial. Existing clinical research evidence is unable to fully prove the renal protective effect of LPD [108–110], although other studies have shown that LPD can delay the decline of renal function [111, 112]. In addition, the American Diabetes Association believes that a short-term (approximately 3–4 months) low-carbohydrate (LC) diet is beneficial for diabetes management [113]. Compared with an ordinary diet, a LC diet contains a higher protein and fat content and ratio. The energy required by the body mainly comes from the metabolism of fat into ketone bodies; therefore, it is also called a ketogenic diet. A LC diet can directly reduce blood sugar levels, and ketone bodies have various functions, such as anti-inflammatory, mitochondrial biogenesis regulatory, and antioxidant activity [114]. However, a long-term LC diet may damage kidney function, which is mainly attributed to its high protein content [113]. Several human physiological studies have shown that a high-protein diet can cause renal hyperfiltration [115–117]. Although the actual cause of this phenomenon remains unclear, studies have attempted to describe the effects related to specific amino acid components as well as dietary advanced glycosylation end products [118, 119].

*5.2. Weight Loss and DN.* In a review on weight loss in coronary heart disease, the GFR and proteinuria in patients with weight loss improved, and the weight loss and CKD index effects of surgical intervention were better than those of drug and lifestyle interventions [120–122]. Miras et al. [123] confirmed this finding by retrospectively analysing data of 84 patients with DN who underwent bariatric surgery over a 12–18-month period. Among them, 32 patients with albuminuria at baseline had a mean 3.5-fold decrease in the postoperative albumin–creatinine ratio, and albuminuria in 32 patients returned to normal levels.

A systematic review and meta-analysis including approximately 30 studies reported the impact of bariatric surgery on renal function. All studies measured the changes in relevant indicators of renal dysfunction within 4 weeks before and after bariatric surgery. Among them, six studies measured a 54% reduction in the risk of postoperative glomerular hyperfiltration, and 16 studies measured a 60%–70% reduction in the risk of postoperative albuminuria and total proteinuria [124]. Cohort studies have reported the benefits of bariatric surgery in improving creatinine levels and the GFR or reducing the incidence of stage 4 ESRD [124–128], which may be related to improved renal tubular damage [129]. Furthermore, surgery-induced weight loss can improve mitochondrial biogenesis and mitochondrial dysfunction [70], which may be an effective treatment for DN [130, 131].

*5.3. Physical Activity and DN.* Moderate aerobic exercise can reduce weight and improve insulin sensitivity, hyperglycemia, hyperlipidemia, and DN [132, 133]. Studies have reported that upregulation of the expression of eNOS and nNOS proteins in the kidney and improvement in NADPH oxidase and $\alpha$-oxyaldehyde levels may reduce early diabetic nephropathy in Zucker diabetic fatty rats. Chronic aerobic

exercise has health benefits and may be utilized as a treatment method for the prevention and development of renal dysfunction in T2D [134]. However, strenuous exercise may aggravate DN progression. Studies have reported that the rates of urinary protein excretion increase after strenuous exercise and tubular proteinuria occurs [107, 135]. A prospective study has demonstrated for the first time that the intensity of physical activity, rather than the total amount, is associated with the occurrence and progression of DN in type 1 diabetes. Moreover, the beneficial relationship between moderate- and high-intensity physical activity and progression of nephropathy is not affected by the duration of diabetes, age, sex, or smoking [136].

This high-intensity, low-volume training program not only increases the content of citrate synthase and cytochrome C oxidase subunit IV with increasing insulin sensitivity but also stimulates mitochondrial biogenesis. The contraction activity can lead to important signal events such as calcium release, AMP/ATP ratio change, cell redox state, and ROS generation. These events activate AMPK and stimulate PGC-1$\alpha$ [137]. PGC-1$\alpha$ can stimulate several genes encoding mitochondrial proteins, mtDNA amplification and proliferation, and oxidative metabolism. In short, the number of mitochondria per cell and their function increased several times in trained subjects compared to those in sedentary subjects. Although the best exercise type, frequency, and intensity for preventing DN or DN progression have not been formally determined, it is recommended to perform moderate-to-high-intensity aerobic exercise for at least 150 minutes and two to three sessions of resistance exercise per week for patients without contraindications [138].

## 6. Conclusion

Recently, mitochondrial dysfunction has been shown to be a critical determinant of the progressive loss of renal function in patients with diabetes. Pharmacological regulation of mitochondrial networking may be a promising therapeutic strategy in preventing and treating DN. Moreover, nontraditional therapies, including herbal medicine and lifestyle interventions, play a renoprotective role in improving mitochondrial homeostasis and function. Overall, the interventional mechanisms of nontraditional therapies for DN are still in their infancy compared with traditional treatments. Elucidating the mechanism of action and efficacy of nontraditional therapies involving mitochondria may facilitate the discovery of novel therapeutic approaches in treating DN and preventing the progression of DN to ESRD.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Ping Na Zhang and Meng Qi Zhou contributed equally to this work.

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*Research Article*

# Combination of GLP-1 Receptor Activation and Glucagon Blockage Promotes Pancreatic $\beta$-Cell Regeneration *In Situ* in Type 1 Diabetic Mice

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Pancreatic $\beta$-cell neogenesis *in vivo* holds great promise for cell replacement therapy in diabetic patients, and discovering the relevant clinical therapeutic strategies would push it forward to clinical application. Liraglutide, a widely used antidiabetic glucagon-like peptide-1 (GLP-1) analog, has displayed diverse $\beta$-cell-protective effects in type 2 diabetic animals. Glucagon receptor (GCGR) monoclonal antibody (mAb), a preclinical agent that blocks glucagon pathway, can promote the recovery of functional $\beta$-cell mass in type 1 diabetic mice. Here, we conducted a 4-week treatment of the two drugs alone or in combination in type 1 diabetic mice. Although liraglutide neither lowered the blood glucose level nor increased the plasma insulin level, the immunostaining showed that liraglutide expanded $\beta$-cell mass through self-replication, differentiation from precursor cells, and transdifferentiation from pancreatic $\alpha$ cells to $\beta$-cells. The pancreatic $\beta$-cell mass increased more significantly after GCGR mAb treatment, while the combination group did not further increase the pancreatic $\beta$-cell area. However, compared with the GCGR mAb group, the combined treatment reduced the plasma glucagon level and increased the proportion of $\beta$-cells/$\alpha$-cells. Our study evaluated the effects of liraglutide, GCGR mAb monotherapy, and combined strategy in glucose control and islet $\beta$-cell regeneration and provided useful clues for the future clinical application in type 1 diabetes.

## 1. Introduction

Pancreatic $\beta$-cell dysfunction and cell mass loss are a pivotal pathogenesis in both type 1 diabetes (T1D) and type 2 diabetes (T2D) [1, 2]. Therefore, it is highly necessary to preserve $\beta$-cell function and expand $\beta$-cell mass for diabetes treatment. Glucagon-like peptide-1- (GLP-1-) based therapies, including GLP-1 receptor agonists and dipeptidyl peptidase-4 inhibitors, have several beneficial effects on pancreatic $\beta$-cells, including upregulating insulin gene transcription and biosynthesis, potentiating glucose-stimulated insulin secretion, and promoting $\beta$-cell regeneration by promoting $\beta$-cell proliferation, inhibiting $\beta$-cell apoptosis, and inducing stem cells to differentiate into $\beta$-cells [3, 4]. Recent researches have proven that *GLP-1* overexpression or GLP-1 receptor agonists promoted $\beta$-cell regeneration *via* $\alpha$- to $\beta$-cell transdifferentiation [5–7]. Although GLP-1-based therapy shows various beneficial effects in T2D animals and humans, these drugs cannot be used alone for T1D treatment [8]. Whether GLP-1-based therapy has similar effects on $\beta$-cell protection, especially for $\beta$-cell regeneration in T1D, needs to be determined, and which therapy should be combined with GLP-1 should be evaluated.

REMD2.59, a fully competitive antagonistic glucagon receptor (GCGR) monoclonal antibody (mAb), has a strong hypoglycemic effect in T1D and T2D rodents and nonhuman primates [9–11]. A randomized clinical trial showed that REMD477, another GCGR mAb that has an affinity for the GCGR equivalent to that of REMD2.59, improved glycemic control in patients with T1D without serious adverse effects [12]. Our previous findings suggested that treatment with the GCGR mAb increased the $\beta$-cell mass by promoting $\alpha$- to $\beta$-cell conversion [13]. However, GCGR mAb substantially increased pancreatic $\alpha$-cell mass and

plasma glucagon levels. Notably, GLP-1 receptor agonists have the ability of inhibiting glucagon secretion. When combined with GCGR mAb, the GLP-1 receptor may avoid hyperglucagonemia.

In this study, we investigated the possible effect of liraglutide, a commonly used GLP-1 receptor agonist, on $\beta$-cell regeneration in T1D mice, and evaluated the combined effect of liraglutide with GCGR mAb. Our study provides new clues for the clinical therapy to maintain glucose homeostasis and promote pancreatic $\beta$-cell neogenesis in T1D patients.

## 2. Materials and Methods

*2.1. Animals and Intervention.* All the animal experiments were conducted at Peking University Health Science Center (Beijing, China) and approved by the Institutional Animal Care and Use Committee. B6.Cg-Tg(Gcg-cre)1Herr/Mmnc (cre expression in pancreatic $\alpha$-cell lineage) and B6.Rosa26-LSL-Cas9-tdTomato/J (when crossed to a cre recombinase-expressing strain, red fluorescence protein (RFP) expression is observed in the cre-expressing tissues) mice were crossed to generate pancreatic $\alpha$-cell lineage-tracing mice, namely, glucagonCre-RFP mice. Eight-week-old male glucagonCre-RFP mice or C57BL/6J mice (Vital River Animal Center, Beijing, China) were injected intraperitoneally with streptozotocin (STZ, 150 mg/kg; Sigma-Aldrich, Saint Louis, MO) in citric acid buffer (0.1 mol/L, pH 4.2) to establish a T1D model. The diabetic condition was confirmed if fasting blood glucose was 11.1 mmol/L or the random blood glucose was 16.7 mmol/L at least twice. If the blood glucose level was higher than 33.3 mmol/L (the upper detection limit of the blood glucose meter), 33.3 mmol/L was recorded. The diabetic C57BL/6J animals were treated for four weeks by intraperitoneal administration of liraglutide (0.2 mg/kg, twice daily; Novo Nordish, Denmark), REMD2.59 (a human GCGR mAb, 5 mg/kg, once a week; REMD Biotherapeutics, Camarillo, CA, USA), a combination of two agents, or saline (as control). There were 6-8 mice in each group. The diabetic glucagonCre-RFP mice were treated for four weeks by intraperitoneal administration of liraglutide or saline. Mice were fasted 8 h for the measurement of fasting blood glucose by the glucose oxidase method. After the four-week treatment, the mice were sacrificed, and then, plasma and pancreas were collected. Specific ELISA kits were used for detecting insulin (Millipore, Saint Charles, MO, USA) and glucagon (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

*2.2. Immunofluorescence Staining.* Pancreases were fixed with 10% formalin at 4°C overnight and embedded in paraffin, and 5 $\mu$m thick sections were prepared. The sections were incubated with primary antibodies at 4°C overnight and secondary antibodies for 1 h at room temperature, followed by washing and staining with DAPI (1 $\mu$g/mL, Sigma-Aldrich). Images were captured under a confocal fluorescence microscope (Leica TCS SP8; Leica Microsystems Inc., Wetzlar, Germany) or an automatic digital slide scanner (Panoramic MIDI, 3D HISTECH, Budapest, Hungary). For cell quantification, 6 to 9 equally spaced sections (which covered the entire pancreas) per pancreas were imaged, and the total numbers of positive staining cells from 6 mice per group were counted by using ImageJ software.

The following antibodies and dilutions were used: rabbit antiglucagon (1 : 800, Cell Signaling Technology, Beverly, MA), mouse antiglucagon (1 : 400, Sigma-Aldrich), mouse anti-insulin (1 : 800, Sigma-Aldrich), guinea pig anti-insulin (1 : 200, Abcam), mouse antiproliferating cell nuclear antigen (PCNA; 1 : 400, Cell Signaling Technology), rabbit anticytokeratin 19 (CK19; 1 : 400, Abcam), rabbit polyclonal antipancreatic and duodenal homeobox 1 (Pdx1, 1 : 200, Abcam), rabbit monoclonal anti-NK6 homeobox 1 (Nkx6.1, 1 : 400 , Abcam), rabbit polyclonal antiproprotein convertase 1/3 (PC1/3, 1 : 400, Millipore), rabbit polyclonal anti-RFP (1 : 200; Abcam), Alexa Fluor 594-conjugated AffiniPure goat antimouse IgG (H+L) (1 : 800, Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 488 or 594-conjugated AffiniPure goat antirabbit IgG (H+L) (1 : 800, Jackson ImmunoResearch Laboratories), and Alexa Fluor 647-conjugated AffiniPure goat antiguinea pig IgG (H+L) (1 : 800, Jackson ImmunoResearch Laboratories).

*2.3. Primary Islets and Islet Cell Line Experiments.* Primary islets were isolated from C57BL/6J mice as previously reported [14, 15]. Briefly, the pancreas was perfused by collagenase V (Sigma-Aldrich), and individual islets were hand-picked and cultured in the RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mmol/L GlutMax, 1 mmol/L sodium pyruvate, and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Mouse pancreatic $\alpha$-cell line $\alpha$TC1 clone 7 ($\alpha$TC1.9) cells (ATCC, USA) were cultured in Dulbecco's modified Eagle medium (5.5 mmol/L glucose; Invitrogen) supplemented with 10% fetal bovine serum, 15 mmol/L HEPES, 0.1 mmol/L nonessential amino acids, 0.02% bovine serum albumin, 2 mmol/L GlutMax, and 1% penicillin-streptomycin. Primary islets or $\alpha$TC1.9 cells were treated with GCGR mAb (100 nmol/L) or IgG for 24 h, and RNA was collected for further analysis.

*2.4. Quantitative RT-PCR.* Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific) and reversely transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). The quantitative RT-PCR was performed using iQ SYBR Green supermix (BioRad Laboratories, Hercules, CA, USA) on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). All experiments were performed in triplicate. Relative quantification for gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primers were synthesized by the Beijing AuGCT DNA-SYN Biotechnology Company (Beijing, China). The primer sequences were as follows: *Glp-1r*: forward: AGCACTGTCCGTCTTCATCA, reverse: AGAAGG CCAGCAGTGTGTAT; *Gapdh*: forward: TGCACCACCAC CAACTGCTTAGC, reverse: GGCATGGACTGTGGTC ATGAG.

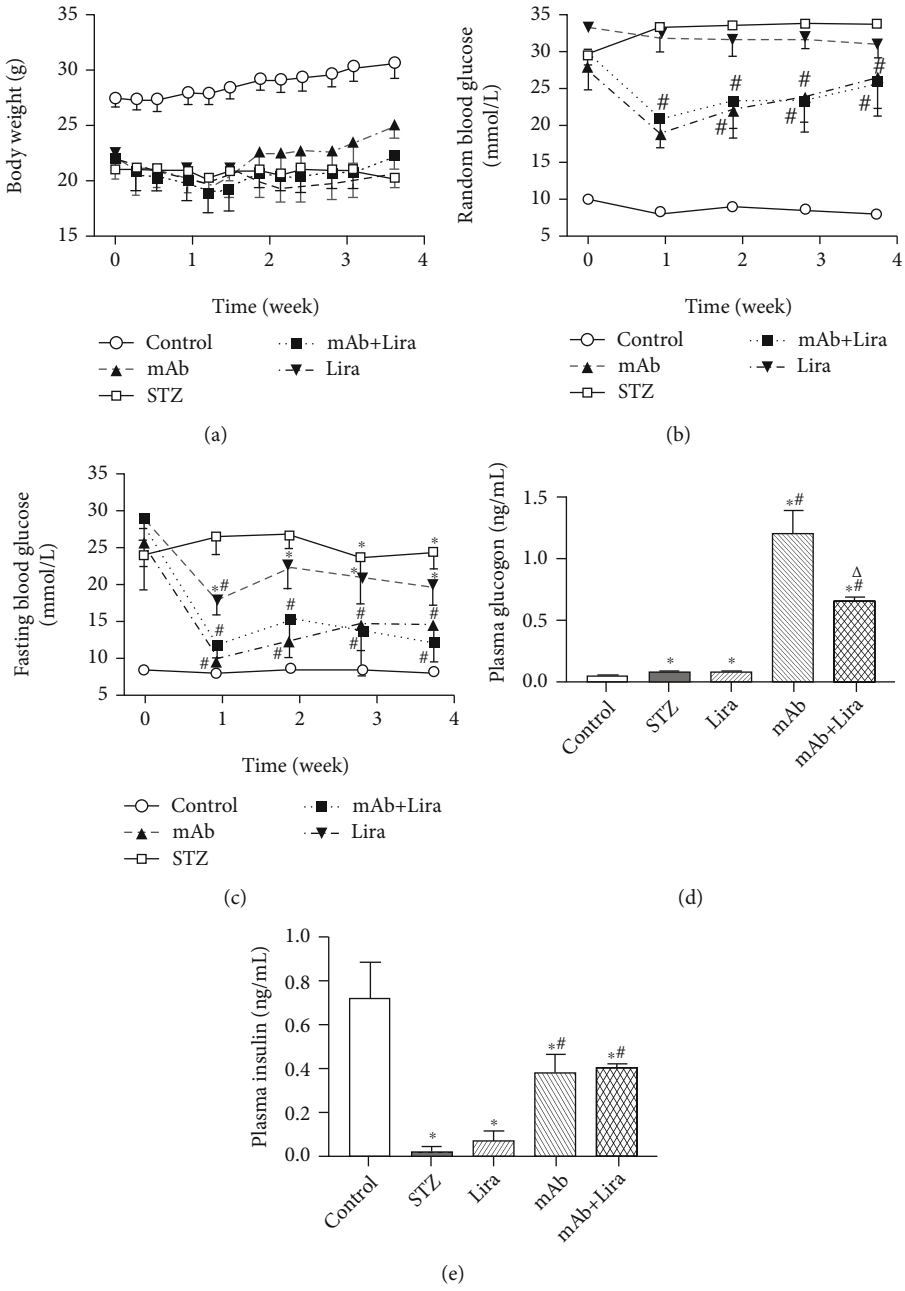


Figure 1: Body weight, blood glucose, plasma insulin, and glucagon levels in T1D and control mice treated with liraglutide, GCGR mAb, or both for four weeks. Eight-week-old male C57BL/6J mice were injected intraperitoneally with 150 mg/kg streptozotocin (STZ) to establish a T1D model. Diabetic mice were treated with liraglutide (Lira, 200 $\mu$g/kg), GCGR mAb (5 mg/kg), or liraglutide combined with GCGR mAb. The littermate C57BL/6J mice were used as normal control: (a) body weight; (b) random blood glucose; (c) fasting blood glucose; (d) plasma glucagon; (e) plasma insulin. Data are presented as the mean ± SEM ($n = 6 – 8$ mice per group). Statistical analysis was conducted by ANOVA. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. STZ group; $^{\triangle}p < 0.05$ vs. GCGR mAb group.

*2.5. Statistical Analysis.* Data are expressed as the mean ± S .E.M. The difference between two groups was analyzed by ANOVA followed by the *post hoc* Tukey-Kramer test or Student's *t* -test (two-tailed) when appropriate. A $p$ value < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA).

## 3. Results

*3.1. Liraglutide and GCGR mAb Treatments Improve Diabetic Phenotype in T1D Mice.* Compared with the normal control group, the body weight of the T1D mice displayed a significant decrease while the blood glucose level showed obvious increment (Figures 1(a)–1(c)). Neither liraglutide

nor GCGR mAb had any effects on the body weights (Figure 1(a)). Liraglutide alone did not decrease the random blood glucose (Figure 1(b)), while displaying a trend to lower fasting blood glucose ($p = 0.17$, Figure 1(c)). The GCGR mAb significantly reduced the random and fasting blood glucose levels (Figures 1(b) and 1(c)). However, the combination of GCGR mAb and liraglutide (mAb+Lira) did not decrease the blood glucose levels further compared to the GCGR mAb group (Figures 1(b) and 1(c)).

After STZ treatment, the plasma insulin level was significantly lower, and the plasma glucagon level was higher in the STZ group than in the control group. Liraglutide did not affect the insulin level or glucagon level (Figures 1(d) and 1(e)). The GCGR mAb treatment significantly upregulated the plasma insulin level and glucagon level when compared with vehicle treatment in T1D mice (Figures 1(d) and 1(e)). The mAb+Lira combination also significantly upregulated the plasma insulin level when compared with the STZ group, but did not show any difference when compared with mAb treatment alone (Figure 1(d)). Notably, although the glucagon level in the combination group was higher than that in the STZ group, it was significantly lower when compared with that in the GCGR mAb group (Figure 1(d)).

*3.2. Liraglutide and GCGR mAb Treatments Increase Pancreatic β-Cell Area of T1D Mice in Varying Degrees.* Histological analysis of the pancreatic islets was carried out by using double-labeled immunofluorescence staining. STZ significantly decreased the entire islet area, with the level less than 1/3 of the normal control (Figures 2(a)–2(c), Table S1). STZ strikingly decreased β-cell area and increased α-cell mass compared with the normal control (Figures 2(d) and 2(e), Table S1). Liraglutide treatment did not change the total islet area and α-cell area, but liraglutide increased the β-cell area, thus having a tendency to increase the β/α-cell area proportion when compared with the STZ group (Figures 2(b)–2(f), Table S1). GCGR mAb treatment increased the total islet area, β-cell area, α-cell area, and β/α-cell area proportion when compared with the STZ group (Figures 2(c)–2(f), Table S1). Similarly, the mAb+Lira combination also greatly increased the total islet area and β-cell area when compared with the STZ group (Figures 2(c) and 2(d), Table S1), but showed no difference with the mAb group. The mAb+Lira combination increased the α-cell area when compared with the STZ group, while decreasing the α-cell area when compared with the mAb group (Figure 2(e), Table S1). Therefore, the mAb+Lira combination increased the β/α-cell area proportion significantly when compared with the STZ or mAb group (Figure 2(f), Table S1).

*3.3. Liraglutide and GCGR mAb Treatments Promote β-Cell Self-Replication in T1D Mice.* As shown above, both liraglutide and GCGR mAb increased the β-cell area. Subsequently, we tried to determine the source of the increased islet cells. We performed costaining of insulin and PCNA in pancreatic sections to detect β-cell proliferation. We found that the proportions of $PCNA^+insulin^+$ cells (the proliferating β-cells) were higher in the liraglutide group and the mAb+Lira group when compared to the STZ group, while the proportion in the GCGR mAb group did not show differences with that in other groups (Figures 3(a) and 3(d), Table S1).

*3.4. Liraglutide Induces Duct-Derived β-Cell Neogenesis in T1D Mice.* Neogenesis from precursor cells is an important approach for the recovery of β-cell mass. Pancreatic precursor cells were often located near or within the adult pancreatic ducts [16]. Our previous study has proved that GCGR mAb could induce pancreatic duct-derived α-cell neogenesis, rather than β-cell neogenesis, in T1D mice [13]. In this study, we costained insulin or glucagon with pancreatic duct marker CK19 to evaluate the cell neogenesis. Results showed that in the liraglutide group or mAb+Lira group, glucagon-positive cells or insulin-positive cells could appear adjacent to $CK19^+$ cells, suggestive of duct-derived α-cell or β-cell neogenesis (Figures 3(b) and 3(c)). However, the glucagon or insulin-positive cells that were located in the duct were rare, so we did not perform quantification further.

*3.5. Liraglutide and GCGR mAb Treatments Induce α- to β-Cell Transdifferentiation in T1D Mice.* To evaluate α- to β-cell transdifferentiation, we performed glucagon and insulin double immunostaining. Compared with the STZ group, the proportion of $glucagon^+insulin^+$ cells were boosted by the liraglutide treatment ($p = 0.031$, Figures 4(a) and 4(c), Table S1). Next, we established pancreatic α-cell lineage-tracing (glucagonCre-RFP) mice. About 80% of glucagon-positive cells were RFP positive, suggestive of the high tracing efficiency, and there was almost no $RFP^+glucagon^-$ cells in the nondiabetic tracing mice, suggestive of no leakage of tracing (Figure S1). Therefore, RFP could be a good tracing marker of α-cells. After liraglutide treatment, the proportion of $RFP^+insulin^+$ cells was significantly higher than that in the STZ group ($p < 0.01$, Figures 4(b) and 4(d)). Notably, we not only found $glucagon^+RFP^+insulin^+$ cells but also found $glucagon^-RFP^+insulin^+$ cells; the latter suggested that the transformed β-cells lost glucagon expression (Figure S2). All the above results confirmed that some newborn β-cells were derived from the transdifferentiation of pancreatic α-cells.

GCGR mAb could promote α- to β-cell transdifferentiation, as indicated by the increased proportion of $glucagon^+insulin^+$ cells ($p < 0.01$), which was consistent with our previous report [13]. Notably, the proportion of $glucagon^+insulin^+$ cells in the mAb+Lira combination group was even higher than that in the liraglutide group ($p = 0.038$, Figure 4(c)).

*3.6. The Regenerated β-Cells Own the Maturity Phenotype.* To further investigate the identity of the neogenerated insulin-expressing cells above, thorough marker gene analyses were performed in all treated animals. Our data indicated that all $insulin^+$ cells (including preexisting and neogenerated) uniformly expressed the bona fide β-cell labels, such as Pdx1, Nkx6.1, and PC1/3 (Figure 5). These analyses led us to conclude that the hyperplastic insulin-expressing cells observed after liraglutide or GCGR mAb treatment owned a β-like cell phenotype.

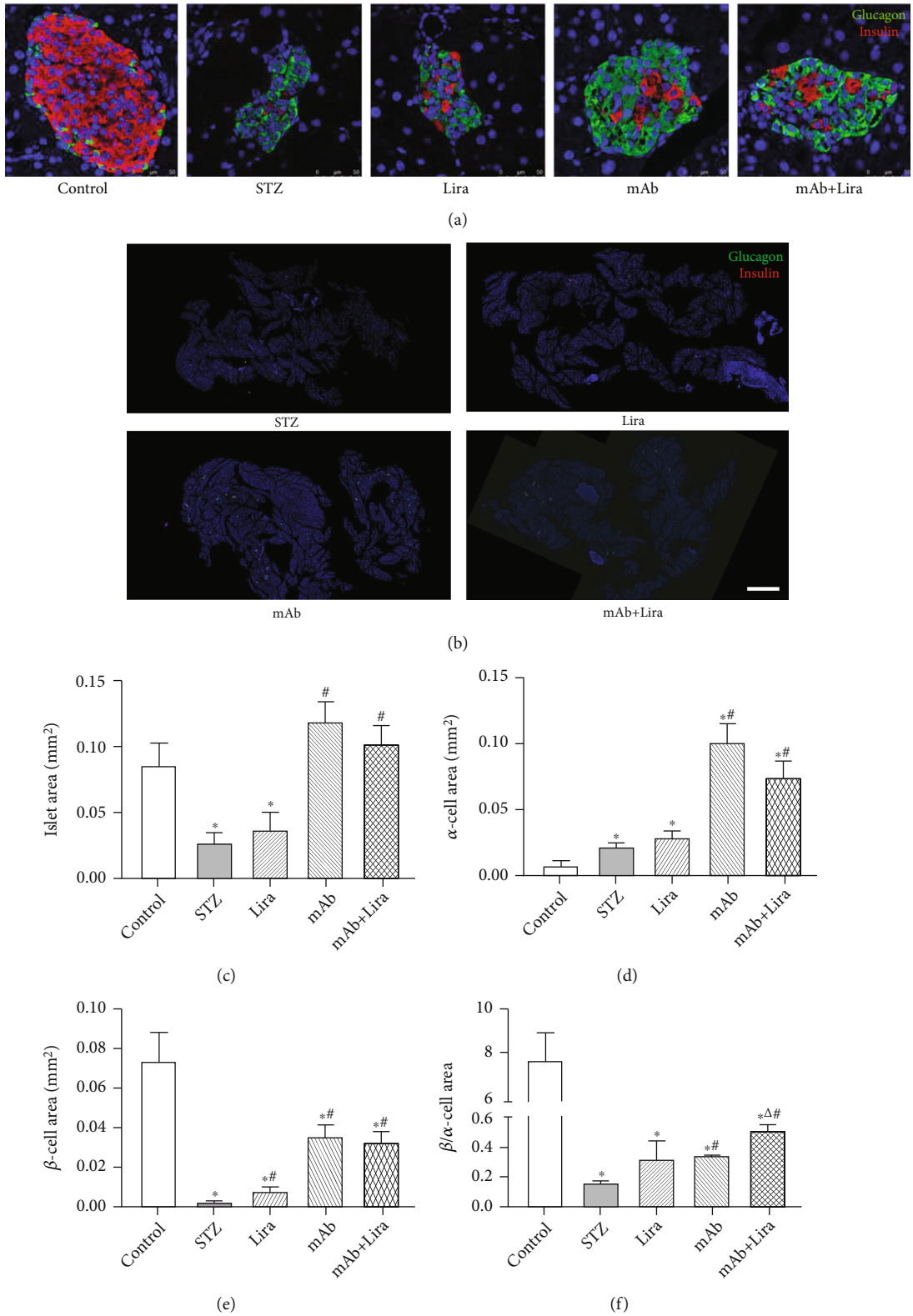


Figure 2: Pancreatic histological analysis of pancreatic $\alpha$-cell and $\beta$-cell mass in T1D and control mice treated with liraglutide, GCGR mAb, or both for four weeks. (a) Representative images of islets immunostained for glucagon and insulin. Scale bar = 50 $\mu$m. (b) Representative images of islets immunostained for DAPI, glucagon and insulin in panoramic view. Scale bar = 2000 $\mu$m. (c–f) Total islet area, $\alpha$-cell area, $\beta$-cell area per pancreatic slice, and the ratio of $\beta$-cell area to $\alpha$-cell area. $n = 6 - 9$ sections/mouse multiplied by 6 mice/group. Data are expressed as the mean ± SEM. Statistical analysis was conducted by ANOVA. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. STZ group; $^{\triangle}p < 0.05$ vs. GCGR mAb group.

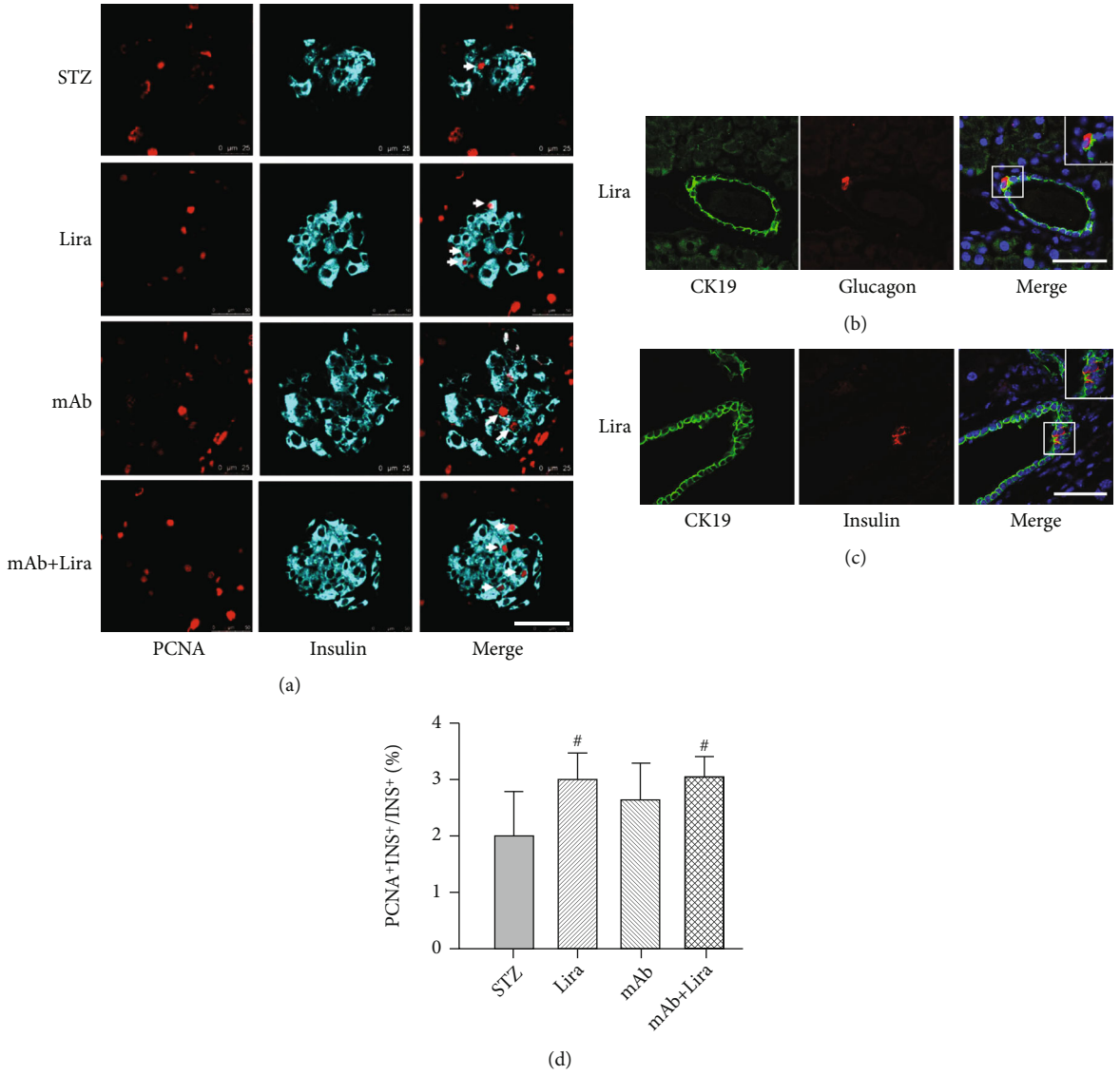


Figure 3: Pancreatic histological analysis of cell proliferation and ductal cell transformation in T1D mice treated with liraglutide, GCGR mAb, or both for four weeks. (a) Representative images of coimmunostaining with insulin and PCNA (proliferating cell nuclear antigen). Scale bar = 50 $\mu$m. (b, c) Representative images of coimmunostaining CK19 (cytokeratin 19) with glucagon or insulin. Scale bar = 50 $\mu$m. (d) Quantification of the proliferating $\beta$-cells. $n = 6 - 8$ sections/mouse multiplied by 6 mice/group. Data are expressed as the mean ± SEM. Statistical analysis was conducted by ANOVA. $^{\#}p < 0.05$ vs. STZ group. Magnified views of box regions are shown in the upper-right panels.

*3.7. GCGR mAb Treatment Upregulates GLP-1 Receptor Expression.* GLP-1 plays diversified effects mainly through the GLP-1 receptor [17]. To explore the potential mechanism of GCGR mAb synergistically promoting $\alpha$- to $\beta$-cell conversion induced by liraglutide, we analyzed the levels of the GLP-1 receptor. In mouse primary islets and $\alpha$-cell line $\alpha$TC1.9, glucagon blockage by GCGR mAb upregulated the mRNA levels of the GLP-1 receptor (Figure 6).

## 4. Discussion

Our results demonstrated that the GLP-1 receptor agonist liraglutide increased pancreatic $\beta$-cell mass in T1D mice through self-replication, differentiation from precursor cells, and transdifferentiation of pancreatic $\alpha$- to $\beta$-cells. Although combination of liraglutide and GCGR mAb did not demonstrate remarkable synergistic effects on the glucose level and $\beta$-cell area, the stimulating effects of GCGR mAb on the $\alpha$-cell area and glucagon secretion were alleviated. Interestingly, transdifferentiation of pancreatic $\alpha$- to $\beta$-cells was also boosted in the combination group. Increased GLP-1 receptor expression might be the possible reason of the synergetic effects of the two drugs. The combination strategy of the GLP-1 receptor activation with glucagon blockage may be beneficial in the T1D context, with good glucose control, $\beta$-cell regeneration, and not-very-high glucagon levels.

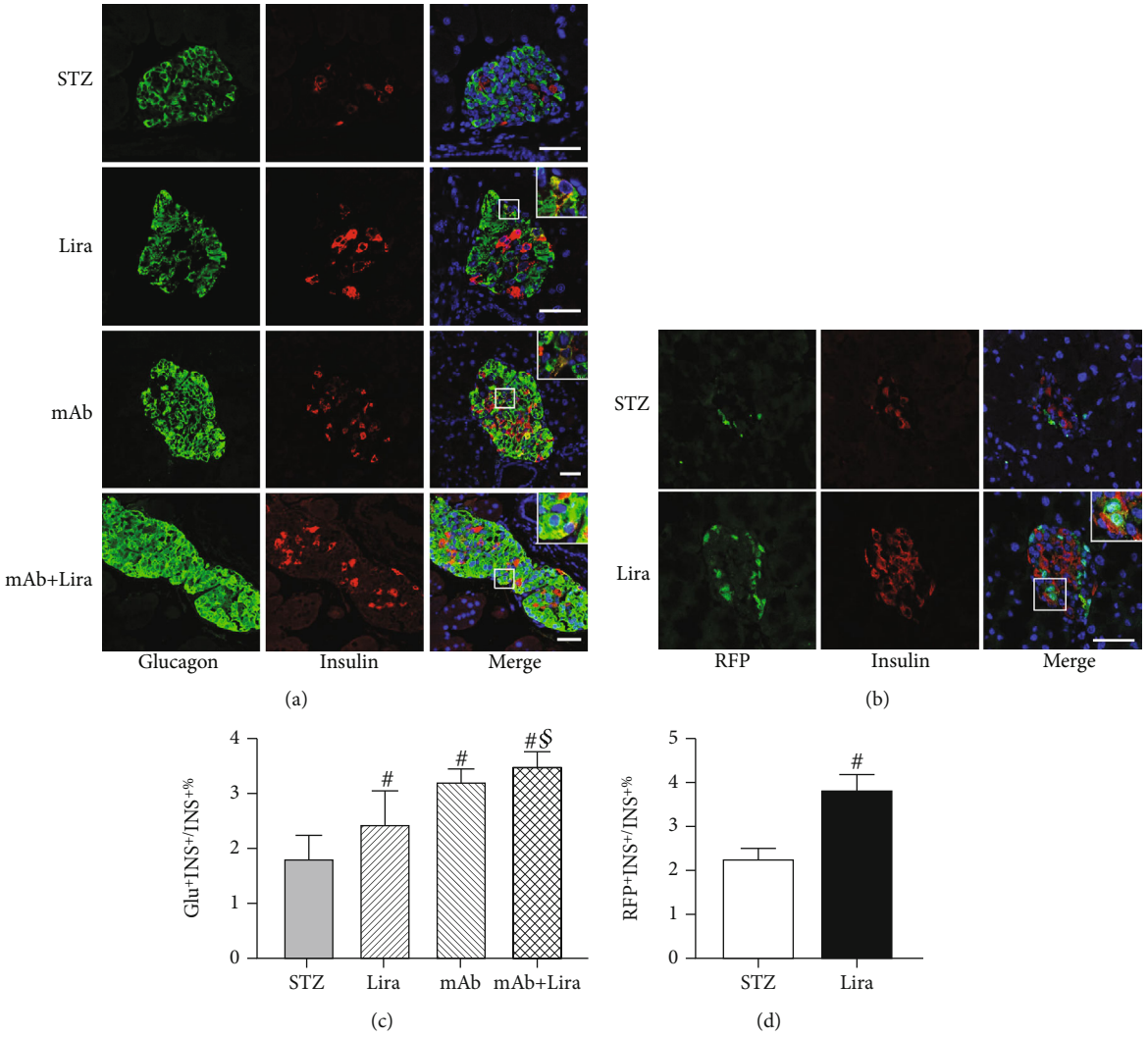


Figure 4: Pancreatic histological analysis of $\alpha$- to $\beta$-cell transdifferentiation in T1D mice treated with liraglutide, GCGR mAb, or both for four weeks. (a) Representative images of insulin and glucagon colocalization. Scale bar = 50 $\mu$m. (b) Representative images of insulin and tracing marker, red fluorescence protein (RFP), and colocalization in pancreatic $\alpha$-cell tracing T1D mice. (c) Quantification of the glucagon+insulin+ cells. Scale bar = 50 $\mu$m. (d) Quantification of the RFP+insulin+ cells. Data are expressed as the mean ± SEM. $n = 6 - 9$ sections/mouse multiplied by 6 mice/group. Statistical analysis was conducted by ANOVA or Student's $t$-test when appropriate. $^{\#}p < 0.05$ vs. STZ group; $^{\$}p < 0.05$ vs. liraglutide group. Magnified views of box regions are shown in the upper-right panels.

The current therapy for T1D is limited, and it is highly needed to evaluate the new nontraditional therapy for glucose control and maybe even for $\beta$-cell regeneration in T1D [18, 19]. GLP-1 receptor agonists have various beneficial effects on pancreatic $\beta$-cells, including promoting $\beta$-cell regeneration [20, 21]. However, most of the conclusions were obtained in T2D models. Our present study showed that a GLP-1 receptor agonist liraglutide increased pancreatic $\beta$-cell area in STZ-induced T1D mice. Moreover, we found that liraglutide not only promoted the proliferation of existing pancreatic $\beta$-cell and induced cells in the duct lining to transform into pancreatic islet cells, but also boosted $\alpha$-cells to transdifferentiate into insulin-positive cells. In this way, we confirmed that all above sources participated in the liraglutide-induced $\beta$-cell renewal. However, liraglutide could not decrease blood glucose in T1D mice. The GLP-1-based therapy cannot be used alone for T1D treatment, and the combination with other drugs is needed.

Our previous study, together with others, has proven that GCGR blockage could decrease blood glucose and improve the phenotype of T1D mice. Strikingly, GCGR mAb increased the number of pancreatic $\beta$-cells and upregulated circulating insulin levels by inducing $\alpha$- to $\beta$-cell transdifferentiation in T1D mice [13, 22]. However, GCGR mAb substantially increased pancreatic $\alpha$-cell mass, which brings a safety concern on the $\alpha$-cell tumor [23]. Notably, GLP-1 receptor agonists have the ability of inhibiting glucagon secretion and inducing $\alpha$- to $\beta$-cell transdifferentiation. In this study, we tried to evaluate the synergistic effect of GCGR mAb and liraglutide in T1D mice. Although the

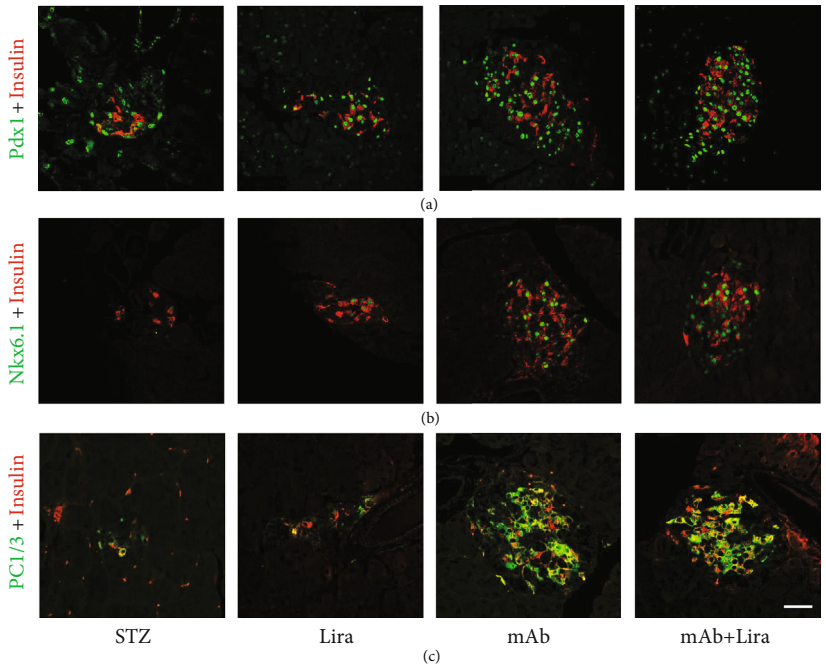


Figure 5: Pancreatic histological analysis of $\beta$-cell phenotype in T1D mice treated with liraglutide, GCGR mAb, or both for four weeks. (a) Representative images of insulin and Pdx1 (pancreatic and duodenal homeobox 1) colocalization. (b) Representative images of insulin and Nkx6.1 (NK6 homeobox 1) colocalization. (c) Representative images of insulin and PC1/3 (proprotein convertase 1/3), colocalization. Scale bar = 50 $\mu$m.

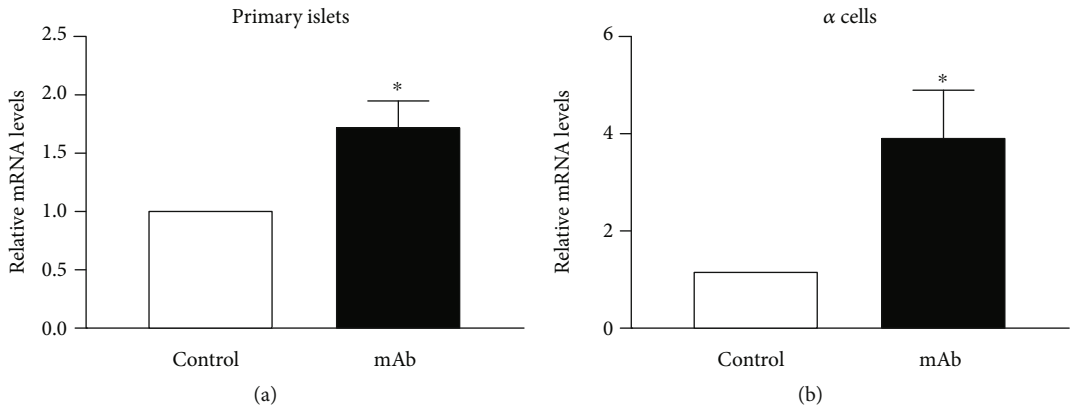


Figure 6: GLP-1 receptor expression induced by GCGR mAb treatment. The primary islets (a) and $\alpha$-cell line $\alpha$TC1.9 cells (b) were treated with GCGR mAb or IgG (as control) for 24 h, and GLP-1 receptor mRNA levels were determined by real-time RT-PCR. Statistical analysis was conducted by Student's t-test. $^*p < 0.05$ vs. control group.

combination did not show obvious advantages in decreasing blood glucose or increasing $\beta$-cell mass, the plasma glucagon level in the combination group decreased significantly and the $\alpha$-cell area showed a downward trend, and notably, the $\alpha$- to $\beta$-cell transdifferentiation was enhanced. Glucagon blockage by GCGR mAb could upregulate GLP-1 receptor expression, which might be the possible reason of the synergetic effects of GCGR mAb and liraglutide.

However, there were some limitations in our research. First, the immunostaining of PCNA with insulin, CK19 with insulin, and glucagon with insulin only displayed the proliferating, neogenetic, and transdifferentiated insulin-positive cells at a time point (before sacrifice). Although the low proportion at a real-time state might not contribute to the increased beta cell mass, the increased beta cells could be much more during the whole treatment period. Because we did evaluate the neogenetic cell proportion during the whole treatment period, so we could not conclude which source contributed most to the regenerated beta cells. Second, precursor-specific lineage tracing mice were needed for verification of stem cell-derived $\beta$-cells. Third, we only found that the mRNA expression of the GLP-1 receptor was

upregulated by GCGR mAb, and its effects on the combination and other underlying molecular mechanisms need to be further confirmed.

In summary, our present study evaluated the synergic effect of GLP-1 receptor activation and GCGR antagonism in T1D mice. Although we did not find better glucose control and $\beta$-cell regeneration, we discovered that combination of liraglutide with GCGR mAb could promote $\alpha$- to $\beta$-cell transdifferentiation, thus attenuating the GCGR mAb-induced $\alpha$-cell hyperplasia and hyperglucagonemia. Our research may provide useful clues for the clinical therapy in T1D.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

## Conflicts of Interest

None of the authors of this paper has any financial or personal relationships with other people or organizations that could influence (bias) the research results. No conflicts of interest exist for the authors of this study.

## Authors' Contributions

L.G. and R.W. designed the research; L.G., T.W., and X.C. performed the research; J.Y., K.Y., and R.W. analyzed the data; L.G. and D.W. wrote the manuscript; and J.Y., R.W., and T.H. revised the paper.

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## Supplementary Materials

Figure S1: the efficiency of lineage-tracing. B6.Cg-Tg(Gcg-cre)1Herr/Mmnc and B6.Rosa26-LSL-Cas9-tdTomato/J mice were crossed to generate pancreatic $\alpha$-cell lineage-tracing mice. Pancreas from eight-week-old mice was coimmunostained of glucagon and RFP. Figure S2: pancreatic histological analysis of $\alpha$-to $\beta$-cell transdifferentiation in $\alpha$-cell lineage-tracing T1D mice treated with liraglutide for four weeks. The arrowhead in the upper lane shows glucagon, RFP (the tracing marker of $\alpha$-cells), and insulin colocalization. The arrowhead in the lower lane shows RFP and insulin colocalization with glucagon loss. Scale bar = 10 $\mu$m. Table S1: quantification of plasma hormone and immunostaining in the pancreas. *(Supplementary Materials)*

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*Research Article*

# Retinal Neuropathy in IGT Stage of OLETF Rats: Another Characteristic Change of Diabetic Retinopathy

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*Aims*. We investigated the changes of retinal structure in normal glucose tolerance (NGT), impaired glucose tolerance (IGT), diabetes mellitus (DM), and diabetic kidney disease (DKD) stages in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Methods*. We assigned OLETF rats to four groups based on their OGTT results and 24 h urinary microalbumin (24 h UMA) levels: NGT, IGT, DM, and DKD groups. We observed the structural and the corresponding pathological changes and quantified the expression of HIF-1$\alpha$, iNOS, NF-$\kappa$B, VEGF, ICAM-1, and occludin in the retina. *Results*. Significant damage to the retinal structure, especially in retinal ganglion cells (RGCs), was observed in the IGT stage. The expression of HIF-1$\alpha$, iNOS, NF-$\kappa$B, VEGF, and ICAM-1 was significantly upregulated, while that of occludin was downregulated. *Conclusion*. Significant retinal neuropathy occurs in the IGT stage. Inflammation and hypoxia may damage the blood retina barrier (BRB), leading to diabetic retinopathy.

## 1. Introduction

With the increasing prevalence of diabetes, chronic complications, especially diabetic kidney disease (DKD) and retinopathy (DR), pose serious social problems threatening human health. The overall prevalence of DR, the primary retinal vascular complication of diabetes mellitus (DM), is 34.6% for any DR in DM. It is a leading cause of vision impairment and blindness in the working-age population [1–3]. DKD is the major cause of end-stage renal disease (ESRD) worldwide. DR is usually accompanied by DKD, and both have similar pathogenesis [4, 5]. Multiple studies have demonstrated a significant correlation between DR and DKD in patients with type 2 diabetes [6–8]. Some studies also believe that DR may precede DKD [9, 10]. Patients may suffer from various degrees of kidney injury before or upon the diagnosis of diabetes [11, 12]. Our previous studies focused on the structural and functional damage to the renal tubule in the impaired glucose tolerance (IGT) stage [13]. As DR may occur at an earlier stage of diabetes, we analyzed the structural alterations of the retina in different stages of diabetes and investigated the pathogenesis of DR in Otsuka Long-Evans Tokushima Fatty (OLETF) rats.

## 2. Materials and Methods

*2.1. Animals and Groups.* The study was performed adhering to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1996) and was approved by the Institutional Animal Care and Use Committee of the Tianjin Medical University.

Thirty-two male OLETF rats were supplied by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). They were housed in an air-conditioned room at 20-25°C with 50%-

Table 1: Biochemical data in the different stages of rats.

| Group | Weight (g) | FBG (mmol/L) | FINS (uIU/L) | 24 h UMA ($\mu$g/24 h) | TNF-$\alpha$ (ng/L) | IL-6 (pg/mL) |
|---|---|---|---|---|---|---|
| Control | 291.63 ± 4.93 | 5.74 ± 0.21 | 23.00 ± 1.60 | 151.68 ± 24.22 | 61.80 ± 12.92 | 43.13 ± 11.27 |
| NGT | 301.00 ± 16.95 | 5.59 ± 0.30 | 24.04 ± 2.52 | 145.73 ± 21.48 | 65.40 ± 10.71 | 41.76 ± 9.83 |
| IGT | 608.38 ± 34.12$^{ab}$ | 5.86 ± 0.33 | 64.66 ± 2.85$^{ab}$ | 163.43 ± 21.78 | 84.88 ± 20.62$^{ab}$ | 115.06 ± 21.69$^{ab}$ |
| DM | 586.50 ± 50.60$^{ab}$ | 6.61 ± 1.05$^{abc}$ | 33.53 ± 4.71$^{abc}$ | 168.07 ± 21.95 | 103.66 ± 23.50$^{abc}$ | 140.05 ± 32.89$^{abc}$ |
| DKD | 563.14 ± 88.92$^{ab}$ | 6.83 ± 0.41$^{abc}$ | 29.35 ± 3.13$^{abcd}$ | 224.21 ± 57.87$^{abcd}$ | 106.42 ± 25.17$^{abc}$ | 176.49 ± 32.30$^{abcd}$ |

$^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. NGT. $^{c}P < 0.05$ vs. IGT. $^{d}P < 0.05$ vs. DM.

70% humidity and a 12 h light-dark cycle and fed a high-fat diet. We allowed the rats free access to food and water. Thirty-two male Long-Evans Tokushima Otsuka (LETO) rats were used as the nondiabetic control model.

We performed an oral glucose tolerance test (OGTT) every 4 weeks. Before the test, the rats fasted for 16 h overnight, and 30% glucose solution (2 g/kg) was administered by gastric gavage. We collected the blood samples from the tail vein before glucose loading and 30, 60, and 120 min after glucose loading. Blood glucose levels were determined using an automatic blood glucose analyser. Plasma insulin concentrations were detected using radioimmunoassay. OLETF rats were classified into four stages according to the OGTT results [14] and 24 h urinary microalbumin (24 h UMA) levels: the normal glucose tolerance (NGT) stage, corresponding to a normal glucose level; the IGT stage, corresponding to a peak level of blood glucose > 16.8 mmol/L, or a blood glucose level > 11.1 mmol/L at 120 min; the DM stage, when both the above criteria were fulfilled; and the diabetic kidney disease (DKD) stage when the amount of 24 h UMA in OLETF rats was significantly greater than that in LETO rats. Tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-6 (IL-6) serum levels were tested using enzyme-linked immunosorbent assay (ELISA). Eight OLETF rats were randomly selected and euthanized in their NGT, IGT, DM, and DKD stages, as the NGT, IGT, DM, and DKD groups, and the same-aged LETO rats were euthanized in the control group.

*2.2. Retina Structure*

(1) Immunohistochemistry (IHC). Retina tissues were immediately fixed in 4% formalin and subsequently embedded in paraffin. The expression levels of HIF-1$\alpha$ (209601-1-AP, Proteintech), iNOS (18985-1-AP, Proteintech), NF-$\kappa$B (ab7970, Abcam), VEGF (19003-1-AP, Proteintech), ICAM-1 (ab124760, Abcam), and Occludin (ab31721, Abcam) were tested in the retina using IHC

(2) Light microscopy. Retina tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue slices were cut at 4 $\mu$m thickness, dewaxed in xylene, rehydrated in decreasing concentrations of ethanol in water, stained by haematoxylin and eosin (H&E), and examined using a light microscope

(3) Transmission electron microscopy. Retina tissues were immediately placed in a fixative (2.5% glutaraldehyde and 1% osmium tetraoxide), dehydrated using graded alcohol and epoxypropane, and embedded in Epon 812. Ultrathin sections were cut using an ELICA ULTRACUT-R ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined using a HITACHI-7500 transmission electron microscope

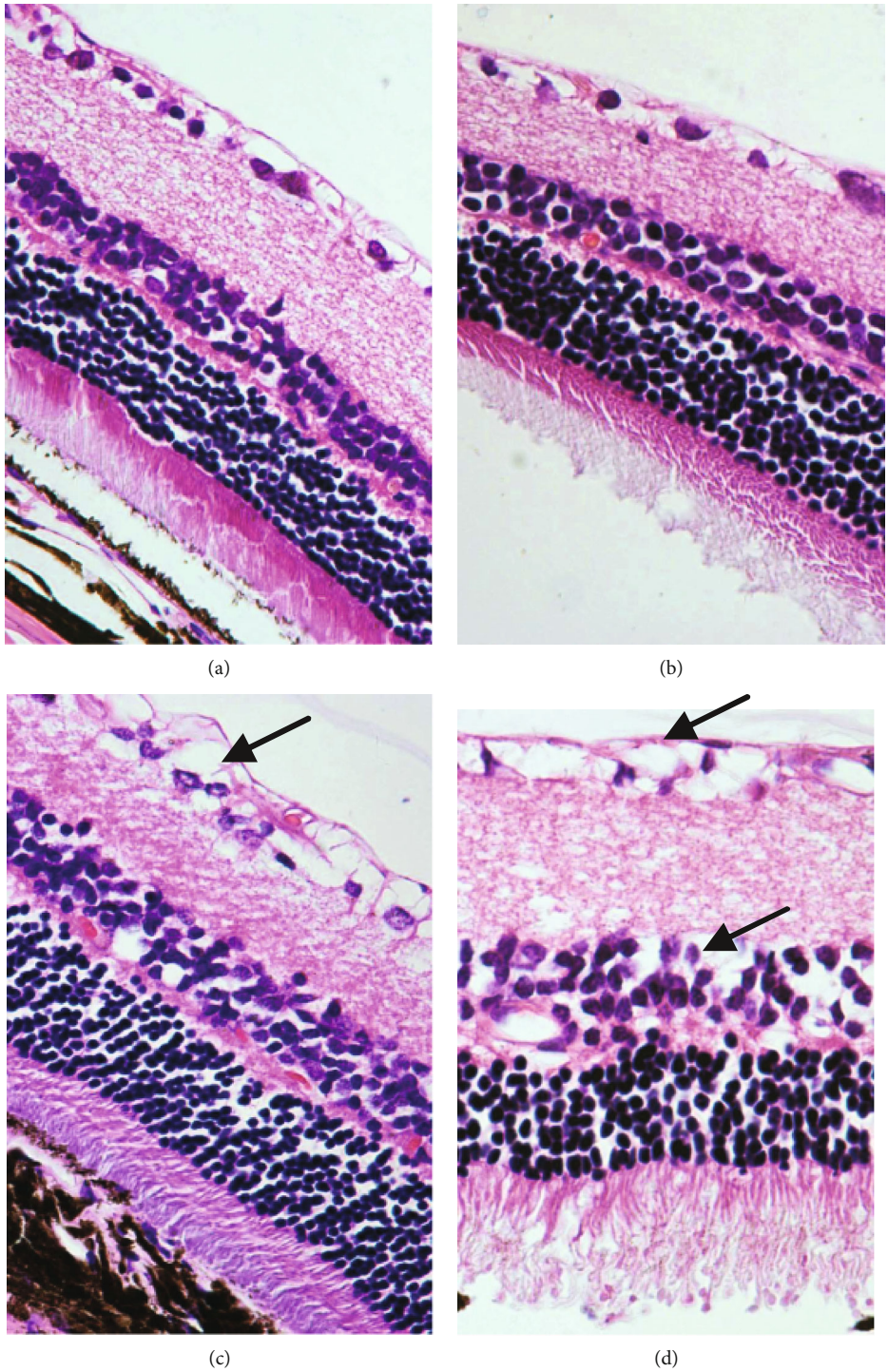
*2.3. Statistical Analysis.* We performed the statistical analysis using the IBM SPSS Statistics 26.0 software. All normally distributed data were expressed as the mean ± standard deviation (SD), and all other data were expressed as the median (range). Differences among the three groups were analysed using one-way analysis of variance for normally distributed continuous parameters. If differences were observed, the LSD $t$-test was used for the comparison between the two groups. Nonnormally distributed data were compared using the Kruskal–Wallis test. If differences were observed, the Mann–Whitney test was used for the comparison between the two groups. A chi-square test was used to compare the differences in the measurement data. Results were considered statistically significant if the two-tailed $P$ value was <0.05.

## 3. Results

*3.1. The Biochemical Data in Different Stage of OLETF Rats.* The fasting insulin (FINS) level and weight in the control and NGT group were not statistically different. However, the levels of the above two indicators were higher in the IGT stage than in the control group. Their levels fell in the DM and DKD groups but were still higher than those in the control group ($P < 0.05$).

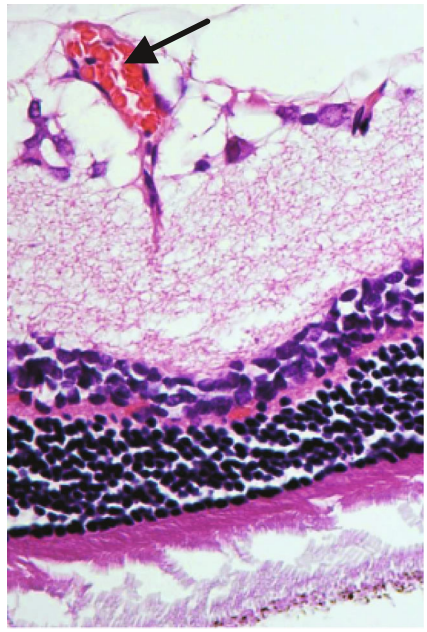
The expression levels of TNF-$\alpha$ and IL-6 were increased in the IGT stage and continued to increase with the progression of the disease ($P < 0.05$). The levels of 24 h UMA were similar in the NGT, IGT, and DM groups but were higher in the DKD group than in the other groups (Table 1).

*3.2. Significant Retinal Neuropathy Occurs in the IGT Stage.* In the control group (Figure 1(a)) and NGT stage (Figure 1(b)), the structures of all the layers in the retina were normal. While in the IGT stage, significant damage had occurred to the retina, including interstitial oedema of the ganglion cell layer, the irregular surface of the inner



(a) (b) (c) (d)

Figure 1: Continued.



(e)

Figure 1: Pathological changes of the retina using H&E (×400). (a) The control group; the inner limiting membrane is clear, and the nerve fibre layer is sparse and regularly arranged; the ganglion cells are neatly arranged in a single layer, and the nuclei are large and round; the inner plexus layer is thicker and has a relatively distinct network structure; the inner granular layer is composed of 3-5 layers of cells; the nuclei are larger and appear slightly darker; the outer plexus layer is distinctly thinner than the inner plexus layer; the outer granular layer is thicker, consisting of 8-10 layers of tightly arranged cells with small, deeply stained nuclei. (b) The NGT group. (c) The IGT group. (d) The DM group. (e) The DKD group.

limiting membrane, and the decreasing of retinal ganglion cells (RGCs) (Figure 1(c)). And in the DM stage (Figure 1(d)), all layers of the retina were structurally disordered, and intercellular substance oedema was conspicuous (as shown by the arrow). In the DKD stage (Figure 1(e)), the retinal ganglion cells were severely degenerated, and a new blood vessel was observed (as shown by the arrow).

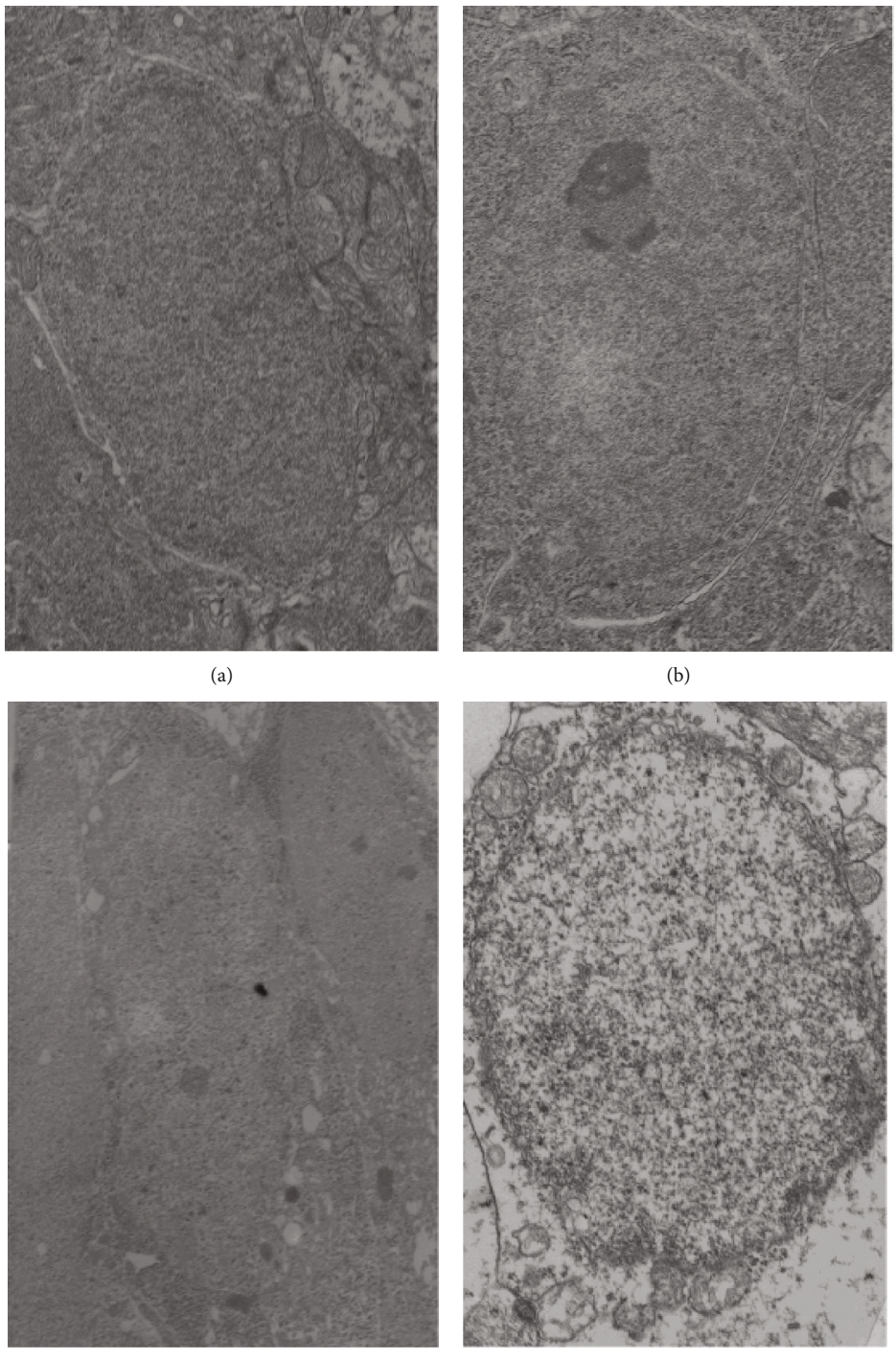
We then observed the ultrastructure of the retina using a transmission electron microscope. Compared with those in the control group, the rats in the NGT group had normal retinal morphology as well as ultrastructure (Figures 2(a), 2(b), 2(f), 2(g), 2(k), 2(l), 2(p), and 2(q)). However, abnormal morphological appeared in the ganglion cells at the IGT stage, including mitochondrial swelling, fuzzy crest, and vacuolization (Figure 2(c)). The cell membrane discs of visible light receptors were blurred and broken (Figure 2(h)), and oedema in the inner granular cells was observed (Figure 2(m)). The outer granular layer cells were irregularly arranged (Figure 2(r)). The above morphological changes gradually aggravated as the disease progressed (Figures 2(d), 2(e), 2(i), 2(j), 2(n), 2(o), 2(s), and 2(t)).

*3.3. Immunohistochemical Staining Alteration of Inflammatory Markers, Cytokines, and HIF-1α in Different Stages.* HIF-1α, NF-κB, VEGF, and ICAM-1 were significantly upregulated in the IGT stage, while occludin was downregulated.

*3.3.1. Expression of HIF-1α and iNOS in the IGT Stage of OLETF Rats.* Same as the control group (Figure 3(a)), HIF-1α was mainly expressed in retinal ganglion cell layer and low expressed in inner granular layer in the NGT group (Figure 3(b)). While in the IGT stage, the expression of HIF-1α increased significantly in inner granular layer (Figure 3(c)), and it further increased in the DM stage (Figure 3(d)). In the DKD stage, HIF-1α was expressed almost in the entire retina, including the outer granular layer (Figure 3(e)). Though the expression of iNOS has no significant difference between IGT stage (Figure 3(h)), NGT stage (Figure 3(g)), and control group (Figure 3(f)), it significantly increased in the DM stage (Figure 3(i)), and in the DKD stage, it was expressed almost in the entire retina (Figure 3(j)). The quantification of HIF-1α and iNOS is shown in Figures 3(k) and 3(l).

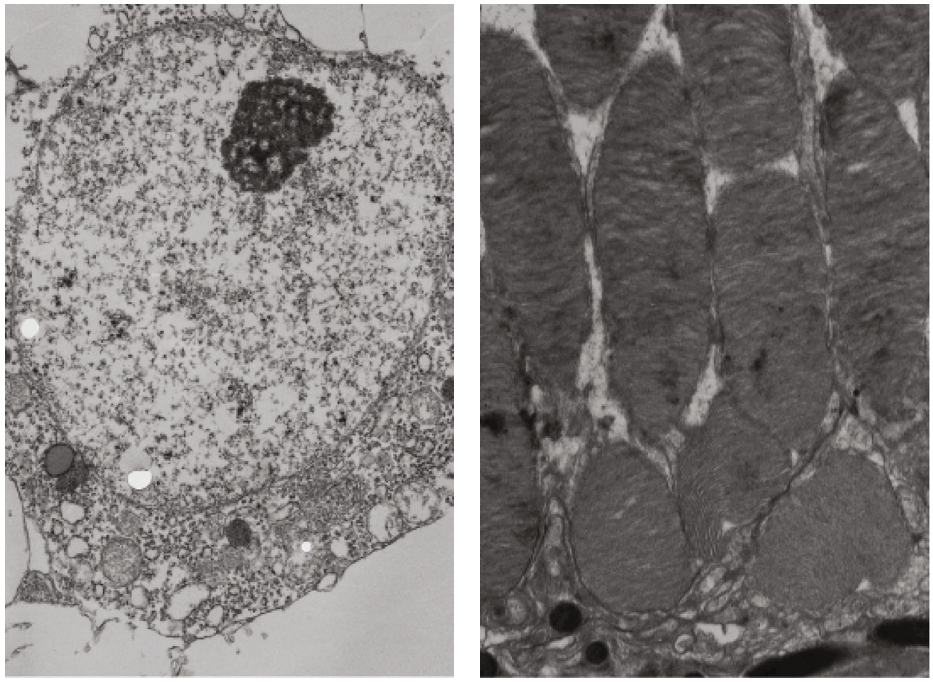
*3.3.2. Expression of NF-κB, VEGF, and ICAM-1 in the IGT Stage of Rats.* NF-κB, VEGF, and ICAM-1 were low expressed in the retinal ganglion cell layer and the inner granular layer in the rats of the control group (Figures 4(a), 4(f), and 4(k)) and NGT stage (Figures 4(b), 4(g), and 4(l)). These inflammatory markers increased markedly in the IGT stage (Figures 4(c), 4(h), and 4(m)). And they further increased in the DM stage (Figures 4(d), 4(i), and 4(n)), and in the DKD stage, they were expressed almost in all the layers of the retina (Figures 4(e), 4(j), and 4(o)).The quantification of NF-κB, VEGF, and ICAM-1 is shown in Figures 4(p)–4(r).

*3.3.3. Occludin Expression in the IGT Stage in Rats.* Consistent with the control group (Figure 5(a)), the occludin was

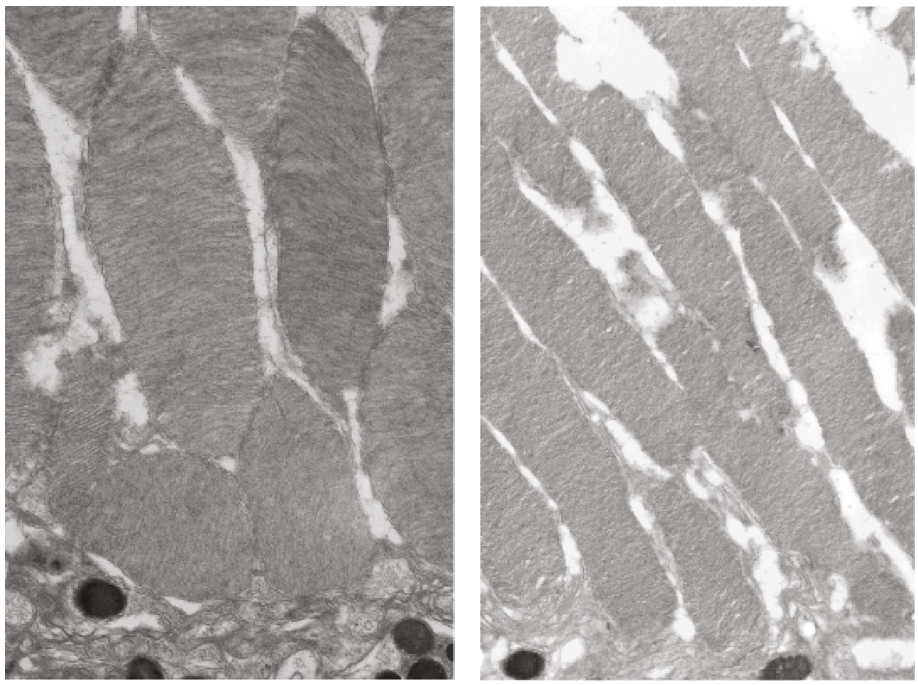


(a) (b) (c) (d)

Figure 2: Continued.



(e) (f)



(g) (h)

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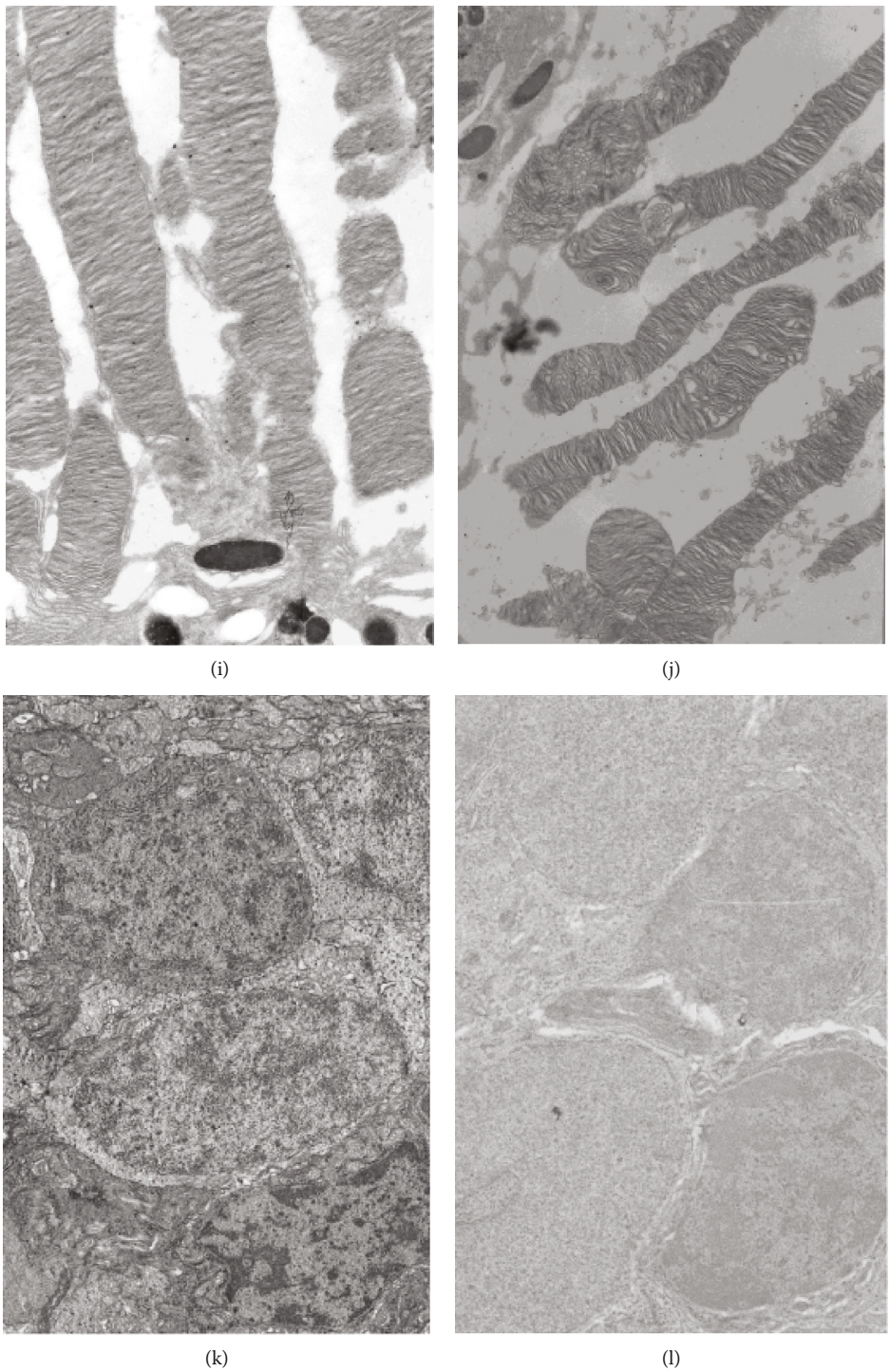

(i) (j)

(k) (l)

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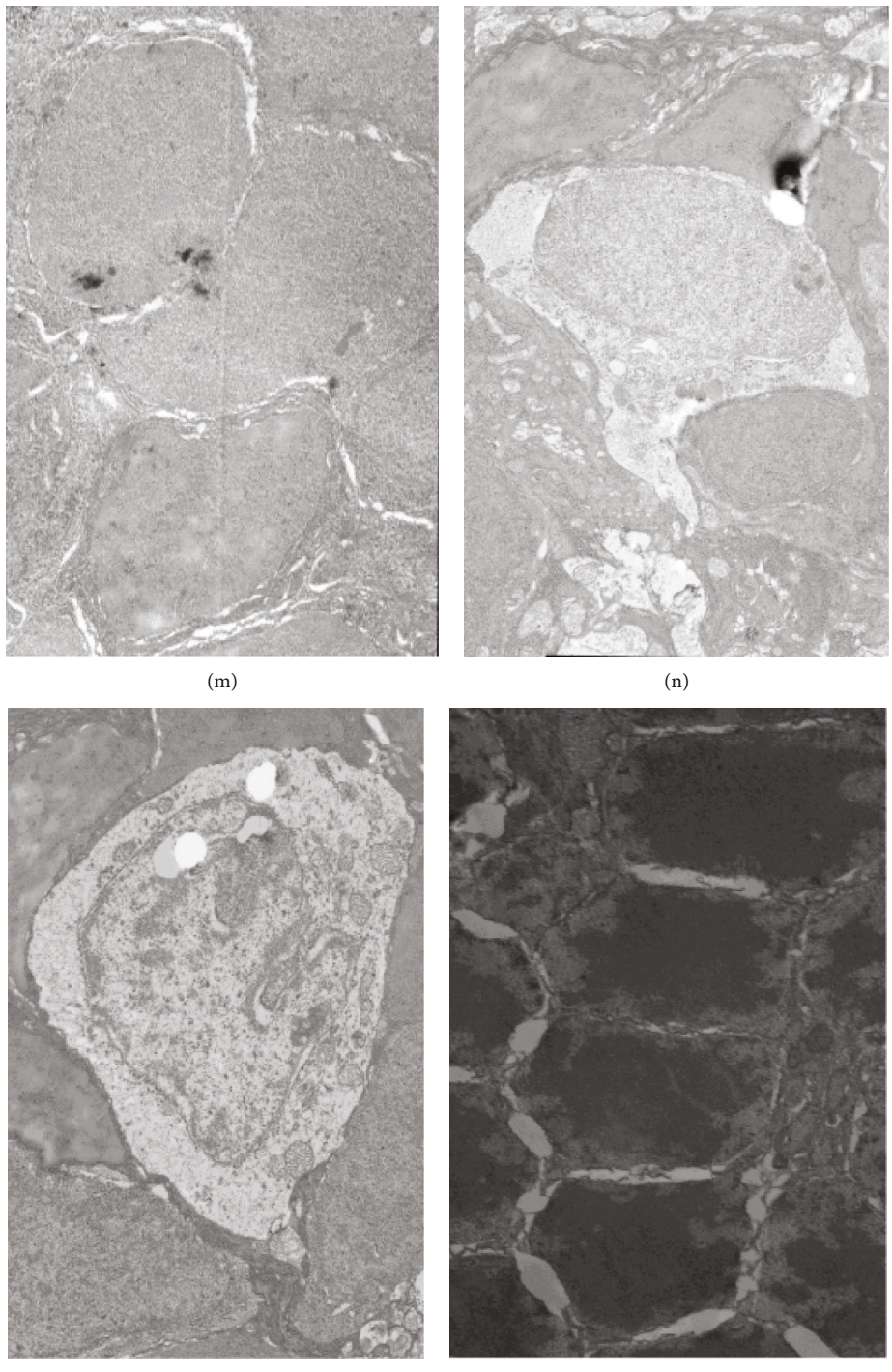


(m) (n)

(o) (p)

Figure 2: Continued.

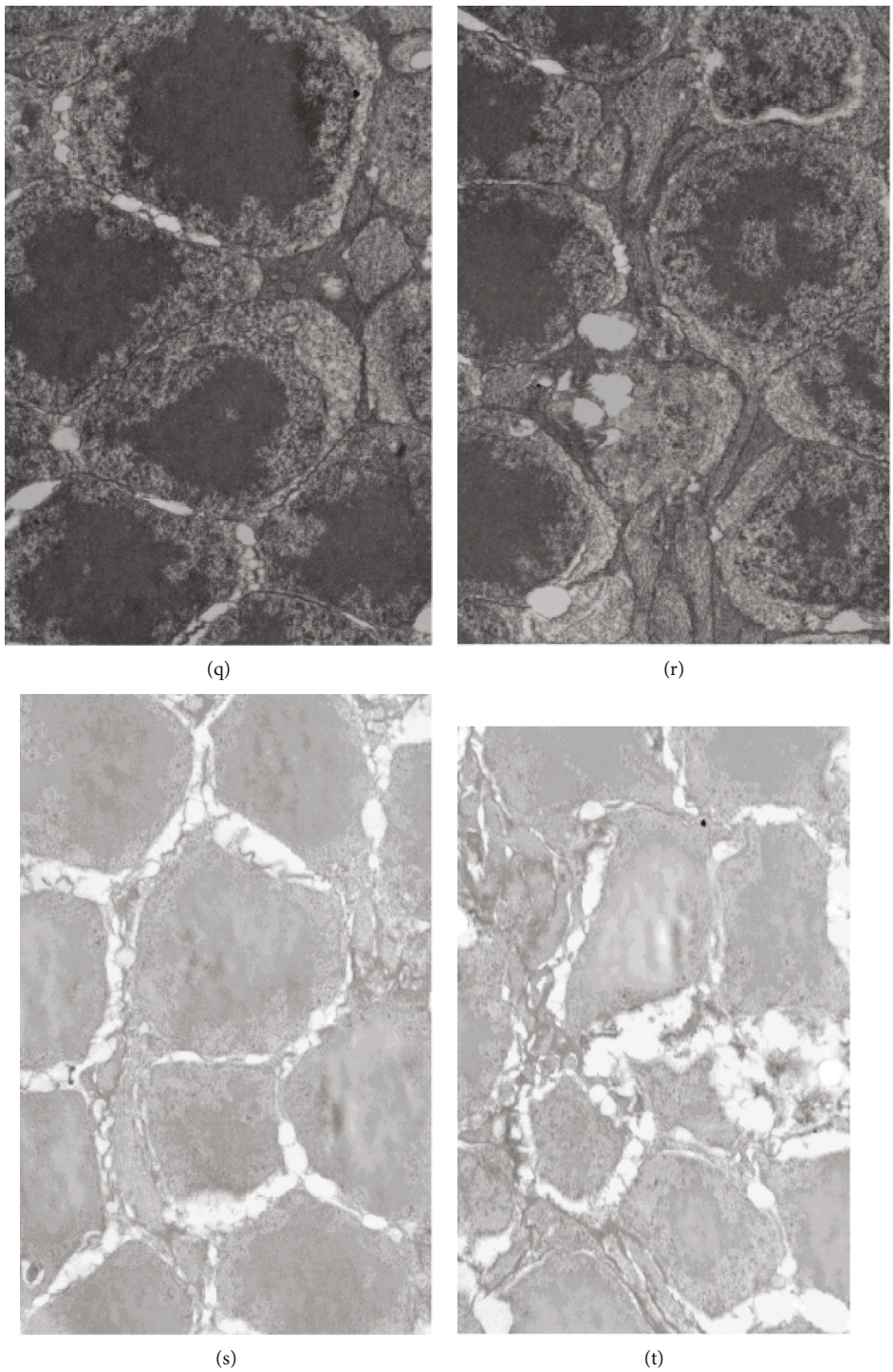
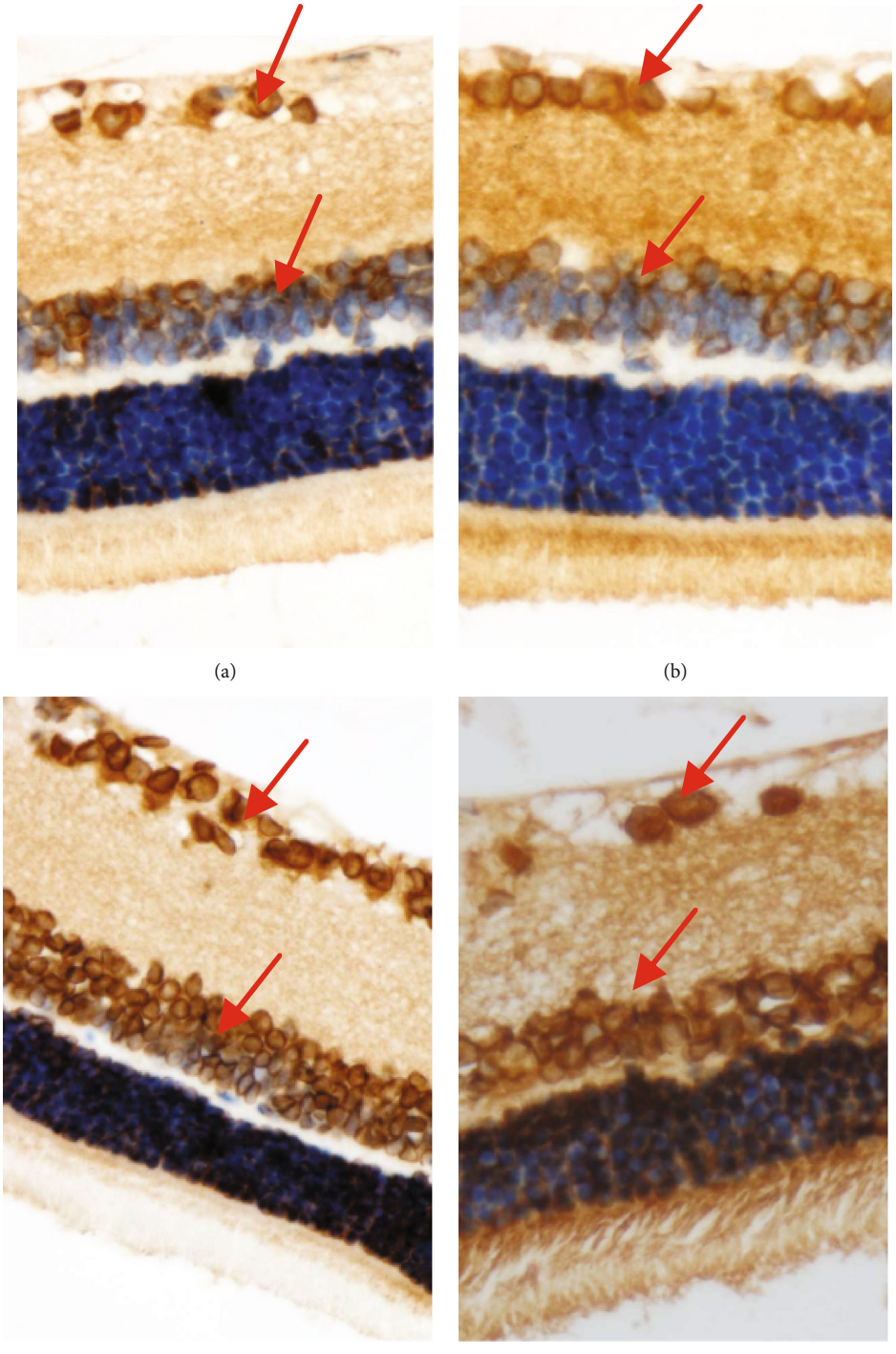



Figure 2: Pathological changes of the retina using transmission electron microscope. (a–e) The ganglion cell (×5000) of control, NGT, IGT, DM, and DKD stage, respectively; (f–j) the retinal disc (×10000) of control, NGT, IGT, DM, and DKD stage, respectively; (k–o) inner granular layer of the retina (×5000) in control, NGT, IGT, DM, and DKD stage, respectively; (p–t) outer granular layer of the retina (×5000) in control, NGT, IGT, DM, and DKD stage, respectively.

mainly expressed in the retinal ganglion cell layer and the inner granular layer in the NGT stage (Figure 5(b)). In the IGT stage, the expression of occludin declined in the ganglion cell layer (Figure 5(c)) and further decreased as the disease progressed (Figures 5(d) and 5(e)). The quantification of occludin is shown in Figure 5(f).

*3.3.4. The mRNA Levels of Inflammatory Markers, Cytokines, and HIF-1α in Retina of OLETF Rats at Different Stages.* Compared with the control group, in the IGT stage, the mRNA expression of HIF-1$\alpha$ (Figure 6(a)), iNOS (Figure 6(b)), NF-$\kappa$B (Figure 6(c)), VEGF (Figure 6(d)), and ICAM-1 (Figure 6(e)) was upregulated by 33.3%,



(a) (b)

(c) (d)

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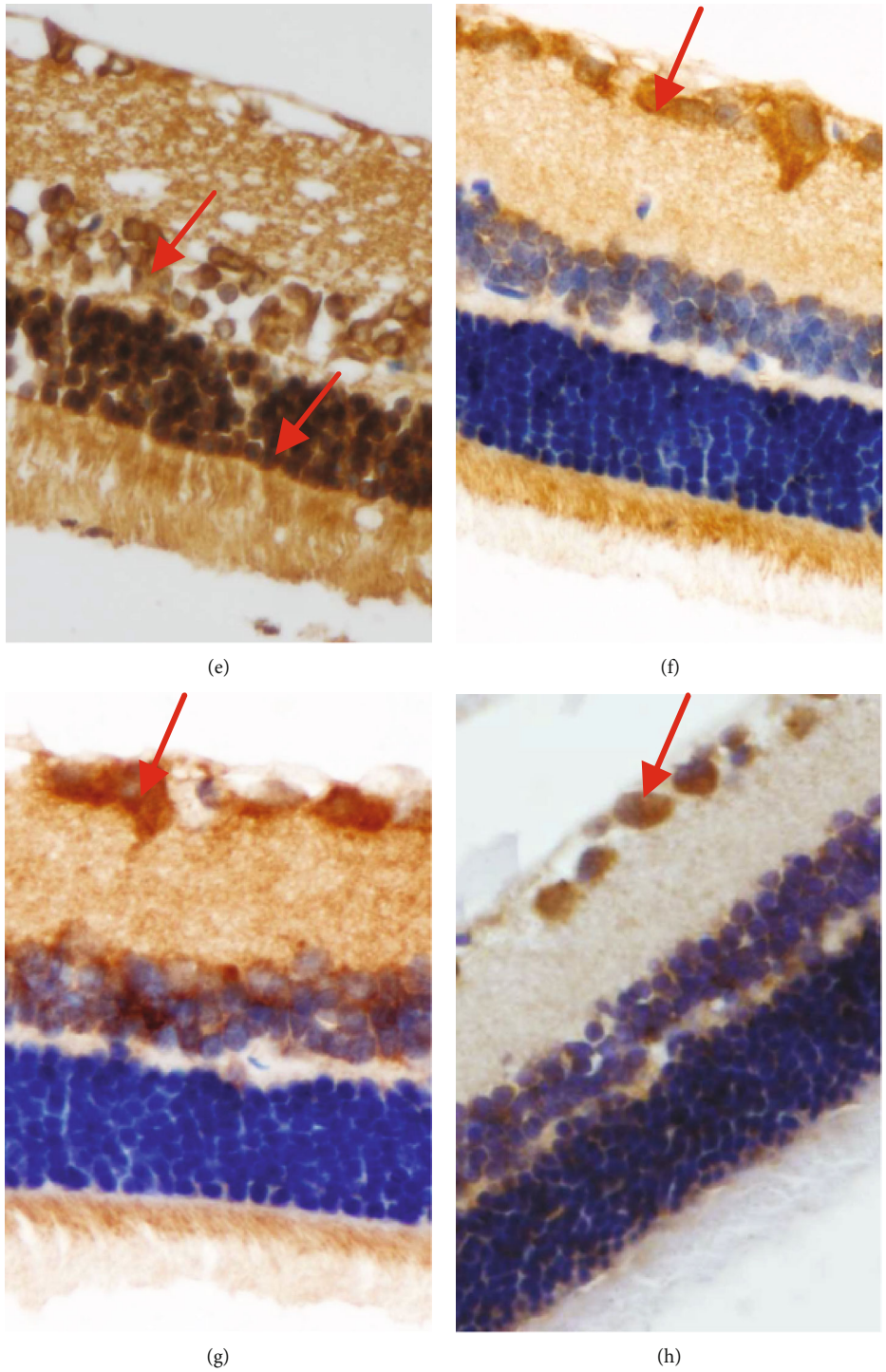


(e) (f)

(g) (h)

Figure 3: Continued.

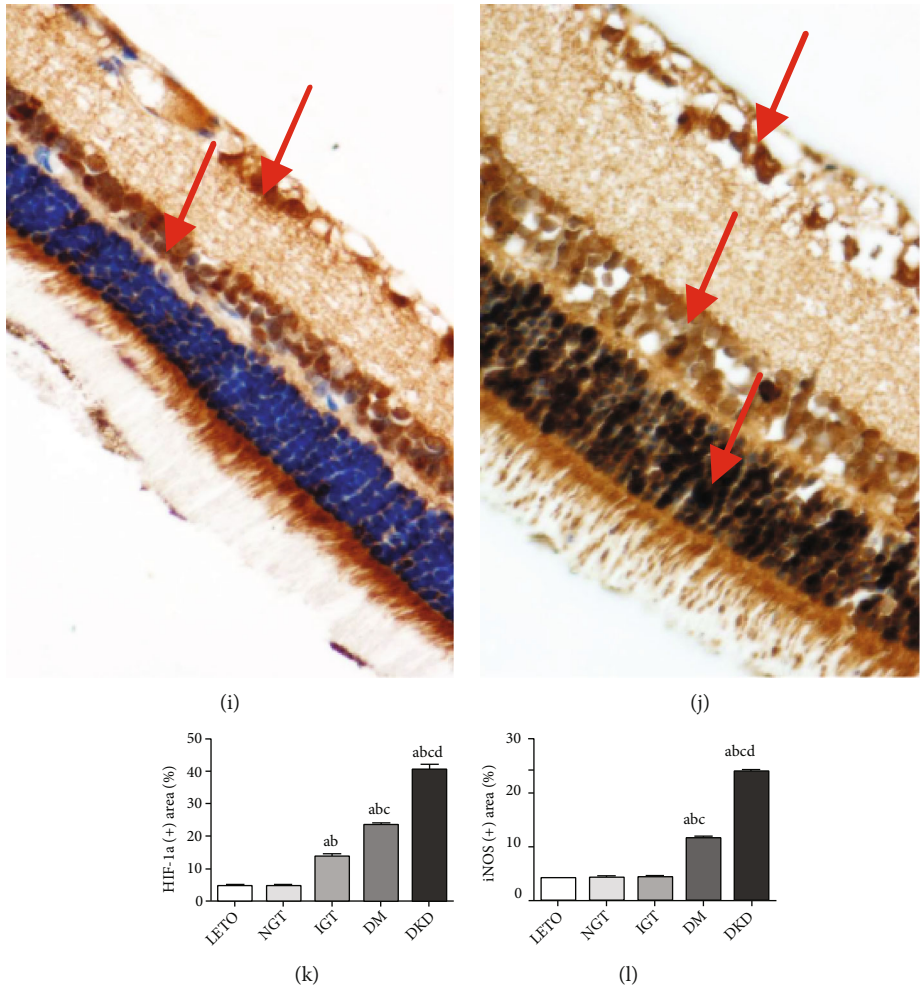



FIGURE 3: The immunohistochemical staining of HIF-1$\alpha$ and iNOS in retina of OLETF rats at different stages. (a–e) The expression of HIF-1$\alpha$ (×400) in the retina of control, NGT, IGT, DM, and DKD stage, respectively; (f–j) the expression of iNOS (×400) in the retina of control, NGT, IGT, DM, and DKD stage, respectively; (k, l) the quantification of HIF-1$\alpha$ and iNOS by Image J. $^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. NGT. $^{c}P < 0.05$ vs. IGT. $^{d}P < 0.05$ vs. DM.

18.2%, 35.8%, 23.4%, 58.2%, respectively, while that of occludin (Figure 6(f)) was decreased by 32.7%.

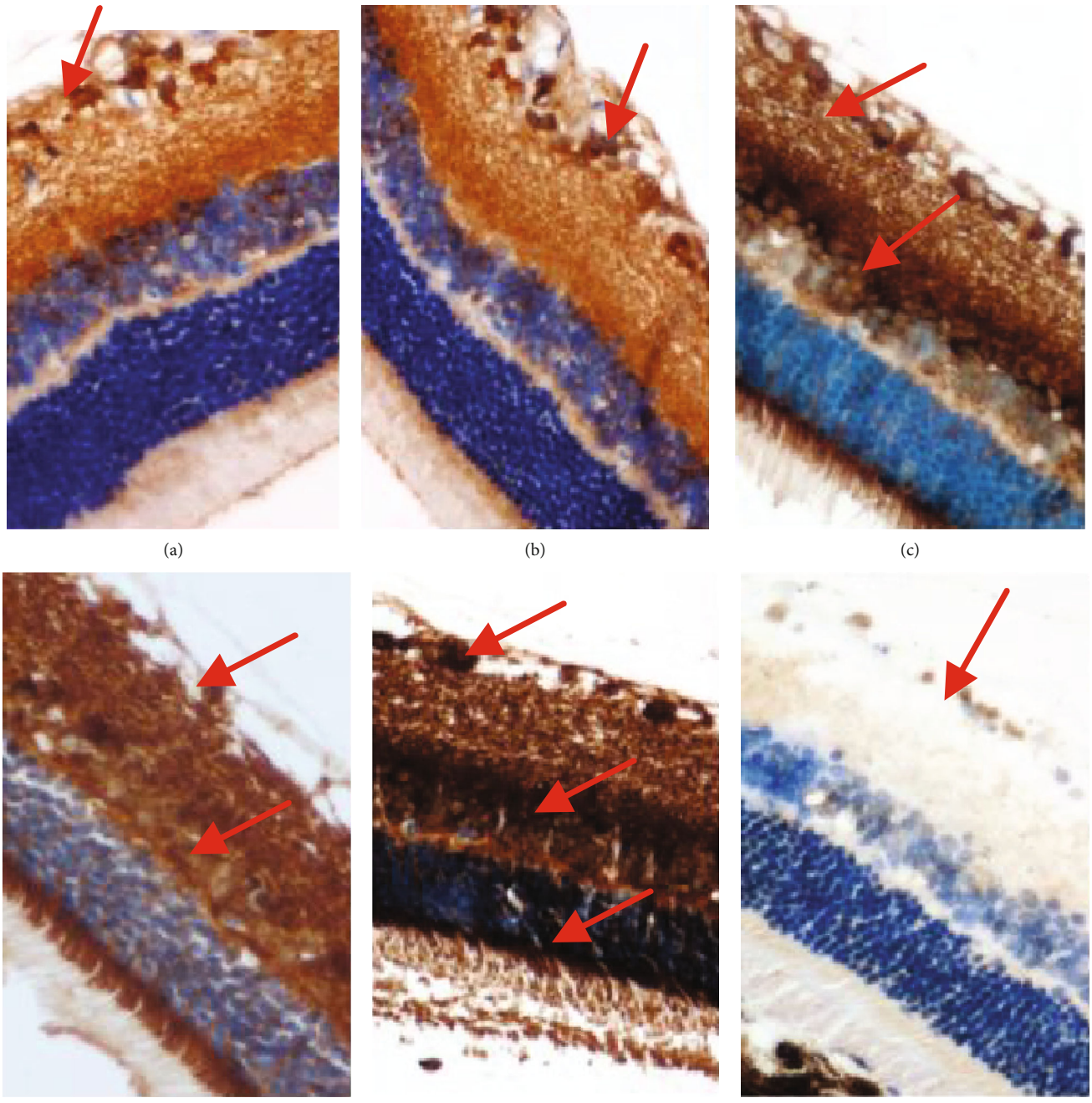
## 4. Discussion

Our study observed the pathological changes occurring in the retina in the IGT stage, focusing on the damage to the ganglion and photoreceptor cells. In the retina, photoreceptors transmit visual information to bipolar cells, which process and pass it to RGCs. Next, the RGC axons travel through the optic nerve, telling the brain about the visual world [15]. The integrity of RGCs promises the normal function of the retina; however, abnormal environment such as hyperglycemia alters the structure or function of RGCs, and this kind of RGCs impairment is progressive in the development of subsequent DR [16].

Diabetic retinopathy is a neurovascular disease, one of the leading causes of severe vision loss. RGCs are damaged in diabetic retinopathy, producing cell function impairment and their subsequent loss [17].

DR was previously considered a microvascular complication of diabetes, but now, it is widely considered a neurovascular disease caused by neurovascular unit (NVU) injury. The recent view is that retinal neurodegeneration occurs before microvascular impairment especially the vulnerable neurons like RGCs; the destruction of the neural unit leads to the vascular unit injury and accelerates microvascular damage [18]. Studies have shown that diabetic retinal neuropathy is caused mainly by retinal photoreceptor lesions [19, 20]. In our study, we observed abnormal structures, including mitochondrial swelling, fuzzy crest, vacuolization in the ganglion cells, and blurred and broken retinal photoreceptor cells in the IGT stage. Interstitial oedema and degeneration of the ganglion cells in the retinal ganglion cell layer were also observed in the IGT stage. Neovascularity was not observed before the DKD stage. These results suggest that neurodegenerative changes may occur in the early stages of diabetes.

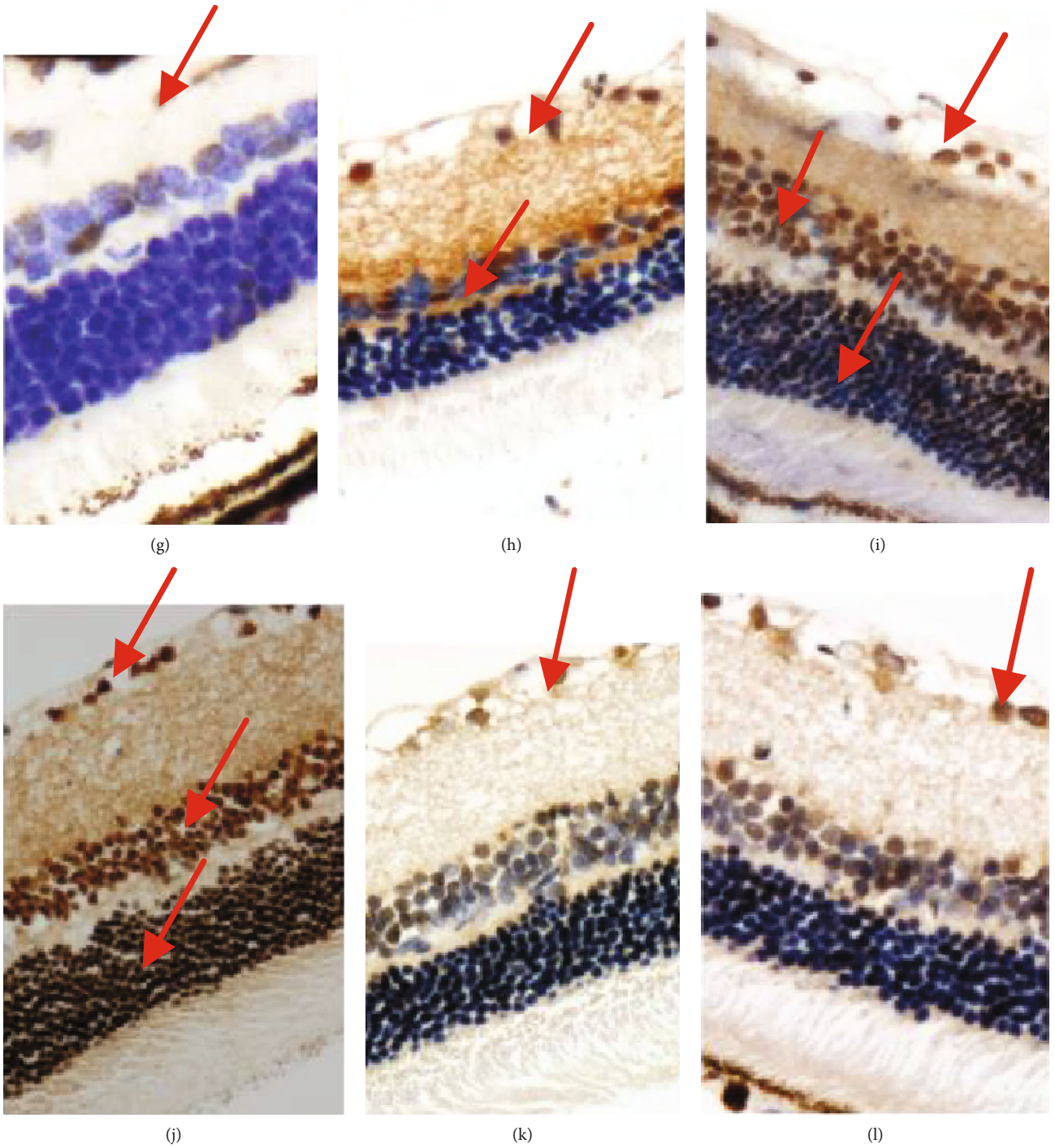
The blood retina barrier (BRB) is a structure that protects retinal cells and optic nerves from damage from cytotoxic substances and provides a stable metabolic



(a) (b) (c)

(d) (e) (f)

Figure 4: Continued.



(g) (h) (i)

(j) (k) (l)

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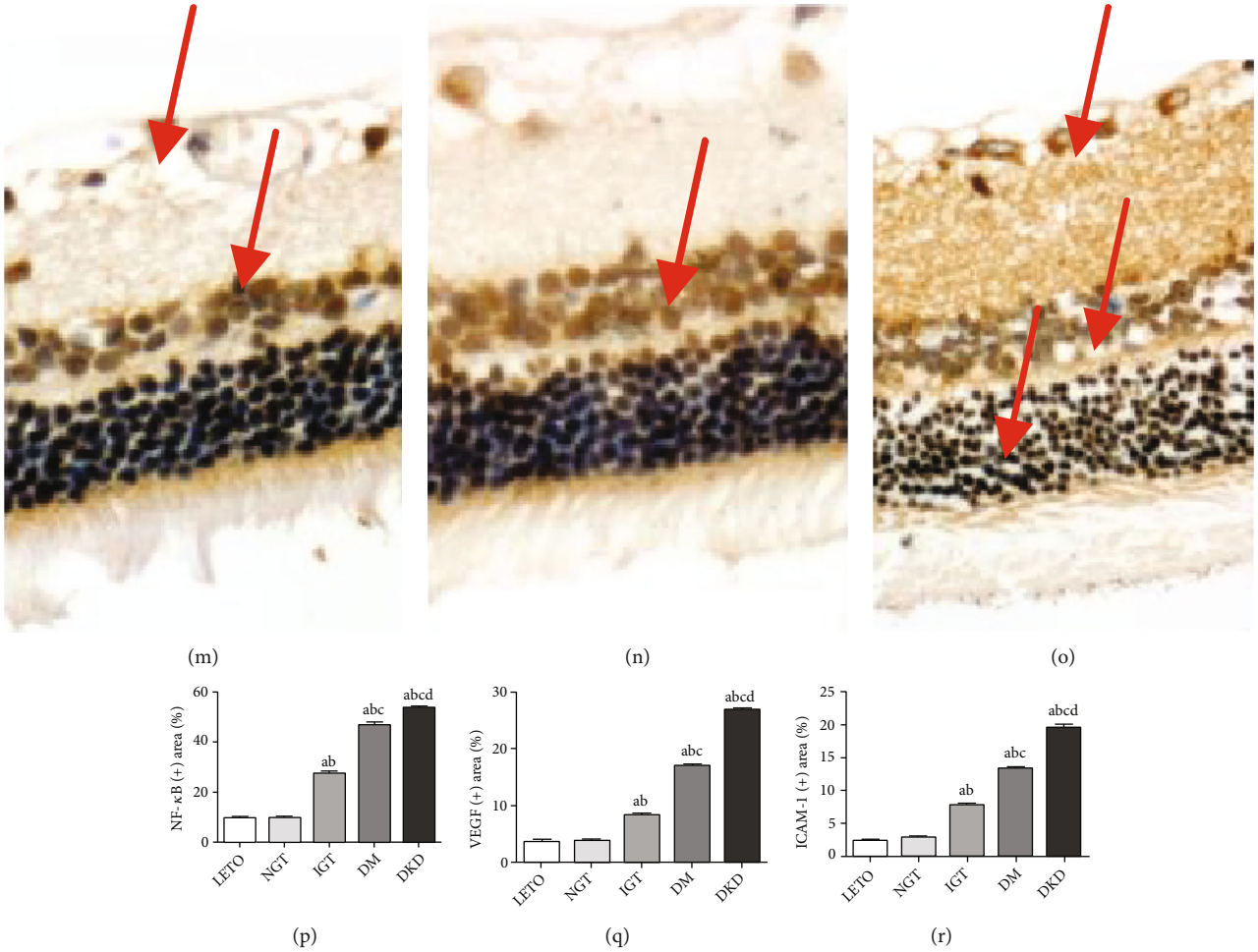
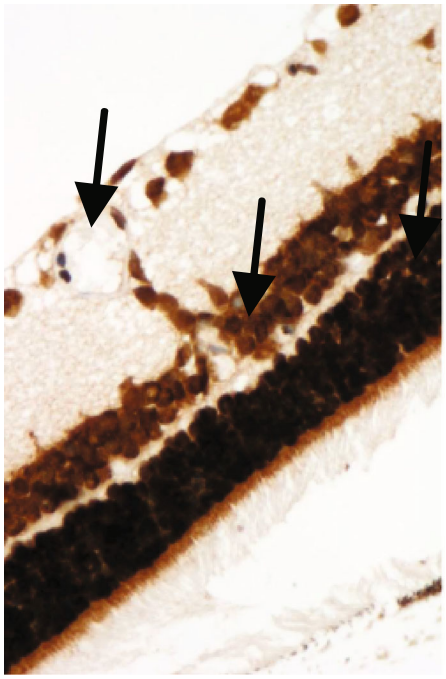



Figure 4: The immunohistochemical staining of NF-$\kappa$B, VEGF, and ICAM-1 in retina of OLETF rats at different stages. (a–e) The expression of NF-$\kappa$B (×400) in the retina of control, NGT, IGT, DM, and DKD stage, respectively, using IHC; (f–j) the expression of VEGF (×400) in the retina of control, NGT, IGT, DM, and DKD stage, respectively, using IHC; (k–o) the expression of ICAM-1 (×400) in the retina of control, NGT, IGT, DM, and DKD stage, respectively, using IHC; (p–r) the quantification of NF-$\kappa$B, VEGF, and ICAM-1. $^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. NGT. $^{c}P < 0.05$ vs. IGT. $^{d}P < 0.05$ vs. DM.

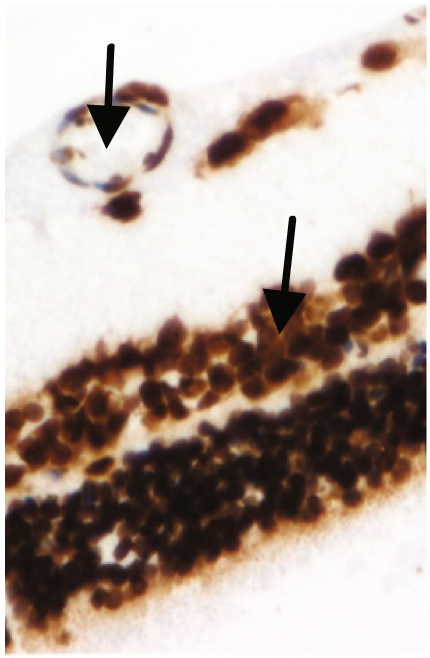
environment for them. Structural damage and increased permeability of the BRB are involved in the pathogenesis of DR. However, the pathogenesis of DR is still unclear, as there is no single mechanism that can explain the destruction of BRB. A series of metabolic abnormalities, such as ischemia, hypoxia, inflammation, oxidative stress, and some cytokines, are thought to be involved in the BRB damage.

Recently, many studies have shown that the local, chronic low-grade inflammation is closely related to the onset and development of DR [21–23]. The activation and release of NF-$\kappa$B and other inflammatory cytokines play an important role in DR. It has been demonstrated that the expression of inflammatory markers such as IL-6, iNOS, and TNF-$\alpha$ is upregulated in the retina in DR [24–27].

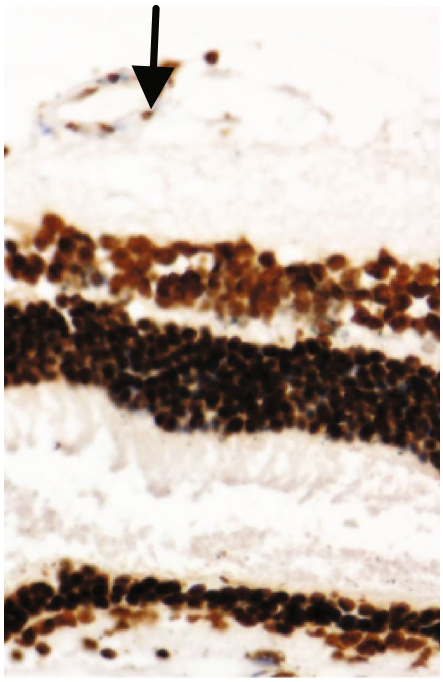
Similarly, in our study, the expression of TNF-$\alpha$ and IL-6 was significantly increased in the IGT stage and continued to increase as the disease progressed. Normally, the levels of VEGF are low in the retina to maintain the integrity of retinal blood vessels. Abnormally, high levels of VEGF can stimulate endothelial cell migration and vascular proliferation, causing retinal oedema and exudation and leading to haemangioma. In our study, the expression of VEGF, ICAM-1, and NF-$\kappa$B was significantly upregulated in the IGT stage and continued to increase as the disease progressed. VEGF is one of the important cytokines that boosts the onset and development of DR. Of the many factors capable of upregulating VEGF, hypoxia is the most direct acting factor. Under hypoxic conditions, the expression of HIF-1$\alpha$ was upregulated, thus promoting the expression of VEGF. We also observed that the expression of HIF-1$\alpha$ and iNOS in the retina increased gradually, consistent with previous studies [28, 29]. As the disease progresses, the expression of VEGF, ICAM-1, and TNF-$\alpha$ gradually increases in the retina, which in turn downregulates the expression of occludin. As occludin is involved in the formation of tight junctions, it is closely related to the BRB. Reportedly, the expression of occludin can be significantly decreased in the DM rat model and in retinal vascular endothelial cells stimulated by high glucose [30], damaging the BRB and causing vascular leakage. We observed a decreased occludin



(a)



(b)



(c)

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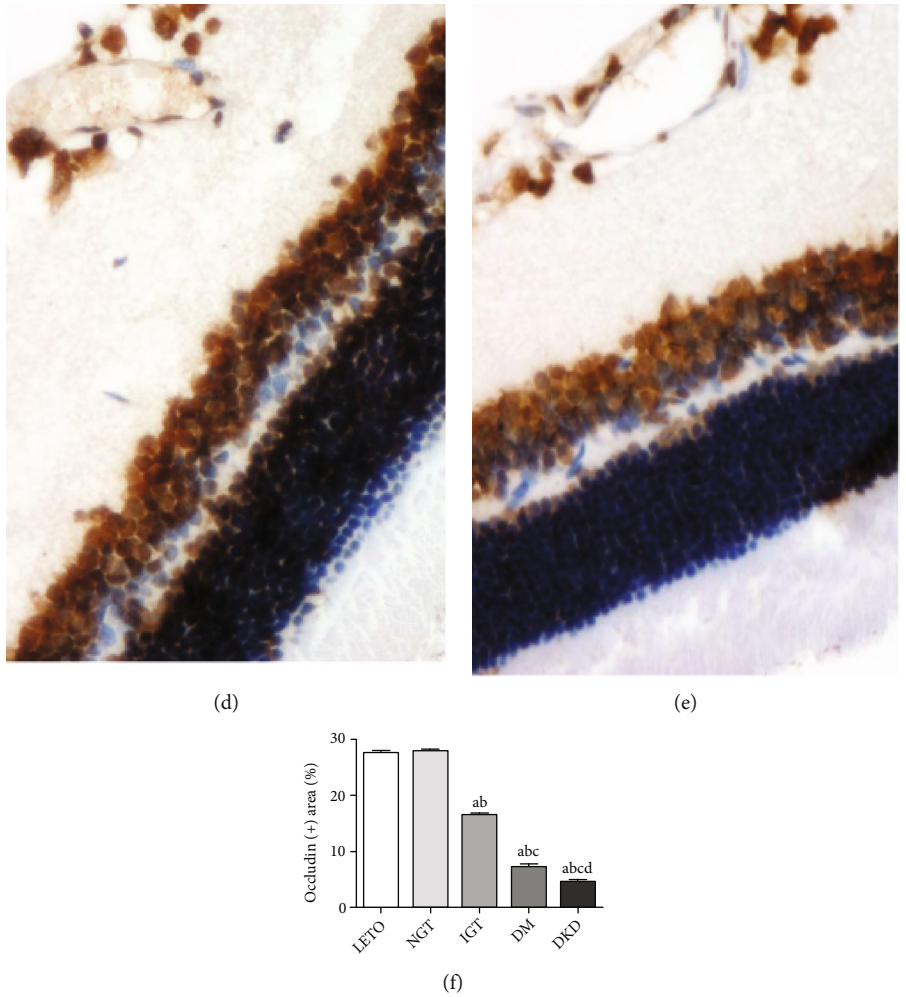


Figure 5: The immunohistochemical staining of occludin in retina of OLETF rats at different stages. (a–e) The expression of occludin (×400) in the retina of control, NGT, IGT, DM, and DKD stage, respectively, using IHC; (f) the quantification of occludin. $^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. NGT. $^{c}P < 0.05$ vs. IGT. $^{d}P < 0.05$ vs. DM.

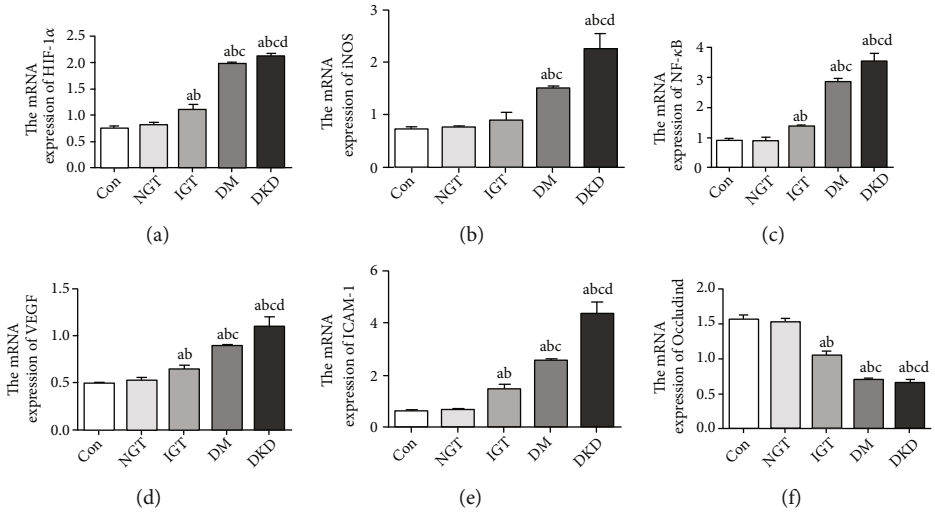


Figure 6: The mRNA levels of inflammatory markers, cytokines, and HIF-1$\alpha$ in retina of OLETF rats at different stages. $^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. NGT. $^{c}P < 0.05$ vs. IGT. $^{d}P < 0.05$ vs. DM.

expression in the retina in the IGT stage, which further decreased as the disease progressed, suggesting damage to the BRB. The above results suggest that inflammation and hypoxia may together participate in BRB damage, leading to DR. However, its exact pathogenesis needs to be investigated further.

In conclusion, the retinal neurodegeneration begins at an early stage. Therefore, early prevention and intervention are important for the treatment of diabetic retinopathy.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare no duality of interest associated with this manuscript.

## Acknowledgments

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*Research Article*

# Understanding the Activation of Platelets in Diabetes and Its Modulation by Allyl Methyl Sulfide, an Active Metabolite of Garlic

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*Background.* Diabetes mellitus (DM) is a chronic metabolic disorder associated with higher risk of having cardiovascular disease. Platelets play a promising role in the pathogenesis of cardiovascular complications in diabetes. Since last several decades, garlic and its bioactive components are extensively studied in diabetes and its complications. Our aim was to explore the antiplatelet property of allyl methyl sulfide (AMS) focusing on ameliorating platelet activation in diabetes. *Method.* We used streptozotocin- (STZ-) induced diabetic rats as model for type 1 diabetes. We have evaluated the effect of allyl methyl sulfide on platelet activation by administrating AMS to diabetic rats for 10 weeks. Flow cytometry-based analysis was used to evaluate the platelet activation, platelet aggregation, platelet macrophage interaction, and endogenous ROS generation in the platelets obtained from control, diabetes, and AMS- and aspirin-treated diabetic rats. *Results.* AMS treatment for 10 weeks effectively reduced the blood glucose levels in diabetic rats. Three weeks of AMS (50 mg/kg/day) treatment did not reduce the activation of platelets but a significant ($p < 0.05$) decrease was observed after 10 weeks of treatment. Oral administration of AMS significantly ($p < 0.05$) reduced the baseline and also reduced ADP-induced aggregation of platelets after 3 and 10 weeks of treatment. Furthermore, 10 weeks of AMS treatment in diabetic rats attenuated the endogenous ROS content ($p < 0.05$) of platelets and platelet macrophage interactions. The inhibition of platelet activation in diabetic rats after AMS treatment was comparable with aspirin treatment (30 mg/kg/day). *Conclusion.* We observed an inhibitory effect of allyl methyl sulfide on platelet aggregation, platelet activation, platelet macrophage interaction, and increased ROS levels in type 1 diabetes. Our data suggests that AMS can be useful to control cardiovascular complication in diabetes via inhibition of platelet activation.

## 1. Introduction

Diabetes mellitus (DM) is one of the chronic and complex disorders among all metabolic diseases. "Diabetes mellitus" is characterized by persistent hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Changes in lifestyle and rapid urbanization have increased the incidence of diabetes and its prevalence day by day. According to the International Diabetes Federation (IDF), 463 million adults (20–79-year age) are diabetic in the year 2019 with a record of 4.2 million deaths. Further, IDF interprets that there could be still more diabetic patients by the years 2030 and 2045 with an estimation of 578.4 and 700.2 million cases, respectively [1].

Type 1 diabetes is a condition that resulted from insufficient production of insulin by the pancreas while type 2 is due to improper utilization of produced insulin and ultimately leads to higher glucose levels in the circulatory system [2]. Both type 1 and type 2 diabetes require careful monitoring and control of blood glucose levels, if not this uncontrolled condition over time may root to several complications including cardiovascular disease (CVD). Cardiovascular complications in diabetes occurring as the disease progresses and leads to premature mortality [3–5]. IDF has suggested that diabetic patients are having 2-3 times more

probability of getting CVDs than nondiabetic patients [6]. The central mechanism for these cardiovascular complications in diabetes includes an imbalance in the systems which maintain the homeostasis of blood coagulation and fibrinolysis [7]. This imbalance results in diabetic thrombocytopathy [8], a condition that majorly affects platelet function and ultimately results in heart attacks or stroke in diabetes [9–12]. Scientific research has shown that antiplatelet therapy can reduce cardiovascular complications in diabetes and premature death [13, 14].

Despite having many drugs and therapies for the management of diabetes, it is still under the category of life-threatening diseases because of its complications. Reducing these cardiovascular complications in diabetes has become more challenging than controlling the disease itself. This has made an immense impact on scientific researchers for exploring new strategies for diabetic treatment. Dietary therapy in diabetes is one among them showing a tremendous effect on preventing as well as controlling diabetes [15, 16].

Among large numbers of nutritional diets, garlic has been showing a very promising effect on diabetic as well as diabetic complications [17–21]. An antiplatelet property has been observed in raw garlic, preventing cardiovascular complications in diabetes [22–24]. Scientific literature strongly supports that garlic is showing these beneficial effects mainly due to its sulfur-containing compounds [25–27]. Allyl methyl sulfide (AMS), an active metabolite observed inside the body after oral administration of raw garlic, has been shown to effect diabetes by reducing glucose levels, increasing insulin levels, and reducing hepatic oxidative stress caused by glucotoxicity in diabetes [28, 29]. However, the effect of AMS on diabetic complications in thrombosis especially on platelet activation and aggregation is yet to be reported.

Therefore, in this present study, we aim to understand the various altered parameters of platelets isolated from diabetic rats and the effect of garlic metabolite, allyl methyl sulfide, on altered parameters of diabetic platelet.

## 2. Materials and Methods

*2.1. Reagents.* Allyl methyl sulfide (cat no. A34201-25G) and phorbol 12-myristate 13-acetate (PMA) (cat no. P8139-1MG) were obtained from Sigma (St. Louis, Missouri, USA). Flow cytometry antibodies such as CD61 FITC antibody (cat no. 104305) and APC anti-mouse/rat CD62P (P-selectin) antibody (cat no. 148303) are from Bio Legend (San Diego, California, USA), and CD14 PE antibody (cat.no: 561707) is from BD Bioscience (Franklin Lakes, NJ, USA). Adenosine diphosphate (ADP) was obtained from Hi-Media (cat no. RM437-1G). $H_2$DCFDA dye (cat no. C6827) was from Invitrogen. Cell culture reagents such as RPMI-1640 (cat no. 31800-014), antibiotic-antimycotic (100X) (cat no. 15240062), and fetal bovine serum (cat no.10270106) are from Gibco.

*2.2. Animals and Study Design.* Wistar rats 200-250 g were used to develop diabetes and evaluate the effect of AMS on altered platelet function in diabetes. Animals were procured from Jeeva Life Sciences (Hyderabad, India). The study was approved by the Institutional Animal Ethics Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), Guwahati, India (NIPER/BT/2020/37). The animals were housed in individually ventilated cages (IVC) at an animal house facility of NIPER, Guwahati, under standard conditions (temperature 23 ± 1 °C, 50 ± 15% relative humidity, and 12 h light/dark cycle). Wistar rats were allowed free access to food and water ad libitum during the study. Post seven days of acclimatization, animals were randomly allocated into four groups ($n = 6$): group 1: control; group 2: diabetes (STZ 35 mg/kg); group 3: diabetes+AMS (50 mg/kg); and group 4: diabetes+aspirin (30 mg/kg). All these rats were maintained for 10 weeks. Every week, the body weight of all animals was recorded to understand the body weight gain or loss during the experimental period and glucose levels were monitored by using a glucometer (Accu Chek Active, Roche). The doses of AMS and aspirin were chosen from our previous work and scientific literature [30, 31].

*2.3. Induction of Diabetes.* Diabetes was chemically induced in Wistar rats (weight 200-250 g) with streptozotocin. After 6 hours of fasting, adult Wistar rats were administered with a single intraperitoneal (I.P) injection of a freshly prepared solution of streptozotocin (STZ) in ice-cold citrate buffer (0.01 M, pH 4.5) at a dose of 35 mg/kg body weight. Animals were then monitored for the next seven days for their blood glucose levels by using a glucometer (Accu Chek Active, Roche). The induction of diabetes was confirmed by monitoring fasting blood glucose. The rats with >250 mg/dl of blood glucose levels were considered diabetic.

*2.4. Oral Dosing.* Allyl methyl sulfide was administered orally at a dose of 50 mg/kg in corn seed oil for 10 weeks in diabetes Wister rats and referred to as the 'diabetes +AMS' group. Aspirin was given at a dose of 30 mg/kg in 0.5% carboxy methyl cellulose (CMC) orally to another group of diabetes Wistar rats and referred to as the 'diabetes+aspirin' group. The control group was referred to the rats feed with corn seed oil.

*2.5. Cell Culture.* Human macrophages (THP-1 cells; an acute monocytic leukemia cell line) were a kind gift from Translational Health Science and Technology Institute (THSTI, Faridabad, India). The cells were cultured in the RPMI 1640 medium enriched with fetal bovine serum (10% *v*/*v*) and antimicrobial agents (antibiotic and antimycotic) (100 U/ml) and cultured under standard conditions that are 37°C and 5% $CO_2$ incubator (eppendorf CellXpert C170).

*2.6. Blood Collection and Isolation of Platelets.* The platelets were isolated from blood by dual centrifugation. It has been carried out at room temperature. Preventive measures have been taken to avoid platelet activation during the process. Initially, the animals were anesthetized with the help of isoflurane. Through retroorbital plexus using capillary tube, blood was collected into a tube containing 3.8% sodium citrate (9 : 1 ratio) and centrifuged at 500 rpm for 15 minutes at

20°C temperature. The centrifugation results in isolation of platelet-rich plasma (PRP) as the upper layer in a tube. Almost 3/4$^{th}$ of this layer was taken into a fresh tube using a wide-bore pipette tip. Further, platelets were pelleted by centrifuging the PRP at 400 g for 10 minutes at 20°C. The platelets pellet was washed, resuspended in HEPES-Tyrode buffer.

*2.7. Measuring the Number of Activated Platelets by Flow Cytometer.* The *in vivo* activated status of isolated platelets was measured by flow cytometry using fluorochrome-tagged antibodies. All these works were carried out within 2 hours of blood collection and took all measures to avoid platelet activation during handling of the sample. 7 $\mu$l of PRP was taken in a tube and diluted by adding 50 $\mu$l of HEPES-Tyrode's buffer having 1% BSA. Samples were added with antibody mix having FITC anti-mouse/rat CD61 antibody (platelet surface marker) and APC anti-mouse/rat CD62P antibody (platelet activation marker) diluted with HEPES-Tyrode's buffer (with 1% BSA). Stained platelets were incubated in dark for 30 minutes at room temperature. Samples were immediately analyzed by using Attune™ NxT Flow Cytometer, where analysis was done by taking 50,000 events for each sample. The compensation was done using individual antibody-stained cells and unstained cells in order to avoid spillover from one channel to other. Platelets were gated on forward light scatter (FSC) vs. side light scatter (SSC) plot, and the percentage of CD62P positive cells is enumerated among platelets positive for CD61 cells which give % activated platelets.

*2.8. Measuring Platelet Aggregation in Presence of ADP, a Platelet Agonist, by Flow cytometry.* With a few modifications to previously mentioned procedure, the hypersensitivity of platelets in presence of platelet-aggregating agents (ADP) has been observed by measuring the percentage aggregation of platelets by flow cytometry [32]. 50 $\mu$l of freshly isolated platelet-rich plasma (PRP) was resuspended in 450 $\mu$l of HEPES-Tyrode buffer. Platelets were activated by adding 20 $\mu$M ADP as a final concentration and incubated for 10 minutes at 37°C under shaking conditions at 1000 rpm using a thermoshaker. Diluted PRP without agonist was used as a negative control. Cells were analyzed by flow cytometry for the presence of platelet aggregation. Platelets were differentiated from platelet aggregated by gating cells on forward light scatter (FSC) versus side light scatter (SSC) plot where the platelet aggregates are having increased size and density.

*2.9. Analysis of Platelet and Macrophage Interaction by flow cytometry.* The heterogeneous interactions of platelets with macrophage were analyzed by flow cytometry method using phorbol 12-myristate 13-acetate- (PMA-) differentiated THP-1 macrophages [33, 34].

For monocyte differentiation to macrophage, THP-1 monocytes ($1.5 \times 10^6$ cells) were seeded in 6-well plate having 1 ml of Roswell Park Memorial Institute medium (RPMI-1640) which was supplemented with 100 $\mu$g/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum. The monocytes were differentiated to macrophages by adding 50 ng phorbol 12-myristate 13-acetate (PMA) and incubated for 48 hours in an incubator maintaining 5% $CO_2$ at 37°C under humidified conditions. After 48 hours of incubation, media was changed to fresh RPMI-1640 complete media without the addition of PMA and incubated for 48 hours in an incubator.

For macrophage and platelet coculture study, isolated PRP of 7 $\mu$l was added in a culture plate having macrophages derived from THP1 and incubated for 30 minutes in a $CO_2$ incubator. After successive coculturing of platelets, each well has been washed with PBS to remove floating platelets. Cells were trypsinized by adding 0.25% trypsin EDTA and incubated in a $CO_2$ incubator for 2 minutes. The detached cells were collected in an individual tube and centrifuged at 400 g for 10 minutes at 20°C. The supernatant was discarded; the pellet was washed with PBS and fixed by incubating with 4% paraformaldehyde for 10 minutes. The cells further were washed and resuspended in PBS for labelling with fluorescently conjugated antibodies of macrophage surface marker (CD14 PE) and platelet-specific marker (CD61 FITC). The cells were incubated in dark for 30 minutes at room temperature and analyzed by Attune™ NxT Flow Cytometer. Analysis was done by gating the macrophage population under FSC vs. SSC plot. To compensate for the overlapping spectra single-stained cells were used. Finally, we measured the mean fluorescence intensity of CD61-FITC in macrophage (CD14+) population inorder to quantify platelet macrophage interaction.

*2.10. Measurement of Endogenous ROS in Platelets.* Intracellular ROS was measured by using 2′,7′-dihydrodichlorofluorescein diacetate, $H_2$DCFDA, dye as per the procedures mentioned in articles [35, 36]. The freshly isolated PRP (7.0 $\mu$l) was diluted in 100 $\mu$l of HEPES-Tyrode's buffer and incubated with 10 $\mu$M of $H_2$DCFDA dye under dark conditions for 30 min at 37°C. Later, samples were further diluted with 200 $\mu$l HEPES-Tyrode's buffer and mixed gently with a pipette. The tubes were centrifuged at 400 g for 10 minutes at 20°C for pelleting the cells and discarding the supernatant. Further, the pellet was resuspended by adding 100 $\mu$l of HEPES-Tyrode's buffer. The unstained samples were used as a negative control. All of these samples were analyzed using Attune™ NxT Flow Cytometer by taking 10,000 events where the mean fluorescent intensity of DCFDA in platelet population was determined for each sample.

*2.11. Statistical Analysis.* All the statistical analysis was performed using GraphPad Prism version 8.0.2(263) (GraphPad Software, San Diego, CA, USA), and its comparisons were made utilizing ANOVA test followed by Tukey's test post hoc analysis. Results were expressed as mean ± standard error, and a $p$ value $< 0.05$ was considered significant.

## 3. Results

*3.1. Body Weight and Blood Glucose Level Changes in Rats.* We monitored body weights and blood glucose levels of

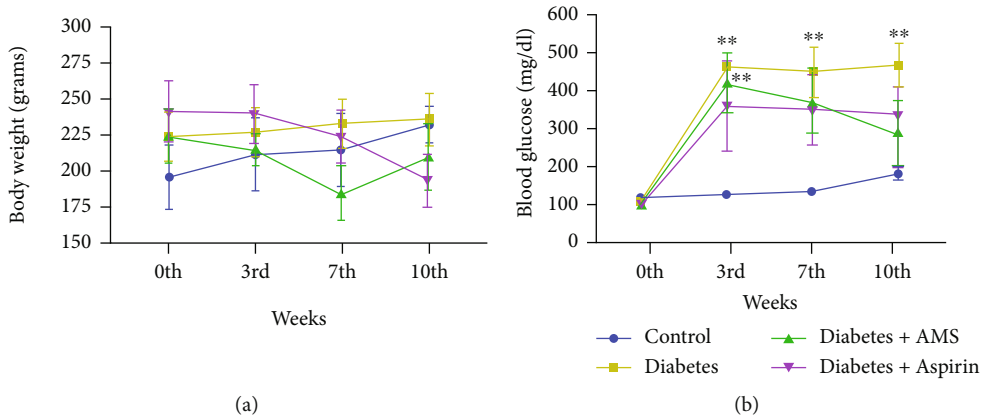


Figure 1: (a) Body weights and (b) blood glucose levels of the control, diabetes, AMS-treated, and aspirin-treated rats at the end of the $0^{th}$, $3^{rd}$, $7^{th}$, and $10^{th}$ weeks. All values are represented as mean ± SEM ($N = 4$-6). $^{**}p < 0.01$ vs. the control.

experimental rats until 10 weeks. The mean values of body weight in grams of all study groups were compared in $0^{th}$, $3^{rd}$, $7^{th}$, and $10^{th}$ week and plotted as a graph. As shown in Figure 1(a), we did not observe any significant body weight changes among all four groups. The blood glucose levels of the diabetes group significantly increased at all ($3^{rd}$, $7^{th}$, $10^{th}$ weeks) the time points when compared to the control. However, AMS treatment decreased the glucose levels at 7 and 10 weeks when compared to the $0^{th}$ and $3^{rd}$ week of treatment (Figure 1(b)).

*3.2. Effect of AMS on Platelet Activation in Diabetes.* The *in vivo* activated status of platelets in the control, diabetes, and treatment groups after $3^{rd}$ and $10^{th}$ weeks of study duration has been analyzed by flow cytometry. The graph represents percent CD61 positive on the $X$-axis and CD62P-positive platelets on the $Y$-axis (Figure 2(a)). The dual positive cells are considered activated platelets, and percent of the activated platelets were represented as bar graphs. At the end of $3^{rd}$ week, the percentage of activated platelets was significantly increased in the diabetes group (~32.4%) when compared to the control (~19.11%). We did not observe any significant changes in the AMS and aspirin-treated groups (Figure 2(b)). Similarly, at the end of $10^{th}$ week, the percentage of activated platelets was increased significantly in the diabetes group (~47.19%) when compared to the control group (~35.24%). Moreover, both aspirin and AMS treatments significantly decreased this activation of platelets (~30.15 and 25.22%, respectively) induced in diabetes (Figure 2(c)).

*3.3. Effect of AMS on ADP-Induced Platelet Aggregation.* To assess the effect of AMS on platelet aggregation, flow cytometry analysis was performed on platelets derived from all the experiment groups in the absence or presence of ADP at the $3^{rd}$ week and $10^{th}$ week. Figure 3(a) represents FSC vs. SSC density plots showing the $3^{rd}$ week platelet aggregation with and without ADP where aggregates are gated as R2. In the absence of ADP, we did not observe any significant change in the percentage of platelet aggregation in the diabetes group compared to the control. However, a significant decrease (3-fold) in percent aggregation was observed in the AMS- and aspirin-treated diabetes groups compared to diabetes (Figures 3(a) and 3(b)). Similarly, as shown in Figures 3(a) and 3(c), we observed a significant induction (~1.6-fold) in platelet aggregation by ADP in the diabetes group when compared to the control. With AMS and aspirin treatment, we observed a significant decrease (~3.8- and 4.2-fold, respectively) in platelet aggregation when compared to the diabetes group.

Similar flow cytometry analysis in the presence and absence of ADP was performed at the end of the $10^{th}$ week, and results were represented as FSC vs. SSC density plots (Figure 4(a)). In the absence of ADP, we observed that the percentage of platelet aggregation increased 1.2-fold in diabetic rats when compared to the control (Figures 4(a) and 4(b)). A significant ($p < 0.05$) decrease in the percentage of platelet aggregation was observed after AMS and aspirin treatment (~2.3- and 3.3-fold, respectively) when compared to diabetes. Similarly, ADP-induced aggregation was increased by 1.1-fold in diabetes while it was decreased by 1.1- and 1.5-fold in the AMS and aspirin treatment groups, respectively (Figures 4(a) and 4(c)).

*3.4. Effect of AMS on Diabetes-Induced Reactive Oxygen Species Generation in Platelets.* After platelet activation and aggregation study, we measured the reactive oxygen species (ROS) levels in the platelets isolated from the control, diabetic, and treated diabetic rats at $10^{th}$ week of the study duration. ROS levels in platelets were measured by using $2',7'$-dihydrodichlorofluorescein diacetate ($H_2$DCFDA) dye and analyzed it by flow cytometry. $H_2$DCFDA dye is a direct measure of amount of ROS present in cells, and the results are expressed as the mean fluorescent intensity (MFI) of DCFDA (Figure 5). Platelet ROS levels was significantly ($p < 0.05$) increased in the diabetic group when compared to the control. However, the ROS levels was decreased significantly ($p < 0.05$) in platelets isolated from the AMS-treated diabetic rats when compared to diabetes rats. We did not observe any significant change in ROS levels in platelets isolated from the aspirin-treated diabetic rats.

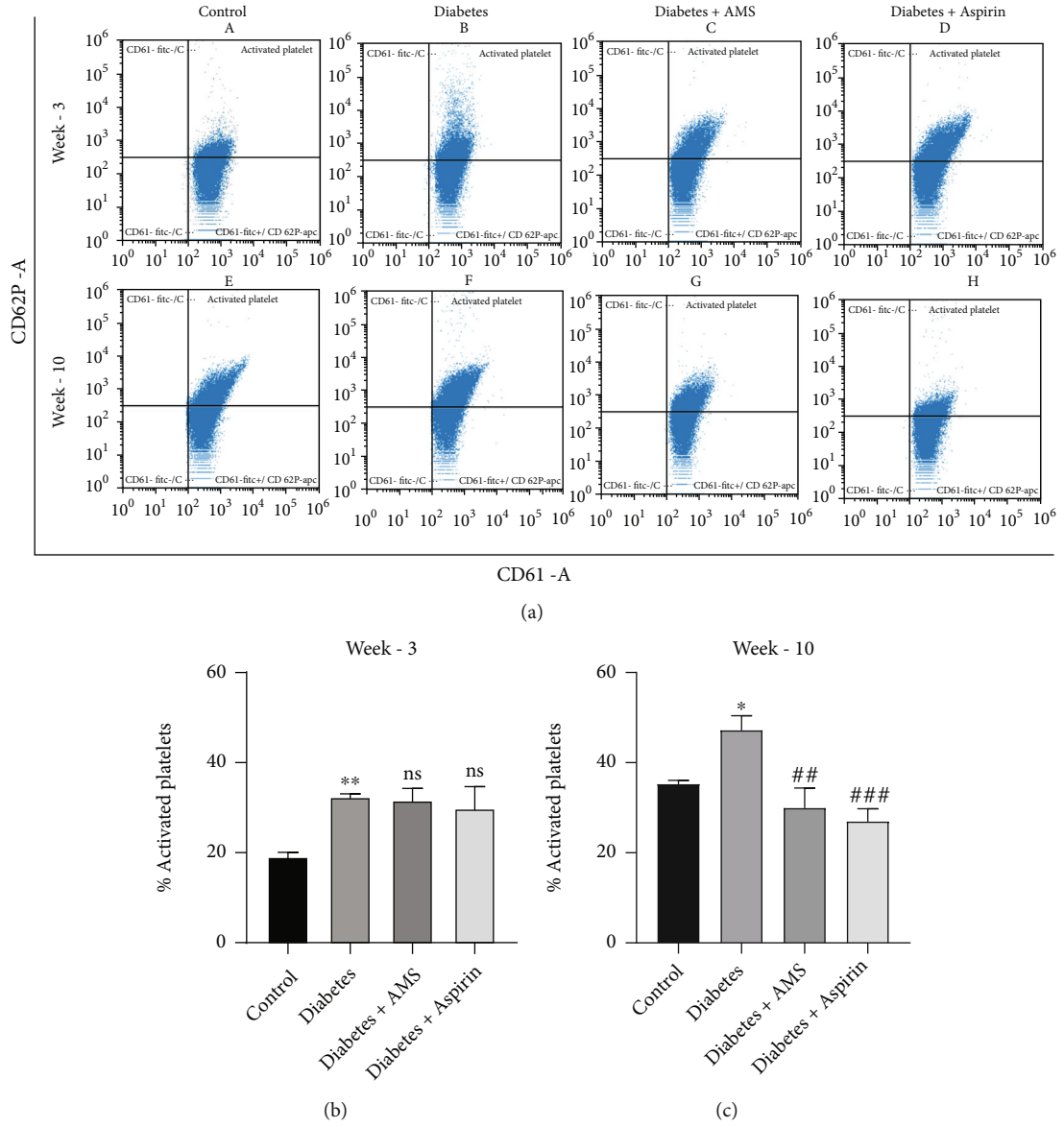


Figure 2: Flow cytometry analysis of platelet activation showing percentage dual positive cells (CD61+, CD62P+). (a) Representation of the scattered plots of percentage platelet activation in the control (A, E), diabetes (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups after 3 and 10 weeks of study. Bar graph showing percentage platelet activation at the end of the 3rd week (b) and 10th week (c). All values are represented as mean ± SEM ($N = 4$-5). $^{*}p < 0.05$ vs. the control; $^{**}p < 0.01$ vs. the control; $^{\#\#}p < 0.01$ vs. diabetes; and $^{\#\#\#}p < 0.001$ vs diabetes; ns: nonsignificant.

*3.5. Effect of AMS on Diabetes-Induced Macrophage and Platelet Interaction.* Platelet macrophage interaction has been considered an important phenomenon for platelet activation in diabetes [37, 38]. To look at this interaction, macrophage and platelet were incubated together and FACS analysis was performed. THP 1, a well-known monocyte cell, were differentiated with phorbol 12-myristate 13-acetate (PMA) and later cocultured with platelets collected from the control, diabetes, and AMS-/aspirin-treated rats at $10^{th}$ week of study duration. The results are shown in mean fluorescent intensity of CD61-FITC (marker of platelets) among CD14-positive THP1 macrophages. The data was represented as a bar graph (Figure 6). The increased mean fluorescent intensity was considered an aggregate cell of macrophages and platelets. In our data, the significant ($p < 0.05$) increase (~2.4-fold) in mean florescent intensity of CD61-FITC was observed in macrophages cocultured with diabetes platelets when compared to the control. Although we observed a decrease in macrophage platelet interactions in the AMS and aspirin treatment groups (~1.2- and 1.5-fold) when compared to the diabetic group, these changes were not significant.

## 4. Discussion

Diabetes mellitus is not a single clinical entity but a spectrum of diseases with various diabetic complications [39–41]. Several reports have shown a strong relation

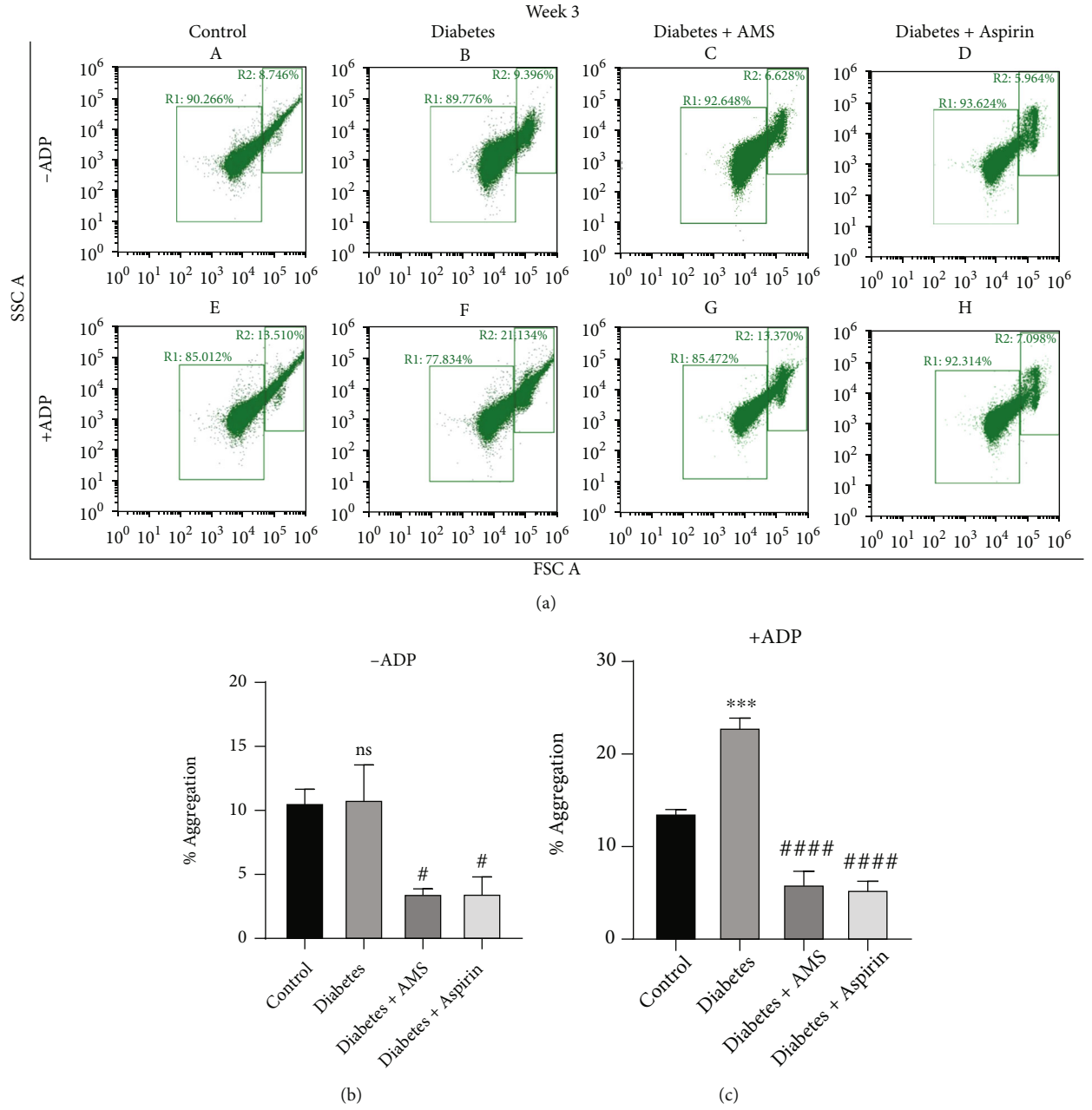


Figure 3: Flow cytometry analysis of platelet aggregation after 3-week treatment. (a) Scatter plot of forward vs. side scatter of platelets from the control (A, E), diabetic (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups, representing percentage of platelet aggregation in the absence and presence of ADP. Bar graph represents percentage of platelet aggregation observed in absence (b) and presence (c) of ADP. All values are represented as mean ± SEM ($N = 3$). $^{***}p < 0.001$ vs. the control; $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs. diabetes; ns: nonsignificant.

between diabetes mellitus and premature cardiovascular events [4, 5]. The underlining mechanisms of cardiovascular complication in diabetes include several physiological and pathological changes in the heart, blood vessels, blood cells, and kidneys [42]. Previous scientific literature identified numerous risk factors i.e., hyperglycemia [43–45], dyslipidemia [46, 47], inflammation [48, 49], endothelial dysfunction, and oxidative stress [50, 51], which together can induce several complexities including cardiovascular complication in diabetes. Studies have also identified that alteration of normal platelet function as one of the major risk factors of diabetic complication and characterized by increased thromboxane synthesis [52], reduced membrane fluidity [53], and increased expression of activation-dependent adhesion molecules (e.g., GpIIb-IIIa and P-selectin) [54]. All of these changes make platelets more reactive and create a prothrombic environment in a diabetes patient [55, 56]. Nevertheless, the studies focusing on platelet dysfunction in type 1 diabetes (T1DM) and its modulation by pharmacological agents are limited. Recent literature says that among all the popular natural remedies, organosulfur compounds from garlic have shown a potential antidiabetic

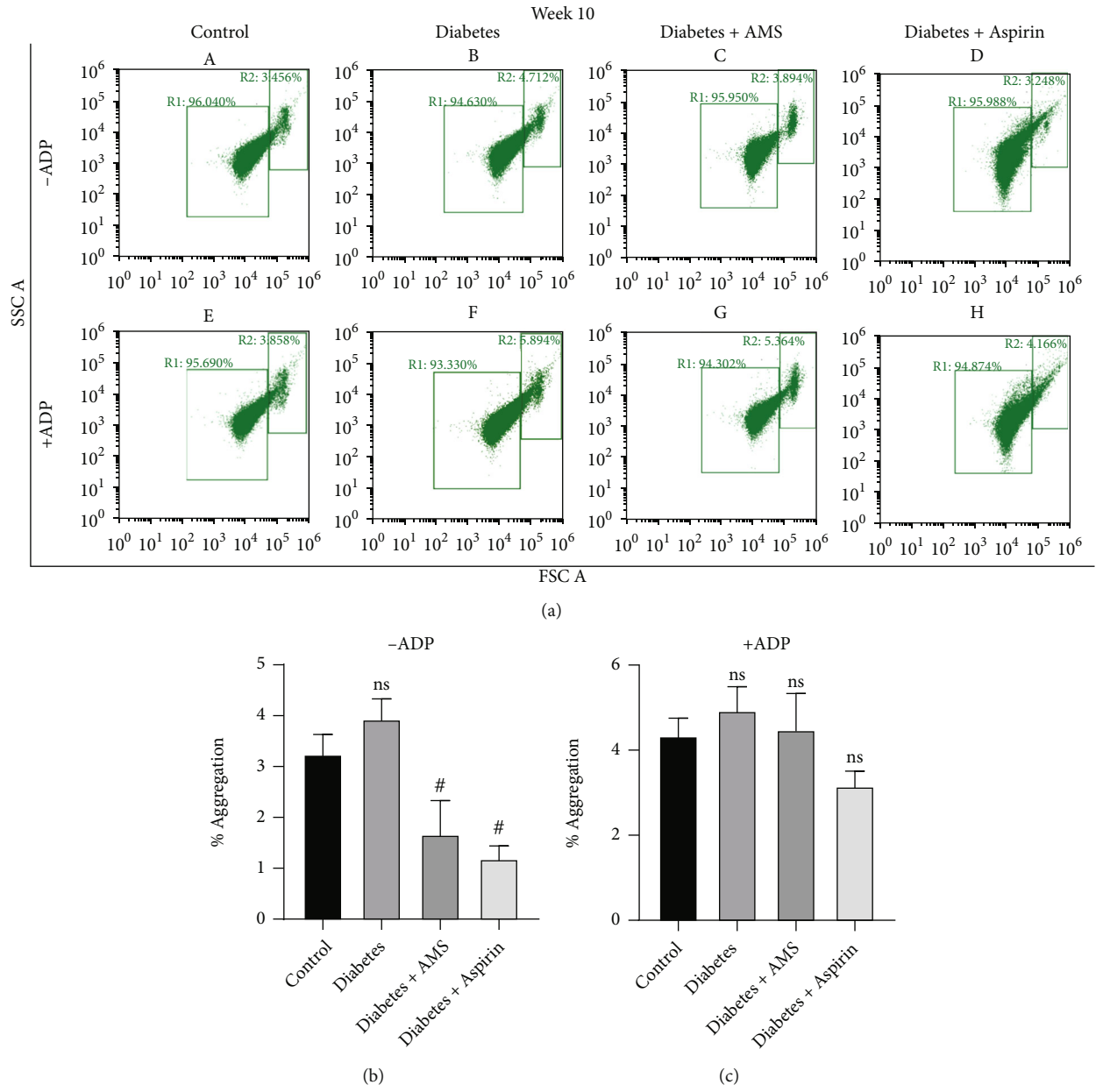


Figure 4: Flow cytometry analysis of platelet aggregation after 10-week treatment. (a) Scatter plot of forward vs. side scatter of platelets from the control (A, E), diabetic (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups, representing percentage of platelet aggregation in the absence and presence of ADP. Bar graph represents percentage of platelet aggregation observed in absence (b) and presence (c) of ADP. All values are represented as mean ± SEM ($N = 3$). $^{\#}p < 0.05$ vs. diabetes; ns: nonsignificant.

and as well as antithrombotic effect in diabetic individual [17, 18, 22, 23, 57]. Previous research work also supported the role of garlic to attenuate cardiovascular complications in diabetes [18]. Allyl methyl sulfide (AMS) is one of the important sulfur compounds obtained from garlic, and studies showed that AMS is a major metabolite that is detected in the human breath and plasma [28]. Our previous study on AMS suggested that chronic administration of AMS is safe in control rats, where the body weight, food, and water intake along with the histopathology of major organs and serum biomarkers remained normal [30]. Further, the same study showed a beneficial effect of AMS on isoproterenol-induced cardiac fibrosis and dysregulated extracellular matrix (ECM) deposition in the myocardium [30]. Also, our recent finding showed a cardioprotective effect of AMS in pressure overload-induced cardiac hypertrophy and heart failure by ameliorating endogenous antioxidants and mitochondrial function [31]. Further research identified the therapeutic role of AMS on type 1 diabetes where different parameters like blood glucose, HbA1c, oxidative stress, inflammation, and insulinotropic activity were normalised after AMS treatment. All the above parameters remained normal in control rats after AMS administration [29, 58]. However, there is no study to find the effect of AMS on platelet activation. Therefore, in the current study, we determined the major platelet alterations in STZ-induced diabetic

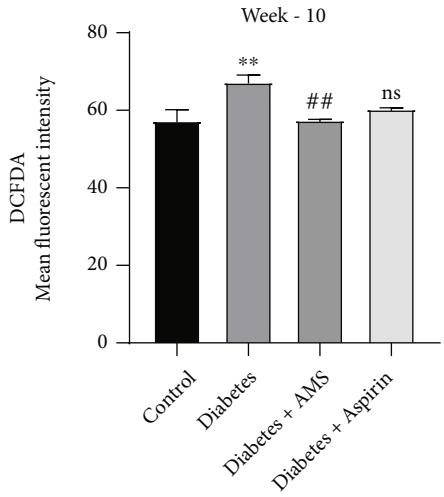


Figure 5: Comparison of ROS levels among the control, diabetes, and treatment groups. All values are represented as mean ± SEM ($N = 3$-4). $^{**}p < 0.01$ vs. the control; $^{\#\#}p < 0.01$ vs. diabetes; ns: nonsignificant.

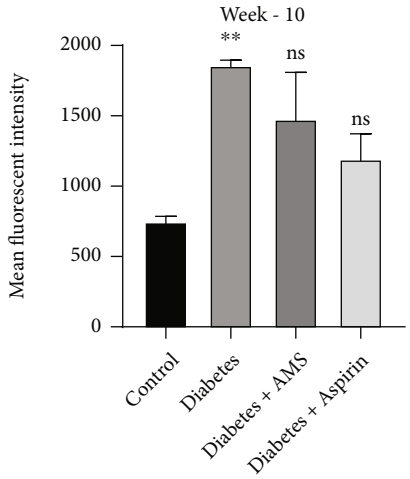


Figure 6: Macrophage platelet interaction in the control, diabetes, and treatment groups. All values are represented as mean ± SEM ($N = 3$-5). $^{**}p < 0.01$ vs. the control; ns: nonsignificant.

rats and explored the antithrombotic effect of AMS, a bioactive derivative of garlic, mainly focusing on platelet activation.

In diabetes, controlling hyperglycemia is the primary goal to reduce complications of diabetes. Poor glycemic control affects platelet activation and vascular dysfunction in diabetes. A previous study reported that a low dose of aspirin reduces blood glucose levels in diabetic rats [59]. In our present study, AMS administration to rats reduced blood glucose levels. The result showed that AMS is more effective to reduce blood glucose level in diabetic rats than aspirin. Our data is supporting the recent studies on AMS, where AMS administered to STZ-induced diabetic rats showed a significant decrease in blood glucose level [58, 60].

Hyperglycemia along with other factors helps to aggregate the platelets in the presence of a small stimulus. Such platelets are referred to as hyperactivated platelets [61]. This hyperactivated platelet has distinct morphology and expresses P-selectin (CD69P) and GP IIb/IIIa receptor on the surface [62–64]. Supporting the previous data, our present study also observed an increase in platelet activation (CD62P levels) in type 1 diabetic rats when compared to nondiabetic rats. This activation of platelets was higher at a later stage (10 weeks) when compared to the initial stage (3 weeks) of diabetes. Moreover, we also observed the effect of AMS on platelet activation. Interestingly, we found a little reduction in platelet activation after initial treatment for 3 weeks with AMS but a significant decrease was found after 10 weeks of treatment. Here, our data suggest that long-term administration of AMS has a superior effect on reduction of platelet activation in diabetes.

Increased platelet activation results in increased platelet aggregation and has been detected in diabetes mellitus [37, 38]. This increased platelet aggregation is a result of increased systemic production of TxA 2 by platelet [65], increased sensitivity of platelets agonist like ADP [66], and impaired production of platelet aggregation inhibitors PGI 2 and NO [67, 68]. The previous finding has shown that the platelet of the diabetes patient was found to be 1.6-fold more sensitive to the ADP-induced aggregation than that of nondiabetes persons [69]. In the present study, we observed the aggregation of platelets (basal level) and its sensitivity to ADP stimuli in both the early ($3^{rd}$ week) and late ($10^{th}$ week) stages of diabetes. At early stages of the disease, there was no difference in the baseline aggregation property of platelets between the diabetes and control groups. However, in the presence of ADP, the diabetic platelets showed an increase in aggregation than the control group. The data suggests that the platelets from diabetic rats were highly sensitive and prone to thrombus formation. A decrease in baseline aggregation was observed in diabetic rats after AMS and aspirin treatment. The percentage of aggregation was also reduced when the platelets were activated by the addition of ADP in treatment groups. The study suggests that AMS has a beneficial effect in reducing the platelet sensitivity and aggregatory properties. Similar to platelet aggregation data in the $3^{rd}$ week, we observed a decrease in baseline platelet aggregation in the $10^{th}$ week after AMS and aspirin treatment. Interestingly, in the $10^{th}$ week of diabetes, we did not observe any significant increase in ADP-induced platelet aggregation when compared to the control. Although we cannot explain the reason for not showing the sensitivity of platelets after ADP addition at week 10, it may be due to desensitization of P2Y1 and P2Y12 ADP receptors of platelets after the long-term interaction with endogenous ADP in chronic diabetes [70]. Further, the control group at $10^{th}$ week showed higher basal platelet aggregation than the control group at $3^{rd}$ week. This can be explained by the fact that age itself may enhance the platelet aggregation in the absence of diabetes [71]. After treatment with AMS for 10 weeks, we observed a decrease in ADP induced aggregation. The data overall indicated that inhibition was less at later stages of diabetes and may be due to the alteration of platelet structure and expression of major protein levels that resist the AMS's beneficial effect.

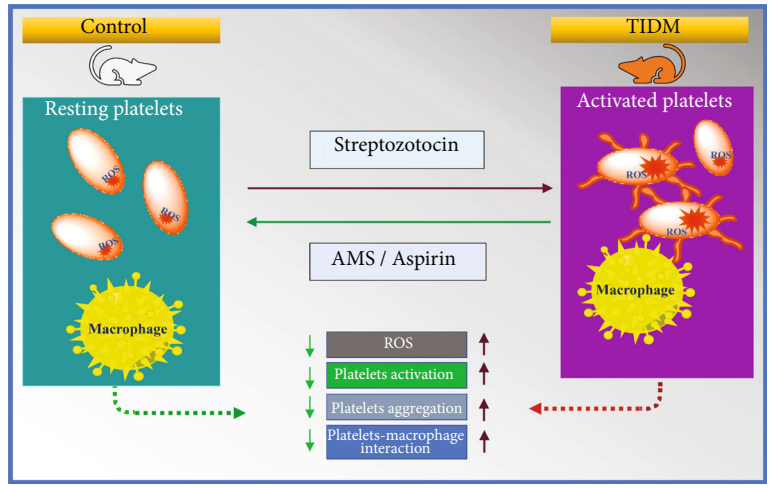


Figure 7: Attenuation of the platelet activation and platelet-macrophage interaction in type 1 diabetes by AMS. AMS: allyl methyl sulfide; ROS: reactive oxygen species; T1DM: type1 diabetes mellitus.

Next, we correlated the platelet aggregation phenotype with intracellular ROS production. Increased ROS has been observed in activated platelets and activates the PKC pathway which led to platelet hyperactivity and aggregation [72]. Supporting the previous literature, our study also found increased ROS content in diabetic platelet when compared to the control. A recent study indicates that AMS administration improved the oxidative stress in STZ-induced hyperglycemic rats [58]. Based on their observation, we evaluated the endogenous ROS generation in platelets after AMS treatment and found an inhibitory effect of AMS on ROS production in platelets.

Increased ROS production can activate the platelets and help to participate in the signaling event of atherosclerosis in diabetes by forming aggregates with monocytes through P-selectin–PSGL-1 interactions [73]. Therefore, platelets play an important role in promoting inflammation in diabetes. The inflammatory condition created by PSGL-1-mediated monocyte activation leads to the synthesis and release of various chemokines, cytokines, and reactive oxygen species. Monocyte platelet interactions also have a role in the coagulation system by a surface expression of phosphatidylserine [74]. Previous literature suggested that increased platelet-monocyte aggregates are the indicator of *in vivo* platelet activation. These aggregates are responsible for the prothrombic stage and play a major role in the development of atherosclerosis in type 1 diabetes [75, 76]. Additionally, macrophage accumulation also plays a vital role in causing diabetic complications [77]. It was reported that a similar interaction between macrophages and platelets causes releases of chemokines and phagocytosis of platelets which further involve in atherothrombosis formation [78, 79].

In the present study, we observed platelet macrophage interactions to evaluate the platelet activation status as well as proinflammatory condition in type 1 diabetes. Here, in the present study, we observed an increase in macrophage and platelet interaction at late stages of diabetes compared to control, whereas after AMS treatment, diabetic rats showed less macrophage and platelet interactions compared to diabetes. The study suggests that AMS can reduce the macrophage and platelet interaction and can further inhibit pro-inflammatory condition which leads to vascular complication in diabetes.

## 5. Conclusion

In this study, we found that allyl methyl sulfide inhibits platelet activation and their aggregation in type 1 diabetes. It has also shown an inhibitory effect on increased platelet-macrophage interaction in type 1 diabetes. The increased platelet activation was associated with elevated levels of ROS. Further, our results showed a decrease in ROS levels in platelets after AMS treatment. The summary of the present study has been represented in Figure 7. Overall, this study provides a piece of strong evidence that treatment with AMS could prevent the phenotypic platelet changes seen in type 1 diabetes and may act as a potential therapeutic molecule for cardiovascular complications especially thrombosis in diabetes.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Ethical Approval

Institutional Animal Ethics Committee (IAEC) Approval (NIPER/BT/2020/37) from National Institute of Pharmaceutical Education and Research (NIPER)-Guwahati to conduct the animal experiment was obtained.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Authors' Contributions

S.K.B., R.A, and N.M have designed the study. Animal handling and dosing were done by N.M. and S.K.U. Blood collection, platelet isolation, and sample preparation for flow analysis were performed by N.M., S.K.U., and V.T. Flow cytometry experiment design and analysis of platelet samples were done by both N.M and E.J. Statistical analysis and writing of the manuscript were done by S.K.B., R.A, M.J.A, and N.M.

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*Research Article*

# Study on the Mechanism of Prunella Vulgaris L on Diabetes Mellitus Complicated with Hypertension Based on Network Pharmacology and Molecular Docking Analyses

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The role of traditional Chinese medicine Prunella vulagaris L in the treatment of tumors and inflammation has been widely confirmed. We found that some signaling pathways of Prunella vulgaris L action can also regulate diabetes and hypertension, so we decided to study the active ingredients, potential targets and signaling pathway of Prunrlla vulgaris L, and explore the "multi-target, multi-pathway" molecular mechanism of Prunella vulgaris L on diabetes mellitus complicated with hypertension(DH). *Methods*. Based on TCMSP(Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform) and CNKI(China National Knowledge Infrastructure), the components and action targets related to Prunella vulgaris L were screened. The OMIM(Online Mendelian Inheritance in Man) and GeneCards (The human gene database) were used to search for targets related to DH. The "gene - drug - disease" relationship map was drawn by Cytoscape_v3.7.2 plug-in. The target was amplified by the STRING platform, and the "protein - protein" interaction relationship (PPI) network of the interacting target was obtained by the STRING online analysis platform and the Cytoscape_v3.7.2 plug-in. Finally, GO enrichment analysis and KEGG pathway enrichment analysis were conducted on David and Metascape platform to study the co-acting targets. Results. 11 active components, 41 key targets and 16 significant signaling pathways were identified from Prunella vulgaris L. The main active components of Prunella vulgaris L against DH were quercetin and kaumferol, etc, and potential action targets were IL-6 and INS, etc and signaling pathways were AGE-RAGE signaling pathway, TNF signaling pathway, MAPK signaling pathway, PI3K-AKT signaling pathway, etc. It involves in biological processes such as cell proliferation, apoptosis and inflammatory response. *Conclusions*. The main molecular mechanism of Prunella vulgaris L against DH is that sterols and flavonoids play an active role by affecting TNF signaling pathway, AGE-RAGE signaling pathway, MAPK pathway, PI3K-Akt pathway related targets such as IL-6 and INS.

## 1. Introduction

The global prevalence of diabetes mellitus (The following abbreviations are diabetes) has continued to grow in recent years. According to the International Diabetes Alliance, the estimated number of diabetes patients worldwide in 2019 was 463 million, and is increasing year by year [1]. In the pathogenesis of Western medicine, diabetes is a metabolic disease characterized by hyperglycemia due to a deficiency in insulin secretion or insulin dysfunction. Continuous hyperglycemia and long-term metabolic disorders can lead to damage to systemic tissues and organs, especially the eye, kidney, cardiovascular and nervous system, and the cause of which has not been fully clarified. About 70-80% of patients with diabetes eventually die from cardiovascular complications [2], hypertension is one of them, both were associated with insulin resistance.People with diabetes are twice the risk of hypertension as non-diabetic patients, and

epidemiological studies prove that hypertension is significantly higher in the diabetic population than in the general population [3],40%~50% of patients with type 2 diabetes have hypertension and almost 100% when diabetes combined with extensive renal impairment. When diabetes mellitus complicated with hypertension, the incidence and severity of coronary heart disease (including myocardial infarction) increased significantly [4].

In traditional Chinese medicine, diabetes and hypertension are attributed to vertigo, wasting-thirst, deficiency of liver-yin and kidney-yin is the common pathological basis of both [5, 6]. Thirst elimination and dry to hurt the lung and stomach, consume body fluid, Yin blood is damaged, liver-yin and kidney-yin are damaged; liver is easy to fire-evil, damage of pubic fluid, liver-yin and kidney-yin nourish each other, [7]. If the condition can not be effectively controlled, a long disease can cause damage of Yin and Yang, with the loss of Yin and Yang. The onset of the two is not successively divided, can appear at the same time, in the process of the disease development, and affect each other, that is, the current clinical common diabetes mellitus complicated with hypertension (DH).

As science advances, plant drugs are gaining more attention and many natural plant chemicals have shown great clinical prospects for combating complications of diabetes and diabetes. Through the examination of ancient books, it is found that Prunella vulgaris L was first contained in Sheng Nong's herbal classic, the main chemical components include triterpenes, sterols, flavonoids, organic acids, coumarin and other type compounds, whose functions include but not limited to blood pressure lowering, blood sugar lowering, etc. Many researchers have now used Prunella vulgaris L in the clinical treatment of diabetes and hypertension [8–10]. But its specific molecular mechanism has not been clearly clarified.

Network pharmacology is a new method and new theory developed on the basis of network biology and multi-directional pharmacology, and based on the construction and analysis technology of the network. Because diabetes is very easy to cause hypertension, and the high mortality rate after the combination, there are few studies on traditional Chinese medicine in treating diabetes associated with hypertension, so we choose to explore the molecular mechanism of Prunella vulgaris L on DH. For the complex diseases of DH, its multi-target and multi-pathway characteristics are suitable for exploring the molecular mechanism with network pharmacology. This paper aims to study the molecular mechanism and pathway prediction of diabetes complicated with hypertension through network pharmacology, and provide a theoretical basis for the further study of the treatment of DH. Its innovation lies in discussing not only one disease in diabetes or hypertension, but exploring the effect of Prunella vulgaris L on disease treatment when diabetes combines hypertension and its complications, and studying the role of the signaling pathway of Prunella vulgaris L in diabetes and hypertension. The molecular docking technology was used to make the results more accurate. The results can not only explore the effect of the traditional drug Prunella vulgaris L on to DH, but also through the study on other diseases.

## 2. Materials and Methods

*2.1. Identification, Selection, Search, and Screening of Active Compounds.* The TCMSP database (http://tcmspw.com/tcmspsearch.php) includes networks of chemicals, targets and drug targets for 499 Chinese herbal medicines, as well as associated drug target networks, Oral bioavailability (OB) is one of the most important pharmacokinetic parameters in drug absorption, distribution, metabolism, excretion (ADME), indicating the speed and extent to which the active ingredient or active base is absorbed into the body circulation and is absorbed, and the higher the OB value usually indicates the better drug-likeness(DL) of the bioactive molecule of the drug. Due to the poor bioavailability of some traditional Chinese medicine compounds. Therefore, according to the TCMSP database recommended screening indicators combined with pubmed, CNKI and other database reports, OB $\geq$30% and DL $\geq$0.18 were identified as screening criteria for active ingredients in Prunella vulgaris L, substances meeting active compounds.

*2.2. Target Gene Prediction.* The Uniprot Protein Database (https://www.uniprot.org/)is the most informative protein sequence database to convert the protein ingredients of Prunella vulgaris L active compounds into gene targets in preparation for the next exploration of the relationship with disease.

*2.3. Disease Target Gene Finding.* The OMIM database (http://www.omim.org/), GeneCards database(http://www.genechttp://ards.org/) is recognized as a disease target database online retrieval tool that can provide a full range of genetic information. In the above database, "diabetic mellitus" "hypertension" was retrieved as the search word, and the intersection and obtained the action targets associated with DH.

*2.4. Filter the Key Targets.* The same fraction of the disease target and Prunella vulgaris L targets were taken using the R language. The results are the key targets for "Prunella vulgaris L-DH", and draw the veen diagram.

*2.5. Network Construction.* Information on disease targets, drug active ingredient targets and so on was imported into the Cytoscape software (Version3.7.2) to build a "DH-Prunella vuglaris L-compounds-key target" relationship network. The mechanism of Prunella vulgaris L improving DH was explored by the network.

*2.6. Build the PPI Network.* The key targets of "drug-disease" were imported into the STRING data online analysis platform (https://string-db.org) to expand the gene. The STRING data online analysis platform is a tool capable of analyzing protein interaction networks, able to visualize the relationship between proteins to obtain PPI network maps where high connectivity is called central genes.

Table 1: Active ingredients of Prunella vulgaris L.

| Mol ID | Molecule name | Molecular weight | OB (%) | DL |
|---|---|---|---|---|
| MOL000358 | beta-sitosterol | 414.79 | 36.91 | 0.75 |
| MOL000422 | Kaempferol | 286.25 | 41.88 | 0.24 |
| MOL004355 | Spinasterol | 412.77 | 42.98 | 0.76 |
| MOL000449 | Stigmasterol | 412.77 | 43.83 | 0.76 |
| MOL004798 | Delphinidin | 303.26 | 40.63 | 0.28 |
| MOL000006 | Luteolin | 286.25 | 36.16 | 0.25 |
| MOL006767 | Vulgaxanthin-I | 339.34 | 56.14 | 0.26 |
| MOL006772 | Poriferasterol monoglucoside_qt | 412.77 | 43.83 | 0.76 |
| MOL006774 | Stigmast-7-enol | 414.79 | 37.42 | 0.75 |
| MOL000737 | Morin | 302.25 | 46.23 | 0.27 |
| MOL000098 | Quercetin | 302.25 | 46.43 | 0.28 |

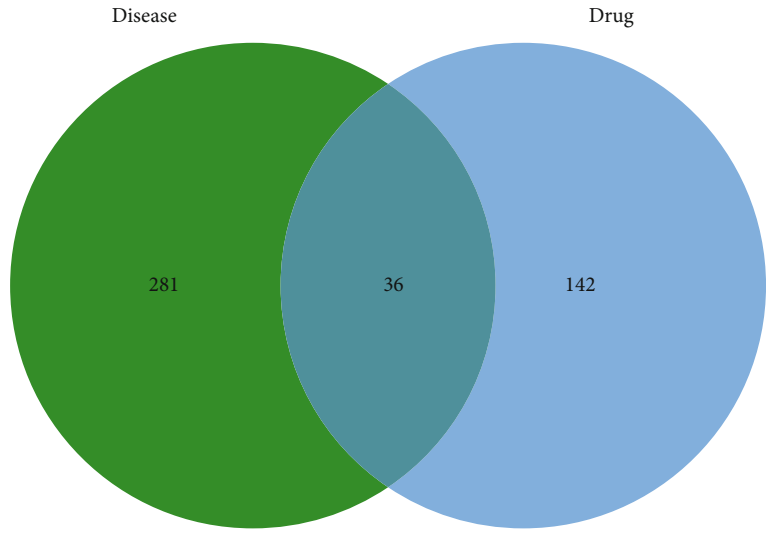



Figure 1: Interintersection of diabetes and hypertension overlap with Prunella vulgaris L.

*2.7. Gene Ontology (GO) Term and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyses.* The GO database classifies genes and gene products in three aspects: biological processes, molecular function, and cell components. The KEGG database is a database integrating information about genomic, chemical, and system functions that presents the signaling pathways in which the screened key genes are located. GO and KEGG analysis of the screened key targets using David (https://david.ncifcrf.gov/), Metascape (http://metascape.org/).

*2.8. Molecular Docking.* Download the target protein structure from the PDB database (http://www.rcsb.org/) and the 2D structure of molecular ligands can be downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), preprocessing and molecular docking of the target protein and active ingredients using Maestro11.5.

## 3. Results

*3.1. Inquiry and Screening of Active Compounds.* Search TCMSP database for "Prunella vulgaris L", set the limits of OB ≥30%, OL ≥0.18 and obtain 11 eligible active compounds such as quercetin and Vulgaxanthin-I (Table 1). It can be considered that the oral bioavailability and drug-likeness is good for these 11 compounds. Myrcene, lauric acid, caffeic acid, phellandrene etc. were abandoned due to inadequate filter criteria.

*3.2. Obtaining Intersection Targets.* The 162 gene targets for the drug active ingredient were obtained through the UNIPROT database gene pairing.The disease targets were crossed between the GeneCards database and 281 gene targets were obtained from the OMIM database. Use R language to map the "disease-drug target" intersection and obtain VENN map (Figure 1), and obtained 36

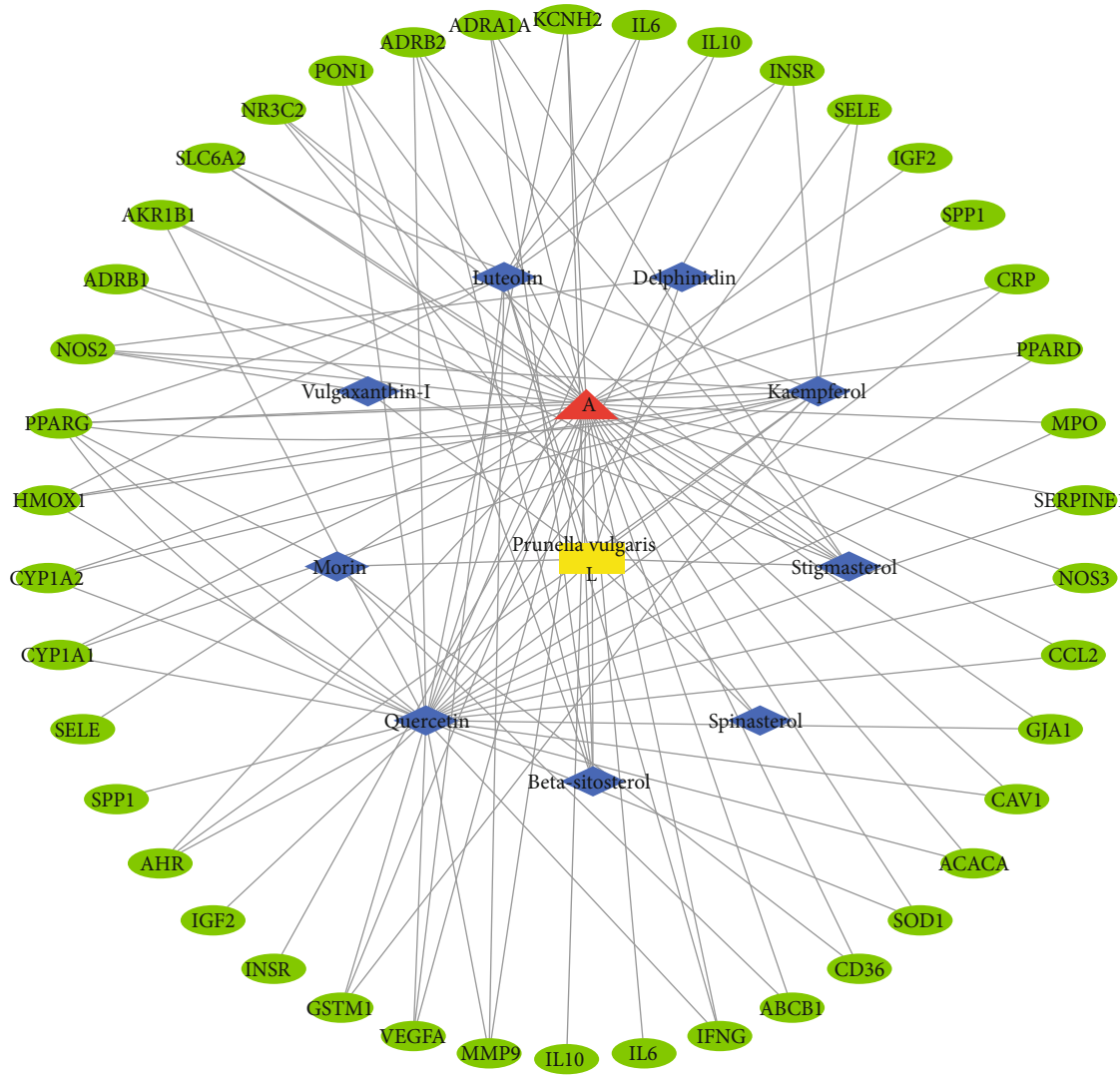



Figure 2: "disease-target-drug- compounds" network diagram of Prunella vulgaris L in the treatment of DH.

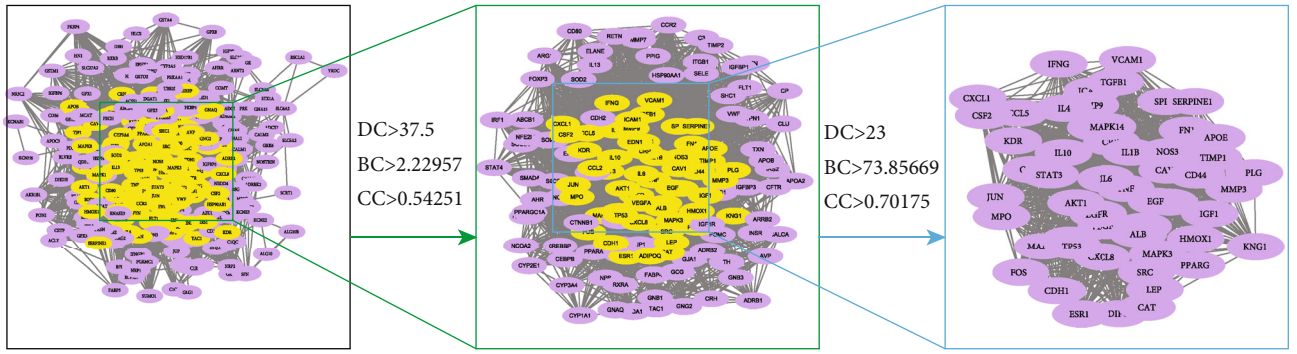



Figure 3: Topology analysis process.

intersection targets for drugs and diseases. The 36 key targets were imported into the Cytoscape to visualize the "disease-target-drug-compounds" relationship network (Figure 2). In the figure, the red node A represents the DH, the yellow node represents the Prunella vulgaris L, the blue node is the drug active compounds, and the green node is the key target of the disease and drug active compounds.

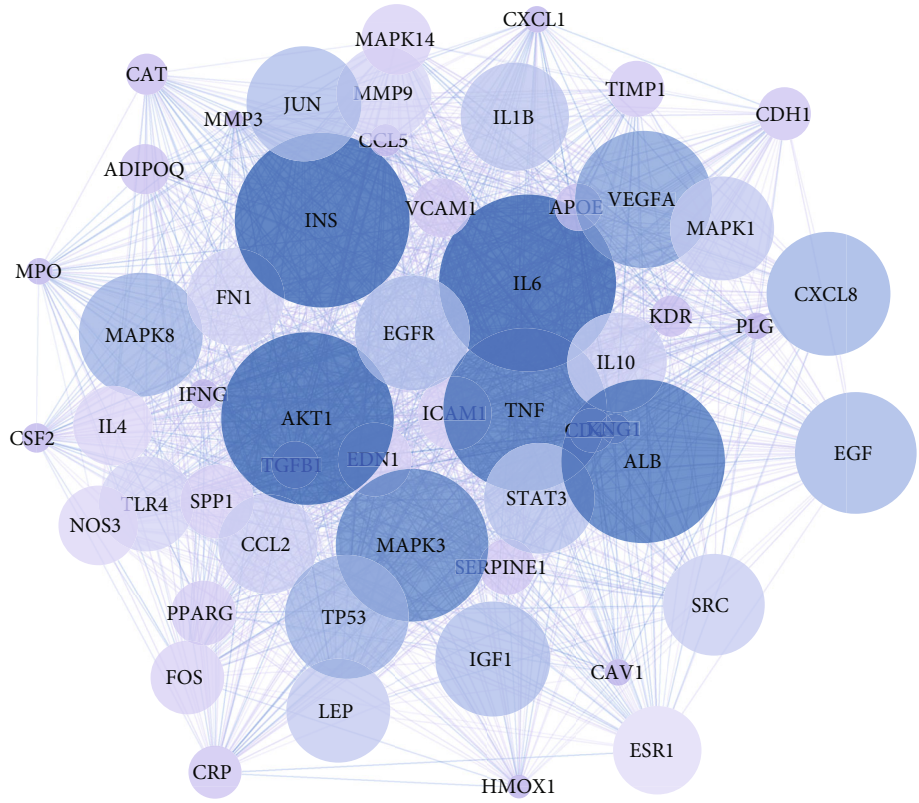



Figure 4: The PPI network diagram.

The 36 key targets were introduced into the STRING analysis platform to obtain 312 genes, hide the disconnected nodes in the network, import the Cytoscape to get a diagram of 304 points, 6369 lines, Use the Cytoscape tool for topological analysis to obtain relevant parameters, selection Degree-centrality(DC), and Betweenness-Centrality(BC), Closeness-Centrality(CC). Set 135 points of DC>37.5, CC>2.5251,3881 lines, BC>73.85669, CC>0.7070175, select 41 core genes and obtain PPI network diagram of 41 points, 748 lines (Figure 3). PPI network diagram of 41 gene targets via Cytoscape (Figure 4).

*3.3. GO Enrichment Analysis.* GO enrichment analysis of 41 target genes through the David database. Making three partial bubble diagram of biological process and cellular component, molecular function at the top 10 enrichment, respectively (Figure 5). It can be seen that the biological process is mainly related to cell proliferation, apoptosis and inflammatory response.

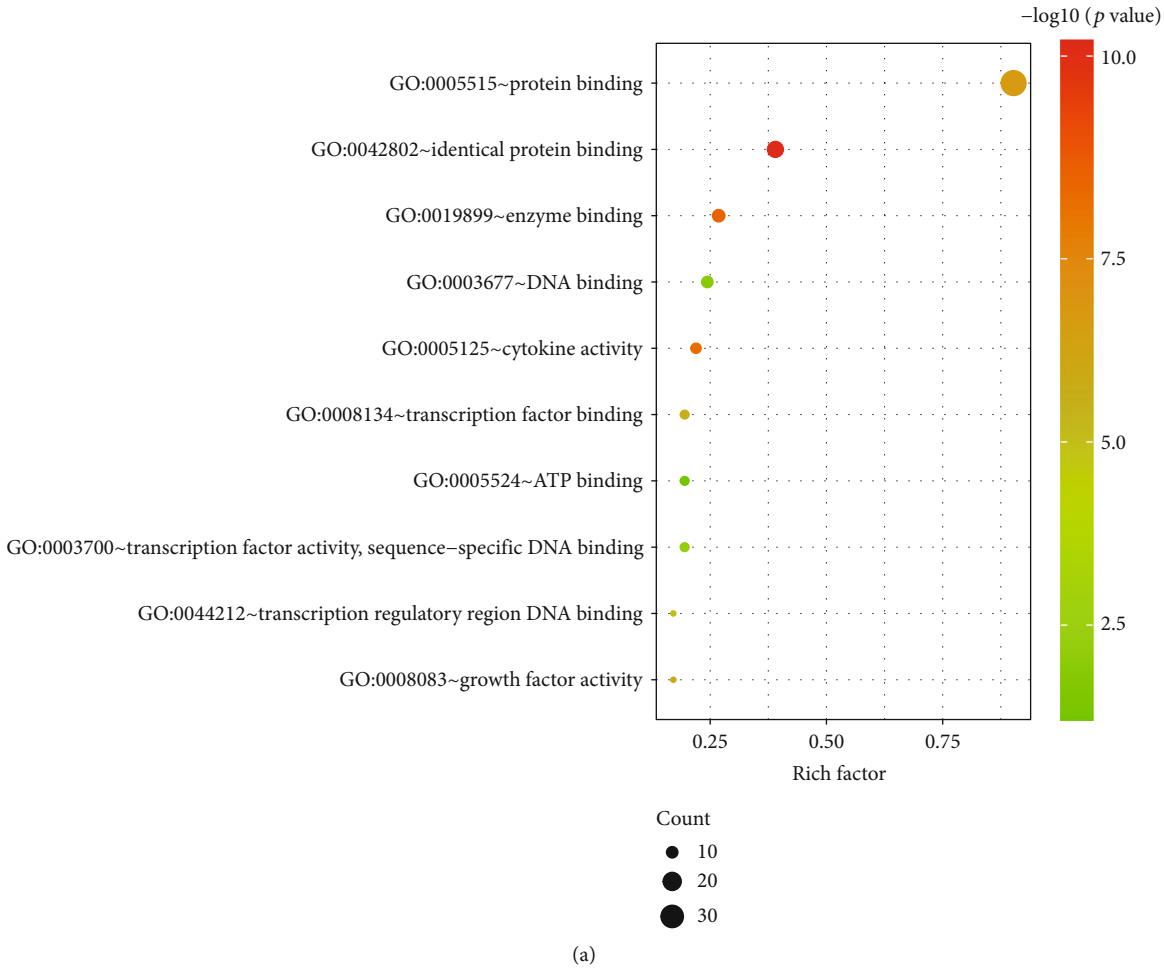
*3.4. KEGG Analysis.* KEGG enrichment information was obtained through the enrichr database (https://maayanlab.cloud/Enrichr/) and the top 11-bit bubble diagram (Figure 6) shows that the signaling path mainly includes TNF, AGE-RAGE, MAPK, and PI3-Akt.

*3.5. Molecular Docking Verification.* Molecular docking of key components using the software Maestro11.5, Verify the combination of IL6, INS, ALB, AKT1 and quercetin, luteolin, kaempferol separately (Figure 7), calculate the combined energy. The validation is as shown in Table 2, compounds and target proteins are rated as kcal/mol. The lower the binding energy, the better the stability. It is shown that these components can bind to the active site of the protein target.

## 4. Discussion

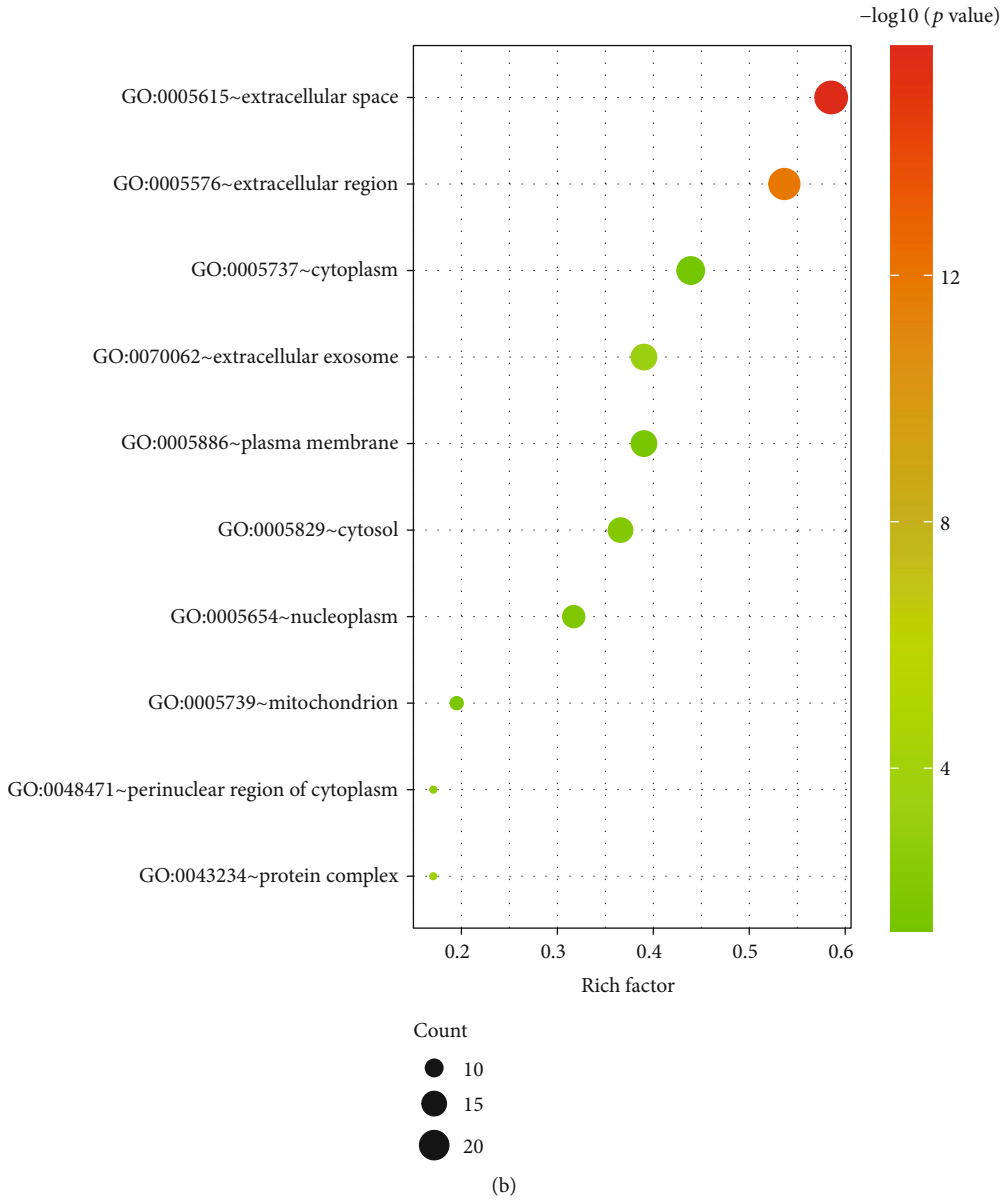
Diabetes has now become a killer of human health, and the mortality rate of diabetes associated with hypertension is far higher than that of simple diabetes.In Chinese medicine, diabetes and hypertension belong to the category of vertigo and wasting-thirst, and liver-yin and kidney-yin deficiency is their common pathogenesis, so the same traditional Chinese medicine can be used for treatment. Prunella vulgaris L from the "Sheng Nong's herbal classic", its pharmacological effects include blood pressure, blood sugar, antiviral and other effects. Currently research is the blood pressure-lowering effect of Prunella vulgaris L. For its hypoglycemic effect is often ignored.This paper analyzes the effective components of Prunella vulgaris L, and creates new ideas for the treatment of DH.

This paper analyzes the effective components of Prunella vulgaris L, and creates new ideas for the treatment of DH. The results of this study show that the key compounds that Prunella vulgaris L plays are quercetin, morin, luteolin, kaempferol, beta-sitosterol, delphinidin, spinasterol, vulgaxanthin-I, etc. Studies show that quercetin is a flavonoid,-Choi,S et al. confirmed that quercletin can acute enhance acetylcholine-induced 2K1C hypertensive rat vascular relaxation, suggesting that quercletin plays an anti-hypertensive




(a)

Figure 5: Continued.

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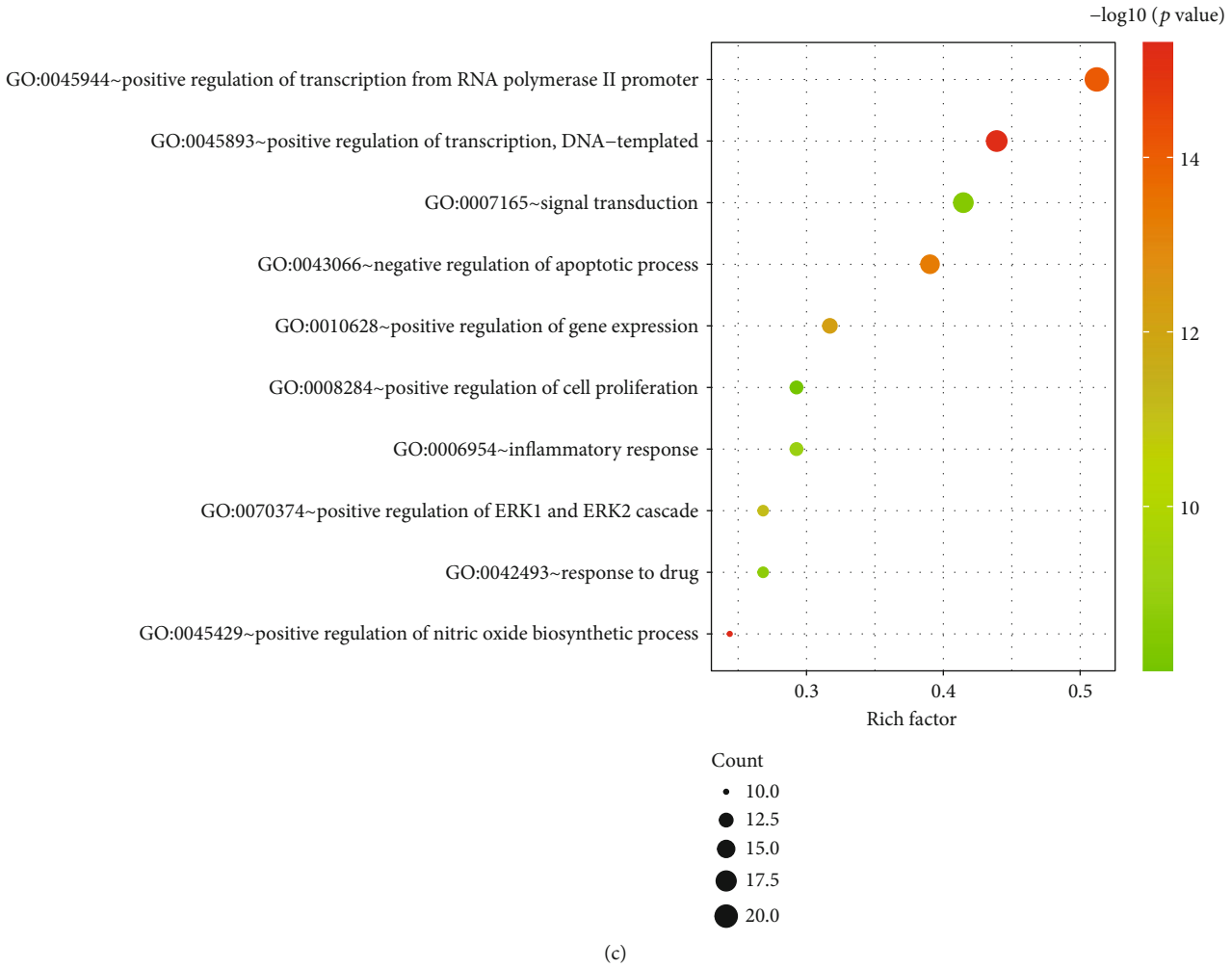



Figure 5: GO enrichment analysis bubble diagram: (a) molecular function; (b) cellular component; (c) biological process.

role by reducing vascular elasticity [11]. Meanwhile, it can inhibit the activity of disaccharidase to achieve the hypoglycemic effect [12]. It is also shown that quercetin can stimulate insulin release and inhibit INS-1beta cell activity, and long-term applications can inhibit cell proliferation and induce apoptosis, most likely achieved by inhibiting PI3K/Akt signaling [13]. Quercetin has been proposed to restore the functional quality of pancreatic $\beta$ cells through multiple targeting, and therefore can be attributed to a prospective treatment strategy for diabetes [14]. WuYT et al. found that luteolin reduces blood pressure-lowering vascular smooth muscle cell proliferation and migration through the growth factor-$\beta$ receptor 1 (TGFBR1) pathway, and has anti-oxidation and anti-inflammatory activity, improving glucose metabolism by enhancing insulin sensitivity and improving $\beta$ cell function and quality[15, 16]. Extensive studies have shown that plant sterols such as $\beta$-glusterol and Spinasterol can prevent and treat hypertension [17, 18]. The researchers found that delphinidin's anti-diabetes mechanism with hypertension may be associated with its strong antioxidant activity, inhibiting $\alpha$-glucossidase and $\alpha$-amylase, angiotensin converting ase (ACE) and direct vascular relaxation or calcium channel regulation [19]. Lai Dengni et al. found that pancreatic $\beta$ cells were immune from apoptosis caused by high glucose stress via the AMPK signaling pathway [20].

We found that the top two gene target, IL6, is a multifunctional cytokine secreted by monocyte macrophages, functioning mainly in immunomodulation. According to research, diabetes, hypertension and other diseases can lead to higher IL6 levels in serum, while multiple reports say that genetic polymorphisms of IL6 are closely related to insulin levels and large blood vessels, and may regulate insulin secretion through pancreatic $\alpha$ cells [21]. IL6 also acts as an inflammatory factor whose expression promotes cardiac fibroblasts, thereby increases collagen synthesis and eventually leading to myocardial fibrosis [22]. INS is a candidate susceptibility gene for diabetes [23]. Carmody D et al. argue that mutations in the INS gene can cause hyperglycemia, hyperinsulinemia, etc. Its dominant mutations can produce a translation product causing an unfolded protein response, causing the endoplasmic reticulum stress and eventually apoptosis and diabetes [24]. Many of the metabolic and angiokinesis actions of INS are mediated by activation of phosphatidylinositol 3-kinase (PI3K) and downstream signaling pathways [25]. The researchers found that INS

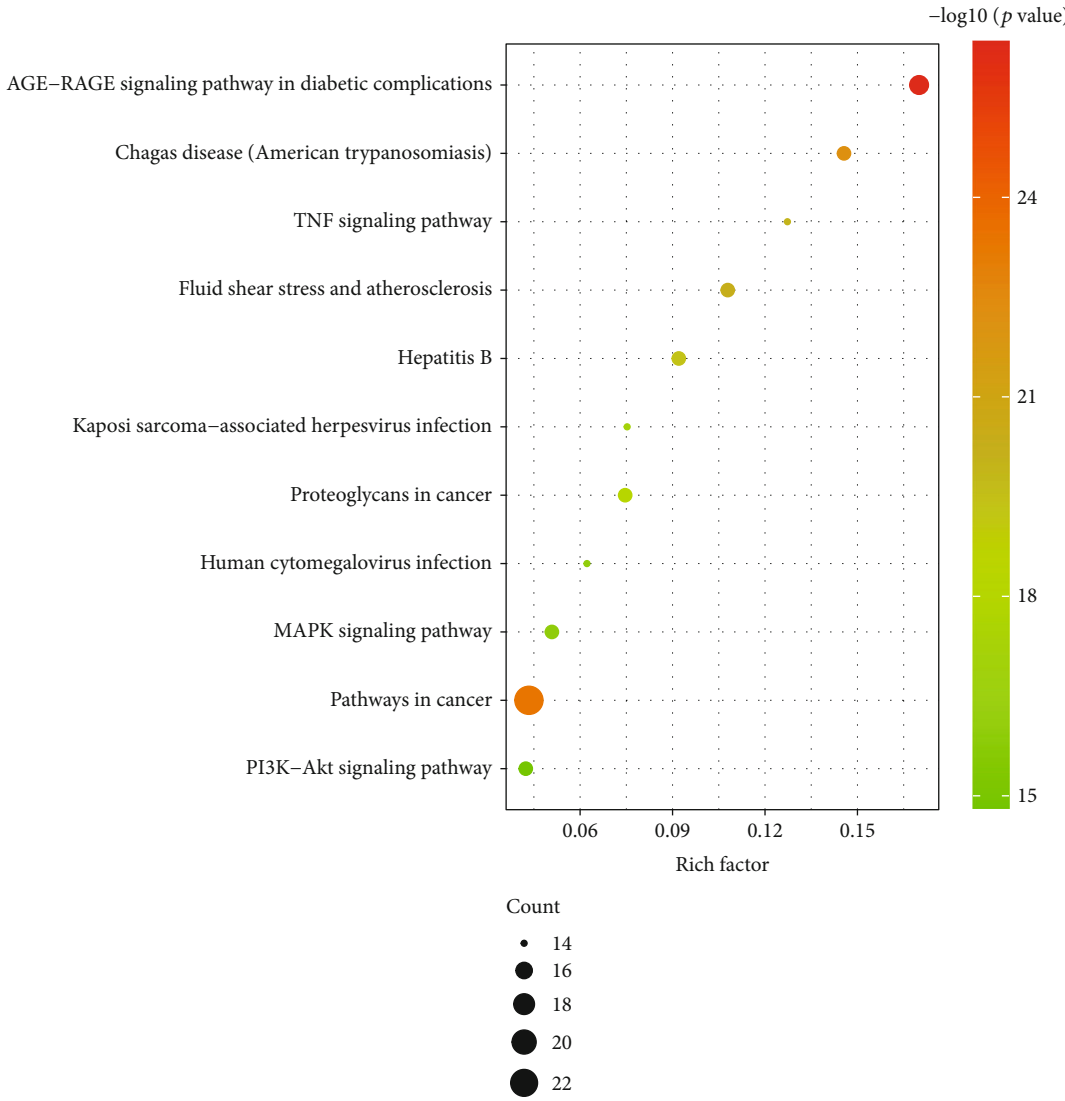



Figure 6: KEGG enrichment analysis.

affected vascular tension by its metabolic effects on endothelial cells [26].

In the AGE/RAGE signaling pathway, AGE and its receptor RAGE interactions trigger oxidative stress, inflammatory response, and thrombosis, thus participating in vascular aging and damage, and may be an important cause of diabetes associated with hypertension [27]. AGE/RAGE signaling induces the activation of multiple intracellular signaling pathways involving NADPH oxidase, protein kinases C and MAPK, enhancing NF-$\kappa$ Bactivity to promote a variety of genes associated with atherosclerosis such as IL-6 [28]. The results of these signaling transduction are possible mechanisms for triggering complications of diabetes. For example, it is reported that AGEs can up-gulate the expression of RAGE by activating NF-$\kappa$B [29]. As previously described, activated NF-$\kappa$B binds to a specific DNA sequence, regulates transcription of corresponding genes and accelerates the occurrence of cardiovascular complications. It is conceivable that positive feedback cycles between downstream paths may produce a vicious cycle that promotes cardiovascular complications of diabetes.

TNFR1 in the TNF signaling pathway mainly regulates cell regulation processes and is associated with insulin resistance and the pathogenesis of type 2 diabetes [30].The study showed that TNF-$\alpha$ levels increased significantly at the generation of oxidative stress [31]. TNF-$\alpha$, as one of the regulators, induces the production of cytokines like IL-1$\beta$, IL-6 involved in oxidative stress and inflammatory response processes.

The Insulin pathway activates the MAPK pathway that causes vasoconstriction through endothelial-dependent mechanisms [32]. A large number of studies show that MAPK signaling pathway participates in biological cell cycle regulation, cell differentiation, metabolism and other processes. In an important process of hypertension, the biological property changes of vascular smooth muscle cells and vascular endothelial progenitor cells are all regulated by MAPK [33]. The MAPK pathway and the PIK3-AKT

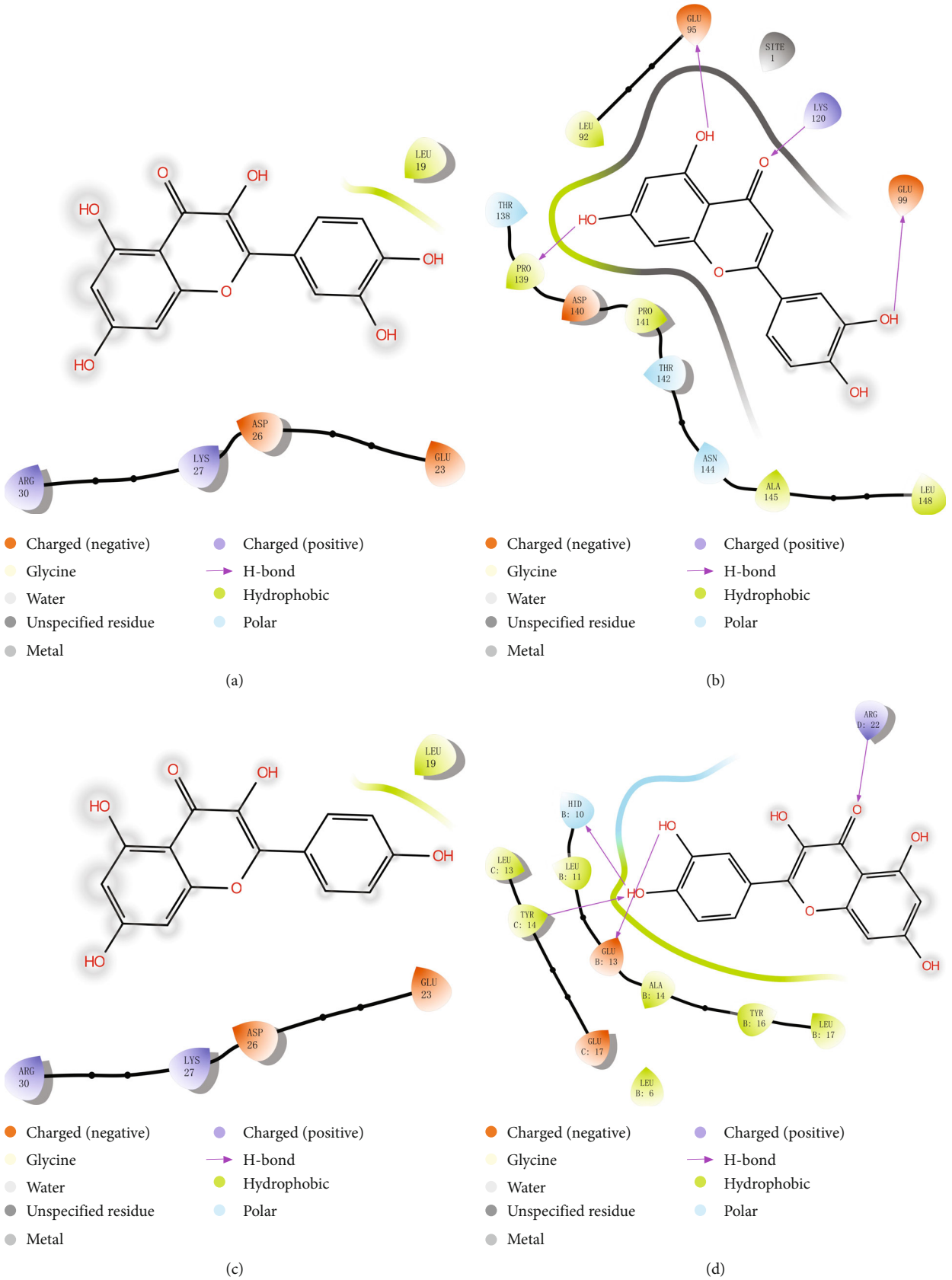
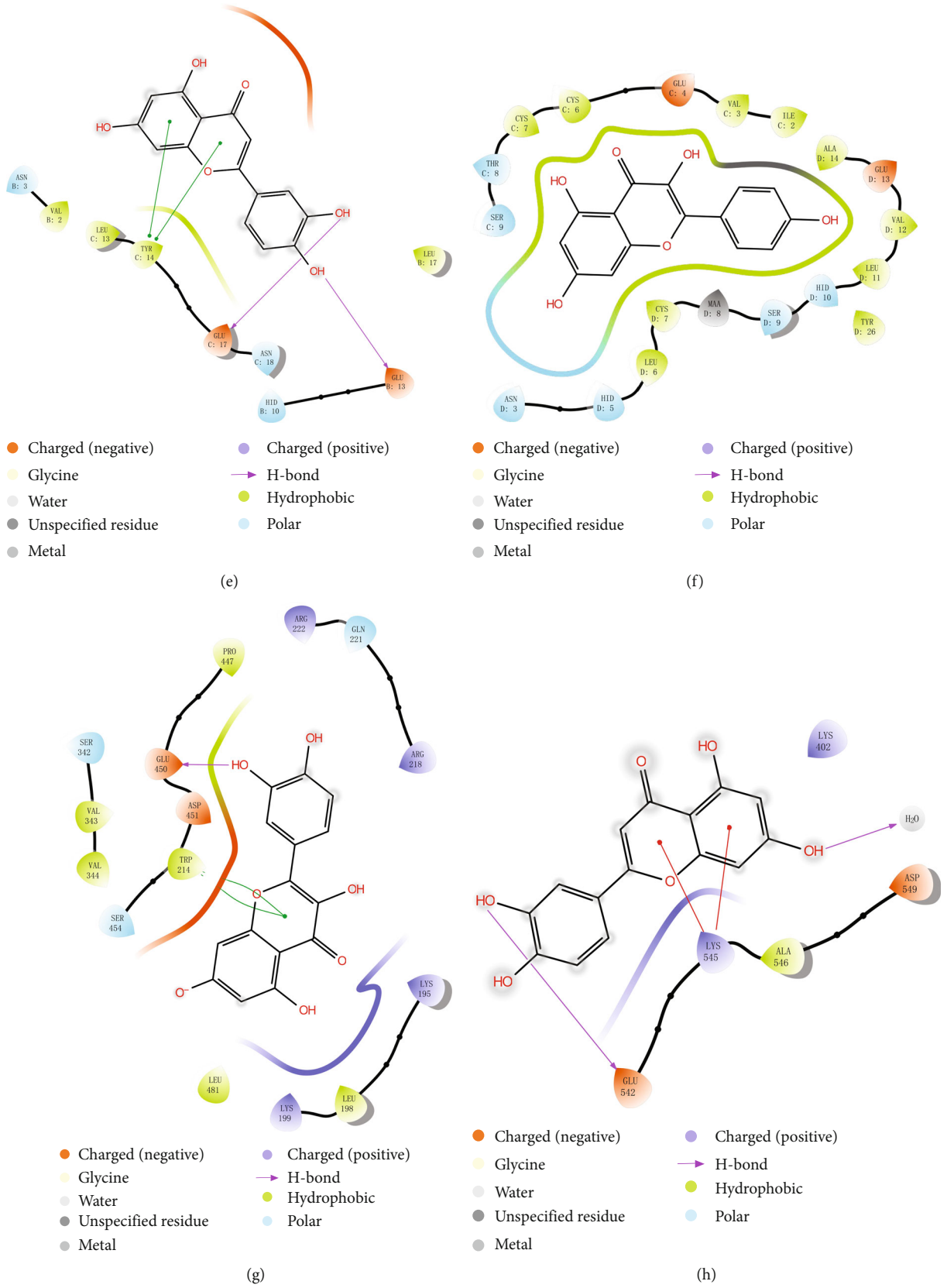



Figure 7: Continued.

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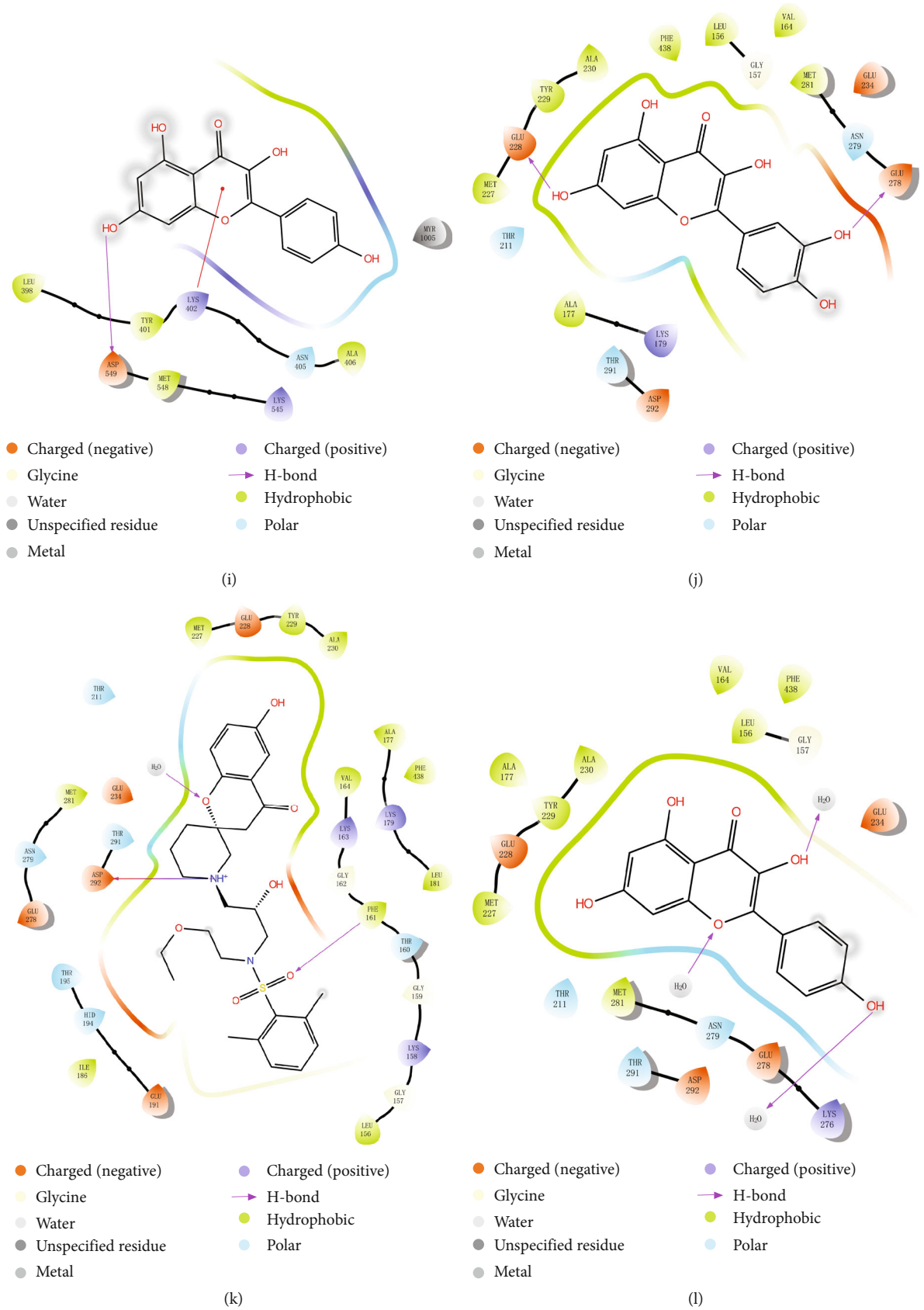


Figure 7: 2 D structure of 12 docking results: (a) IL6 with quercetin; (b) IL6 with luteolin; (c) IL6 with kaempferol; (d) INS with quercetin; (e) INS with luteolin; (f) INS with kaempferol; (g) ALB with quercetin; (h) ALB with luteolin; (i) ALB with kaempferol; (j) AKT1 with quercetin; (k) AKT1 with luteolin; (l) AKT1 with kaempferol.

Table 2: Docking score.

| Key ingredients | Docking score | | |
|---|---|---|---|
| | Quercetin | Luteolin | Kaempferol |
| IL6 | -6.955 | -6.225 | -6.102 |
| INS | -5.169 | -3.724 | -4.907 |
| ALB | -5.022 | -4.964 | -4.505 |
| AKT1 | -6.411 | -3.607 | -5.940 |

pathway can collaborate to regulate TNF-$\alpha$ expression and apoptosis [34].

## 5. Conclusion

This paper analyzes the treatment of Prunella vulgaris L for DH, and verifies the docking activity of small molecular ligand and protein with molecular docking. The eleven active components of Prunella vulgaris L for diabetes combined with hypertension include steroids, flavonoids, etc., 41 key targets IL-6, NOS3, etc., and 21 significant signaling pathways, such as AGE-RAGE, HIF-1, etc., and analyzed the specific role of these targets and signaling pathways. It theoretically explains the potential mechanism of traditional Chinese medicine Prunella vulgaris L in treating diabetes with hypertension, and provides the direction for the subsequent effect of Prunella vulgaris L in the treatment of DH.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare no conflict of interest.

## Authors' Contributions

Xinyi Jiao contributed to conceptualization and writing original draft. Haiying Liu contributed to supervision, review, and editing. Qinan Lu, Yu Wang and Yue Zhao contributed to methodology and software. Xuemei Liu, Fang Liu, and Yaoyao Zuo contributed to supervision.Wenbo Wang and Yujie Li contributed to supervision, funding acquisition, and review and editing.

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*Research Article*

# The Effectiveness of Traditional Chinese Medicine Jinlida Granules on Glycemic Variability in Newly Diagnosed Type 2 Diabetes: A Double-Blinded, Randomized Trial

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This study aimed to evaluate the influence of Jinlida granules on glycemic variability with or without metformin treatment in patients with newly diagnosed type 2 diabetes. This study was a 16-week, double-blinded, randomized, controlled clinical trial. The enrolled patients with newly diagnosed type 2 diabetes were randomly divided into four groups: control, Jinlida, metformin, and combination treatment groups. A retrospective continuous glucose monitoring (CGM) system was used for subcutaneous interstitial glucose monitoring for 3 days consecutively. Hemoglobin A1c (HbA1c), traditional Chinese medicine symptom score, and CGM parameters, including glucose coefficient of variation, standard deviation of blood glucose values, and time in range of glucose 3.9–10.0 mmol/L, were assessed pre-test and post-test. A total of 138 participants completed the entire procedure. Compared with the pre-test, fasting plasma glucose, 2 hour postprandial plasma glucose, HbA1c, and traditional Chinese medicine symptom score all decreased in the four groups at the end of the test, and the combination treatment group showed the most significant decrease. In terms of CGM parameters, time in range of the Jinlida and metformin groups improved after intervention compared with the baseline (Jinlida group: 78.68 ± 26.15 versus 55.47 ± 33.29; metformin group: 87.29 ± 12.21 vs. 75.44 ± 25.42; $P < 0.01$). Additionally, only the Jinlida group showed decreased glucose standard deviation after intervention (1.57 ± 0.61 vs. 1.96 ± 0.95; $P < 0.01$). Jinlida granules can improve glycemic control and glycemic variability in patients with newly diagnosed type 2 diabetes. *Clinical trial registration number*: ChiCTR-IOR-16009296.

## 1. Introduction

The national incidence of type 2 diabetes (T2D) in China is increasing annually. The estimated overall prevalence of diabetes is 10.9% among adults in China [1]. Traditional Chinese medicine (TCM) therapy for T2D has been widely used in China. TCM can provide additional benefits to patients with T2D, such as ameliorating glycemic control, improving insulin resistance and pancreatic islet function, inducing weight loss, and low incidence of adverse events [2–5]. Pharmacological studies have demonstrated that TCM can rehabilitate islet $\beta$-cell impairment, stimulate insulin secretion, and strengthen the utilization of glucose in peripheral tissues [6]. In the Standards of Medical Care for Type 2 Diabetes in China 2019, a section on diabetes and Chinese herbal medicine was first highlighted [7].

Jinlida is a Chinese herbal medicine approved by the China Food and Drug Administration that has been clinically used in China as an anti-diabetic agent. It is an herbal formula nourishing Pi (Spleen) and regulating body fluid of patients with diabetes, based on the TCM theory that Pi (Spleen) deficiency is involved in the pathogenesis of T2D. It is a multi-targeted hypoglycemic medication consisting of danshensu sodium salt, puerarin, salvianolic acid B,

epimedin B, epimedin C, icariin, and ginsenosides Rb1, Rc, and Rb2. Evidence from pharmacological and basic researches has demonstrated its function, including protecting islet $\beta$-cells, anti-oxidative stress, regulating blood glucose-related hormones, and protecting vascular endothelial cells. Additionally, Jinlida can reduce insulin resistance by promoting skeletal muscle gene expression and regulating lipid metabolism, which plays a key role in the anti-diabetic effect. In recent decades, some randomized controlled clinical trials have shown the effects of Jinlida as an add-on therapy to anti-diabetic agents for the treatment of T2D, with the benefits of Jinlida being much safer and more effective than monotherapy with anti-diabetic drugs [8–14].

Besides traditional markers reflecting glycemic levels, such as hemoglobin A1c (HbA1c), glycemic dysregulation also contains markers of glycemic variability, such as glucose standard deviation (SD) and coefficient of variation of glucose levels (%CV), which can be examined and presented in detail by using a continuous glucose monitoring (CGM) system [15]. A few previous studies have reported a positive association between glycemic variability and diabetic macrovascular and microvascular complications [16].

To our knowledge, no specific study has focused on the effect of Jinlida on glycemic variability in T2D, which is correlated with diabetic vascular complications. This study aimed to evaluate the influence of Jinlida granules on glycemic variability with or without metformin treatment in newly diagnosed T2D patients. Additionally, we adopted the time in range (TIR) of glucose 3.9–10.0 mmol/L as a study outcome, because TIR is associated with the risk of diabetic microvascular complications and has been suggested as an acceptable end point for clinical trials [17–19].

## 2. Materials and Methods

*2.1. Trial design and participants.* This study was a 16-week, double-blinded, randomized, controlled clinical trial. Patients referred to the outpatient clinic at the Department of Endocrinology and Metabolism of Shanghai Jiao Tong University Affiliated Sixth People's Hospital were consecutively recruited. We enrolled patients who were newly diagnosed with T2D. The inclusion criteria were as follows: newly diagnosed medication-naive T2D; patients aged 30–70 years, man or woman; body mass index (BMI) $\geq$18.5kg/m$^2$; 7%$\leq$HbA1c$\leq$10.0%; fasting plasma glucose (FPG) $\leq$13 mmol/L, 2-hour postprandial plasma glucose (2h-PG) $\leq$18 mmol/L, and patients who provided informed consent. The exclusion criteria were as follows: 1) type 1 diabetes, gestational diabetes, and miscellaneous diabetes; 2) patients with acute complications, including ketoacidosis, lactic acidosis, hyperosmolar coma, and infection in recent 1 month; 3) patients with severe heart disease, myocardial infarction, stroke, transient ischemic attacks, and peripheral arterial disease; 4) pregnant or lactating women; 5) patients with renal or hepatic dysfunction; 6) patients with systolic pressure >180 mmHg and/or diastolic pressure >110 mmHg; 7) patients with acute or chronic pancreatitis, severe cardiovascular disease, malignancy, and severe mental disease; 8) patients with alcohol or drug addiction; 9) FPG >13 mmol/L and/or 2h-PG >18 mmol/L;10) patients with allergic reactions; 11) patients with hyperthyroidism or hypothyroidism; and 12) patients with medication influencing glucose metabolism, including glucocorticoids, thyroxine and thiazide diuretics.

*2.2. Standard protocol approvals, registrations, and patient consents.* The study protocol was approved by the Ethics Committees of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and was conducted in accordance with the Declaration of Helsinki (1964). Informed consent was obtained from all participants. This trial is registered at http://www.chictr.org.cn with clinical trial registration number ChiCTR-IOR-16009296.

*2.3. Randomization, intervention, and procedure.* After signing the informed consent, each patient underwent blood tests and physical examinations at the screening visit. Venous blood samples were drawn at 0800 h after a 10-h overnight fast. At least 1 week later, the patients who met the screening criteria were randomly divided into four groups according to the random encoder: group A, control group (placebo tablets + placebo granules); group B, Jinlida group (Jinlida granules + placebo tablets); group C, metformin group (metformin tablets + placebo granules); and group D, combination treatment group (Jinlida granules + metformin tablets). The patients were randomly allocated to receive medication for 16 consecutive weeks. The eligible patients underwent CGM, and a TCM symptom score (Supplement 1) was assessed before the study drugs were distributed.

All the enrolled patients were orally administered one bag of granules (Jinlida or placebo) and three tablets of pills (metformin or placebo). The dose of metformin was 500 mg three times a day after three meals from the beginning to the end of the observation period. Patients in all the groups were orally administered one bag of granules (9 g) three times daily with warm water before each meal. The HbA1c, FPG, 2h-PG, and lipid profiles were measured at 0 and 16 weeks. The HbA1c levels were measured using an analyzer (Tosoh HLC-723 G7, Yamaguchi, Japan) using high-performance liquid chromatography. The plasma glucose levels and lipid profiles were measured using an automatic biochemical analyzer (Hitachi 7600, Tokyo, Japan).

The observation period was 16 weeks with every 4-week follow-up visits. In each session, the patients were asked if there were experiencing any adverse events. All patients underwent fasting capillary glucose measurement, physical examination, and compliance with the test drug administration. BMI and blood pressure were also monitored. CGM and TCM symptom score were performed again at the 16-week follow-up visit.

Key withdrawal criteria of the study included severe hypoglycemia and serious adverse events that the investigators considered inappropriate for continuation; severe protocol deviation, including poor drug compliance, inability to continue according to protocol requirements, and unwillingness to follow the study arrangements.

*2.4. CGM parameters.* A retrospective CGM system (ipro2, Medtronic Inc., Northridge, CA, USA) was used for subcutaneous interstitial glucose monitoring for 3 days consecutively at baseline and at the end of the study. Glycemic variability was estimated using the %CV and glucose SD. CV was calculated by dividing the glucose SD by the average of the corresponding glucose readings. The CV values in this study were multiplied by 100 and expressed as %CV. TIR was defined as the percentage of time in the target glucose range of 3.9–10.0 mmol/L during a 24-hour period.

*2.5. Outcomes.* The primary outcomes were changes in HbA1c, glucose SD, and %CV compared with baseline. Secondary outcomes were changes in FPG, 2h-PG, TIR, and TCM symptom score compared with baseline. Safety analysis mainly included the incidence of self-reported hypoglycemia and gastrointestinal adverse events in the four groups.

*2.6. Determination of sample size and statistical analyses.* Since this study was a pilot study, sample size calculation was not performed, and a convenience sample size was adopted. Variables with an approximately normal distribution are presented as mean ± SD, while those with a skewed distribution are shown as medians (interquartile range). Differences in continuous variables among multiple groups were analyzed using the one-way analysis of variance (ANOVA) and post-hoc tests. Differences in the parameters before and after treatment were analyzed using the paired t-test. Skew-distributed variables were tested using the rank-sum test. Comparisons of categorical variables between groups were performed using the chi-square test. Statistical analyses were performed using the Statistical Package for the Social Sciences software (version 17.0; SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

## 3. Results

In this study, 169 patients were screened, of whom six failed to undergo screening and five withdrew consent. In total, 158 patients were randomly assigned, of whom 20 patients discontinued, and 138 patients completed the entire procedure (Figure 1). The participants' characteristics are presented in Table 1. At baseline, there were no significant differences in the clinical parameters, including BMI, blood pressure (systolic blood pressure and diastolic blood pressure), lipid profile (total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol), metrics of glucose level (FPG, 2h-PG, and HbA1c), and TCM symptom score.

*3.1. Glycemic level and TCM symptom score changes pre- and post-test in the four groups.* In terms of glycemic parameters, the FPG, 2h-PG, and HbA1c levels were improved compared with baseline among the four groups (Table 2) after 16-week intervention. The HbA1c levels improved with statistical significance pre-and post-tests ($P < 0.01$). TCM symptom score improved after invention compared with pre-intervention in the four groups. We then compared glycemic changes between the groups to explore the hypoglycemic effect of Jinlida. No significant changes were found between groups A and B and between groups C and D (Figure 2).

*3.2. CGM measurement changes pre and post-test in the four groups.* The TIR of groups B and C significantly increased after the 16-week intervention ($P < 0.01$), while no significant changes were found in group A and group D. Further, %CV of the four groups showed no significant change pre- and post-test. However, in terms of glucose SD, group B showed a significant change after intervention compared with baseline ($P < 0.01$). Figure 3 shows the average CGM measurements of all the groups.

*3.3. Glycemic level, TCM symptom score, and CGM measurement changes in multiple groups.* The ANOVA analysis showed significant differences in the FPG, 2h-PG, and HbA1c levels in multiple groups ($F = 4.972$, 2.763, 10.703, $P < 0.05$). To further explore the effects of Jinlida on glycemic level, TCM symptom score, and CGM measurements, we compared the pre-and post-test changes between groups A and B, and groups C and D with a post-hoc test. However, no significant differences were found in the FPG, 2h-PG and HbA1c levels. In addition, there was no significant difference in the TCM symptom score between the groups (group A vs. group B, group C vs. group D) (Figure 2).

On the contrary, the improvement of glycemic variability assessed by glucose SD showed significant difference between the Jinlida and control groups (glucose SD, -0.39 vs. 0.11 $P < 0.05$). However, compared with the metformin group, no significant improvement in glucose SD post-study was found in the combination treatment group (glucose SD, -0.18 vs. -0.09, $P > 0.05$). Additionally, compared with the control group, the Jinlida group showed a more pronounced improvement in TIR after treatment (23.21% vs. 2.24%, $P < 0.01$). Since there was a significant difference in the metrics of TIR among the four groups at baseline, we compared the percentage increase from baseline in multiple groups after 16 weeks of intervention. No significant difference was found in multiple groups with the ANOVA analysis ($F = 0.796$, $P > 0.05$), or between groups A and B and groups C and group D with post-hoc test ($P > 0.05$) (Figure 4).

*3.4. Adverse events.* During the study period, adverse events (except for hypoglycemia) occurred two times in group A, seven times in group B, 13 times in group C, and nine times in group D. Four cases of gastrointestinal discomfort in group C and three cases in group D were related to the study drugs, while the other adverse events were not related to the study drugs. Hypoglycemia was observed in one patient in group B and in one patient in group D throughout the study, and none of them experienced severe hypoglycemia.

## 4. Discussion

TCM has focused on the treatment of diabetes for thousands of years. The "whole view" and "multi-target" approaches of TCM provide unique advantages in controlling complex metabolic diseases, such as diabetes. It usually focuses on individualized treatments that are based on the differentiation of syndromes, control of balance, and various routes

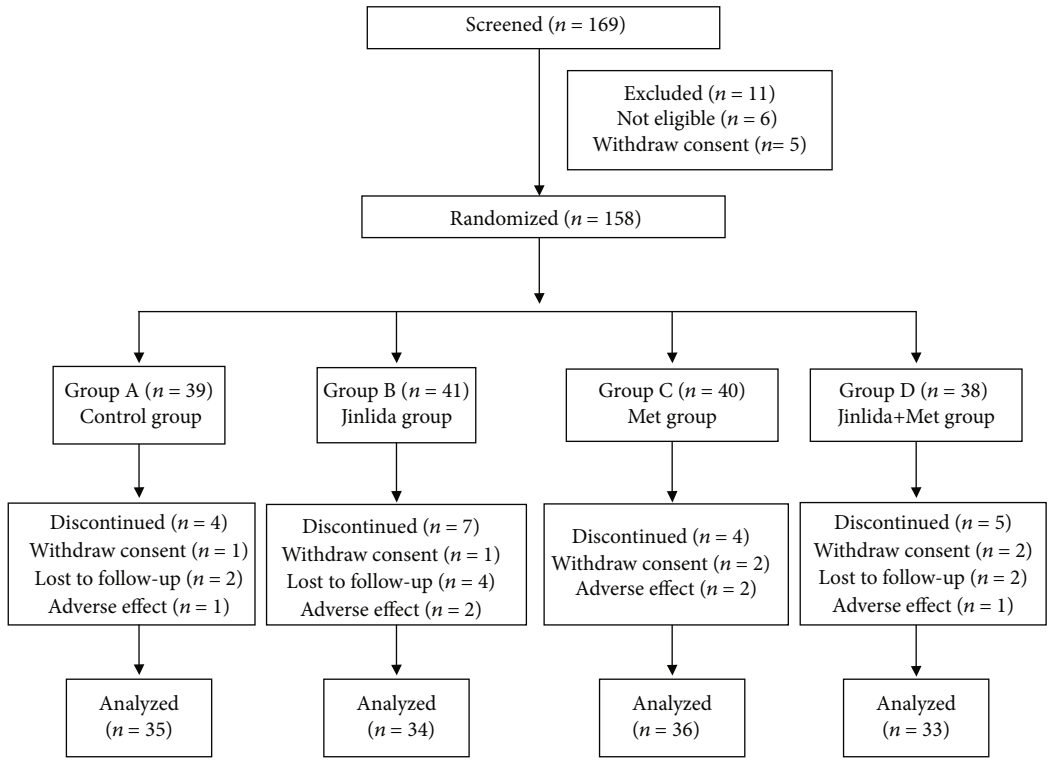


Figure 1: Study design and participant flow diagram.

Table 1: Baseline characteristics of the subjects in the study.

| Variable | Group A | Group B | Group C | Group D | *F* value | *P* value |
|---|---|---|---|---|---|---|
| Samples (n) | 35 | 34 | 36 | 33 | | |
| Age (years) | 56.53 ± 9.18 | 51.59 ± 10.31 | 56.00 ± 9.47 | 54.57 ± 10.54 | 1.733 | 0.163 |
| Sex (male/female) | 23/12 | 25/9 | 24/12 | 22/11 | | 0.892 |
| BMI (kg/m2) | 25.69 ± 3.24 | 25.40 ± 3.97 | 26.60 ± 3.42 | 25.74 ± 2.73 | 0.830 | 0.480 |
| FPG (mmol/L) | 8.77±1.53 | 8.89±2.15 | 8.65±1.48 | 8.33±1.46 | 0.667 | 0.574 |
| 2h-PG (mmol/L) | 13.60±2.83 | 14.35±3.36 | 13.79±2.48 | 13.26±2.86 | 8.834 | 0.478 |
| HbA1c (%) | 7.88 ± 0.78 | 7.83 ± 0.69 | 7.94 ±0.63 | 8.09 ±0.88 | 0.731 | 0.535 |
| FINS (mU/L) | 13.73±7.58 | 14.42±10.31 | 14.65±7.92 | 12.50±6.99 | 0.455 | 0.714 |
| 2h-INS(mU/L) | 63.45±47.01 | 69.40±58.95 | 60.48±31.76 | 46.20±33.54 | 1.663 | 0.178 |
| SBP(mmHg) | 136.06±13.32 | 136.38±15.89 | 137.28±18.39 | 131.30±15.56 | 0.958 | 0.415 |
| DBP(mmHg) | 83.06±7.64 | 84.15±8.99 | 84.61±12.41 | 80.42±9.65 | 1.220 | 0.305 |
| Triglyceride (mmol/L) | 1.66 ± 0.80 | 2.17 ± 1.39 | 1.81 ± 0.91 | 2.15 ±2.12 | 1.136 | 0.337 |
| Total cholesterol (mmol/L) | 4.91 ± 1.03 | 5.17 ± 0.91 | 5.10± 1.00 | 5.32±1.03 | 0.966 | 0.411 |
| LDL-C (mmol/L) | 3.18 ± 1.01 | 2.97 ± 0.86 | 3.19 ± 0.81 | 3.30 ±0.97 | 0.742 | 0.529 |
| HDL-C (mmol/L) | 1.13 ± 0.23 | 1.15± 0.26 | 1.16 ± 0.28 | 1.20 ±0.30 | 0.384 | 0.765 |
| TCM symptom scale | 7.14 ± 4.74 | 6.88 ±5.38 | 7.94 ±6.13 | 8.67±5.67 | 0.729 | 0.536 |
| TIR (%) | 67.15±29.29 | 55.47±33.29 | 75.44±25.42 | 81.87±19.89 | 5.661 | 0.001 |
| SD | 1.74±0.69 | 1.96±0.95 | 1.63±0.57 | 1.55±0.56 | 2.079 | 0.106 |
| CV (%) | 18.87±5.83 | 20.28±8.60 | 18.74±5.15 | 18.94±6.27 | 0.410 | 0.746 |

Group A, control group; Group B, Jinlida group; Group C, metformin group and Group D, combination with Jinlida and metformin group. BMI, body mass index; FPG, fasting plasma glucose; 2h-PG, 2-hour postprandial glucose; HbA1c, hemoglobin A1c; FINS, fasting insulin; 2h-INS, 2 hour insulin; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TCM, traditional Chinese medicine; TIR, time in range; SD, standard difference; CV, coefficient of variation.

Table 2: Glycemic changes pre- and postinvention.

| Variable | Group A $n = 35$ | | Group B $n = 34$ | | Group C $n = 36$ | | Group D $n = 33$ | |
|---|---|---|---|---|---|---|---|---|
| | Pretest | Posttest | Pretest | Posttest | Pretest | Posttest | Pretest | Posttest |
| FPG (mmol/L) | 8.77 ± 1.53 | 8.40 ± 1.77 | 8.89 ± 2.15 | 7.91 ± 2.11 * | 8.65 ± 1.48 | 6.82 ± 0.90** | 8.33 ± 1.46 | 6.90 ± 1.21** |
| 2 h-PG (mmol/L) | 13.60 ± 2.83 | 12.59 ± 3.52 | 14.35 ± 3.36 | 12.02 ± 3.62** | 13.79 ± 2.48 | 10.46 ± 2.84** | 13.26 ± 2.86 | 10.87 ± 3.02** |
| HbA1c (%) | 7.88 ± 0.78 | 7.43 ± 0.79** | 7.83 ± 0.69 | 7.15 ± 1.01** | 7.94 ± 0.63 | 6.61 ± 0.57** | 8.09 ± 0.88 | 6.58 ± 0.61** |
| TCM symptom scale | 7.14 ± 4.74 | 4.40 ± 4.30** | 6.88 ± 5.38 | 3.94 ± 3.50** | 7.94 ± 6.13 | 4.50 ± 3.46** | 8.67 ± 5.67 | 4.55 ± 3.44** |
| TIR (%) | 67.15 ± 29.29 | 70.06 ± 29.77 | 55.47 ± 33.29 | 78.68 ± 26.15** | 75.44 ± 25.42 | 87.29 ± 12.21** | 81.87 ± 19.89 | 87.87 ± 16.69 |
| SD | 1.74 ± 0.69 | 1.85 ± 0.75 | 1.96 ± 0.95 | 1.57 ± 0.61** | 1.63 ± 0.57 | 1.55 ± 0.59 | 1.55 ± 0.56 | 1.42 ± 0.55 |
| CV (%) | 18.87 ± 5.83 | 20.83 ± 6.65 | 20.28 ± 8.60 | 18.62 ± 5.60 | 18.74 ± 5.15 | 19.73 ± 5.63 | 18.94 ± 6.27 | 18.42 ± 5.73 |

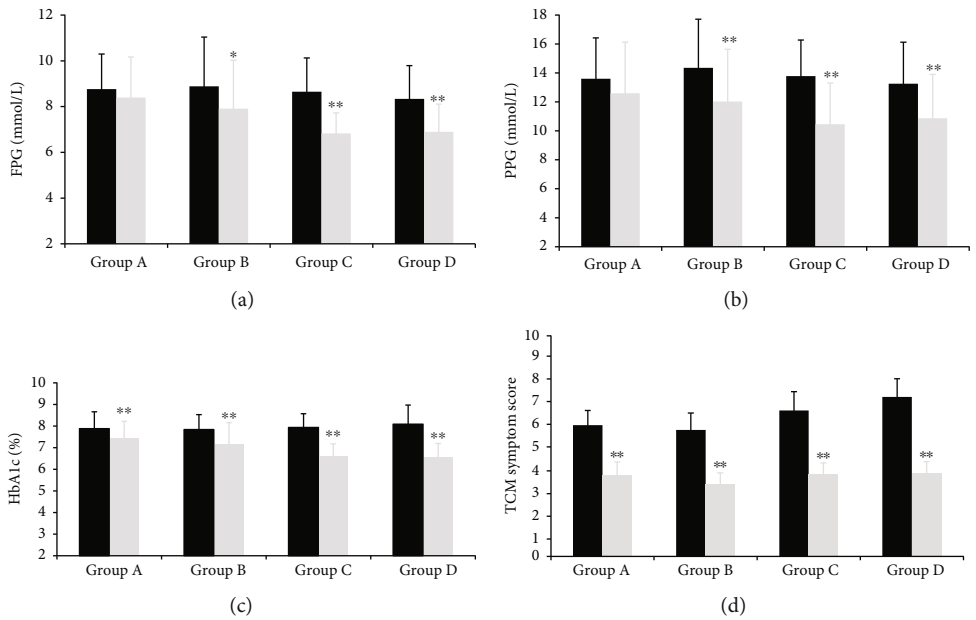


Figure 2: Comparison of Glycemic level and traditional Chinese medicine symptom score at pre-and post-test. (A): Comparison of fasting plasma glucose level at pre-and post-test. (B): Comparison of 2-hour postprandial plasma glucose level at pre-and post-test. (C): Comparison of HbA1c at pre-and post-test. (D): Comparison of traditional Chinese medicine symptom scores at pre-and post-test. Group A: control group; Group B: Jinlida group; Group C: metformin group and Group D: combination treatment group. FPG: fasting plasma glucose; PPG: 2 hour postprandial plasma glucose; TCM: traditional Chinese medicine. *post-test vs. pre-test, $P<0.05$, ** post-test vs. pre-test, $P<0.01$.

of administration. In recent years, large-scale clinical trials have confirmed that TCM has progressed in controlling blood glucose levels [20–24].

Jinlida is an herbal formula originating from the Chinese cognition of the diabetes onset theory, "Pi (Spleen) dysfunction," which was first described in Lingshu, a famous ancient Chinese medical book. Jinlida was made to nourish Pi and regulate body fluid, and consists of ginseng (Renshen), pale white atractylodes rhizome (Cangbaizhu), Poria cocos (Fuling), the root of kudzu vine (Gegen), and radix polygonati officinalis (Yuzhu). Accordingly, the combined herbs tonify the Pi, facilitate the circulation of Qi (energy), and nourish yin [13].

In this study, it was found that after 16 weeks of treatment, the Jinlida, metformin, and combination treatment groups all showed a significant reduction in the HbA1c levels, but there was no significant difference in the magnitude of HbA1c reduction among the three groups. These results are consistent with those of previous studies. Several clinical studies have been conducted in China to assess the efficacy of Jinlida for T2D treatment. Tian et al. conducted a 12-week multicenter double-blind controlled trial to evaluate whether Jinlida can enhance glycemic control in T2D patients with metformin treatment. It was found that HbA1c was reduced by 0.53% with Jinlida in the subgroup with baseline HbA1c ≤ 7.5% [12]. This study showed similar

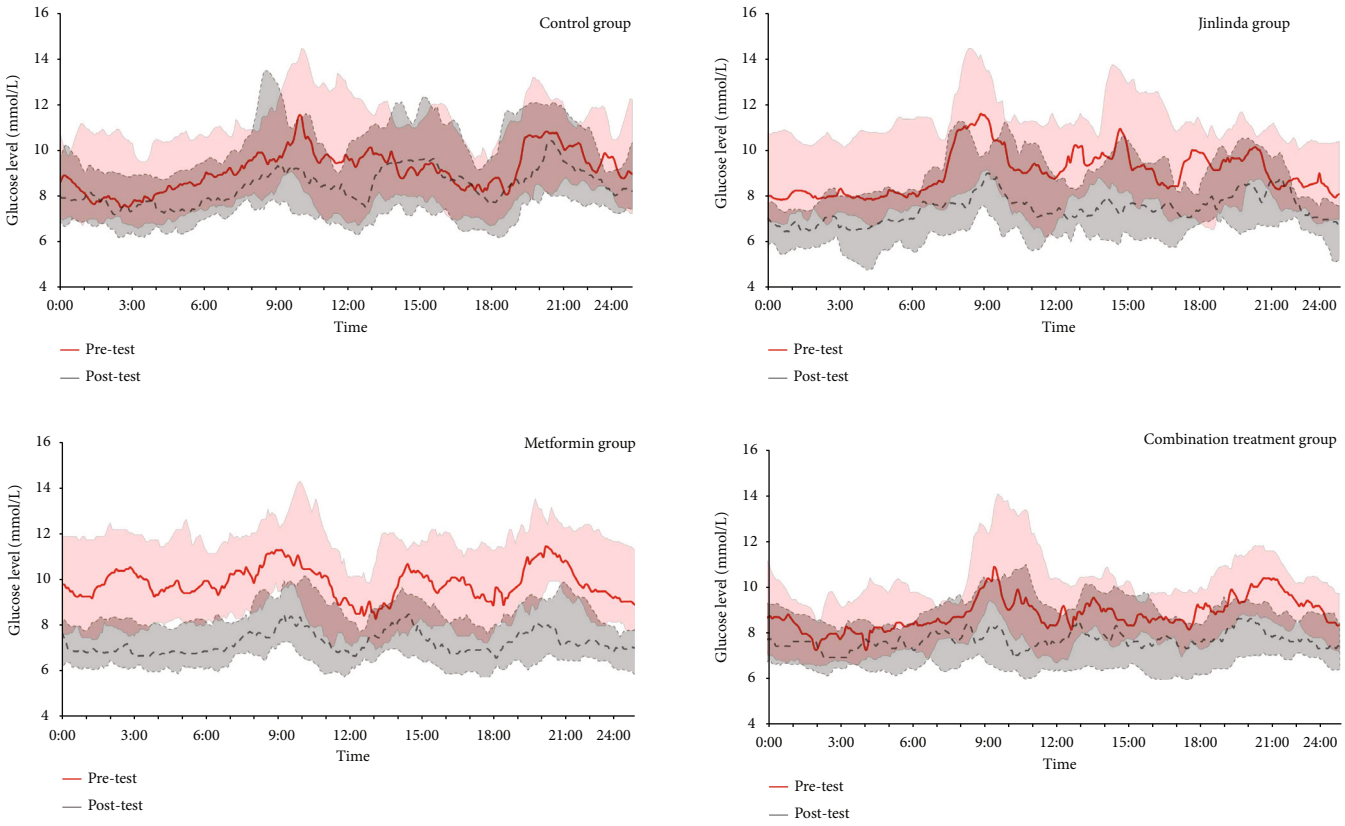


Figure 3: Average continuous glucose-monitoring (CGM) tracings for 72 hours at pre- and post-test. Average CGM values were calculated for each 5 min interval throughout the 3 days for each group.

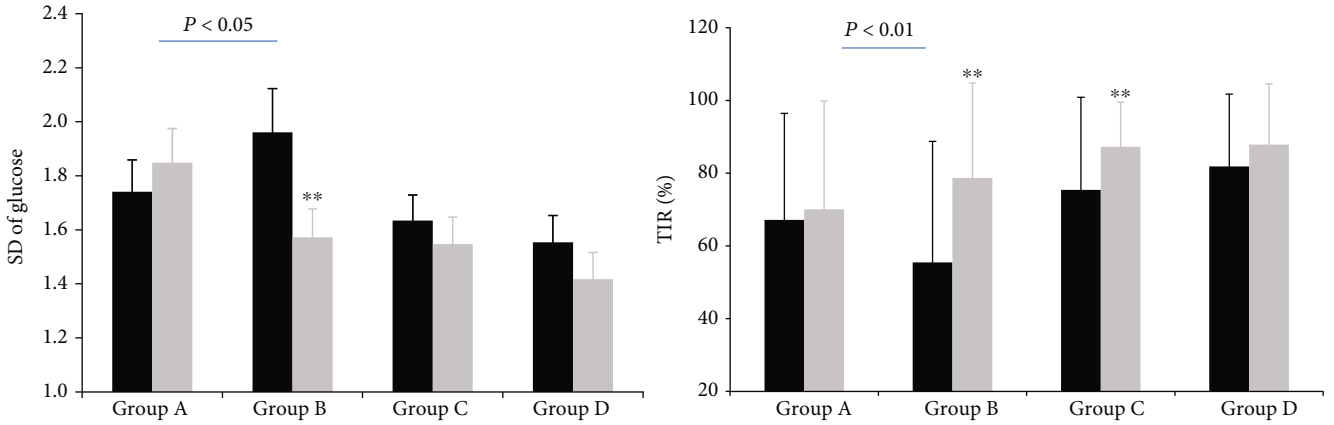


Figure 4: Comparison of standard deviation of glucose and time in range between pre-and post-test. SD: standard deviation; TIR: time in range. *post-test vs. pre-test, P<0.05, ** post-test vs. pre-test, P<0.01. The improvement of SD showed significant difference between control group and Jinlida group (P<0.05). The change of TIR was found significant difference between control group and Jinlida group (P<0.01).

results: HbA1c was reduced by 0.68% with Jinlida monotherapy. Compared with metformin monotherapy, combination treatment with Jinlida reduced HbA1c by 0.18%. The mechanism of the hypoglycemic effect of Jinlida could be related to its protection of pancreatic secretory function through adenosine monophosphate-activated protein kinase activation [6].

To our knowledge, this is the first study to evaluate TIR in all patients with T2D using CGM. TIR is associated with the risk of diabetic microvascular complications and should be accepted as a reasonable glycemic metric to assess glycemic control [25]. In general, a TIR of 3.9–10.0 mmol/L is a useful parameter to evaluate the treatment regimen [17]. Vigersky et al. found a good correlation between TIR and

HbA1c based on 18 randomized controlled trials, including patients with type 1 diabetes and T2D. Further, 70% and 80% of TIR were estimated to be equivalent to an HbA1c level of 6.7% and 5.9%, respectively [26]. We found that Jinlida monotherapy significantly increased TIR by 23% compared to baseline TIR. However, after adjusting for baseline TIR, we did not find an obvious change in the pre-and post-test TIR in multiple groups. The Standards of Care for T2D in China (2019) first included the section on Diabetes and TCM and recommended the addition of Jinlida granules orally in T2D with deficiency of both Qi and Yin and poor efficacy of metformin monotherapy [7]. In this study, we examined the effect of Jinlida granules on TIR improvement and found that Jinlida granules could be an alternative to metformin in newly onset T2D with mild glucose elevation. Additionally, no obvious adverse events were reported, indicating that this herbal medication is safe for clinical use.

Glycemic variability is another part of glucose dysregulation, which also contributes to diabetic vascular complications. Glycemic variability is usually assessed by the parameters of glucose SD and %CV, adopting CGM [16, 27]. In our study, we found that Jinlida granules could reduce glucose SD significantly compared to the control group. Accordingly, the Jinlida granules may improve glycemic variability. In previous research, treatment with Jinlida reduced hepatic oxidative stress, which may lead to the improvement of glycemic variability [2].

This study had some limitations. This was a small sample study with approximately 40 patients in each group. Before performing CGM, all patients did not receive standard dietary guidance. These reasons may lead to the significantly increased TIR levels in group D at baseline and no significant change in TIR in group D after treatment. To further determine the effect of Jinlida granules on glycemic control and glycemic variability, a large sample study is necessary. We only found that Jinlida granules can improve glucose SD significantly compared with the control group ($P < 0.01$), and significant changes in the HbA1c, TIR, and glucose SD levels were not found between the metformin and combination treatment groups. This may be because of the sample size and study period. The baseline difference in TIR may lead to statistical bias, and the sample size should be further expanded. Further study is needed to investigate differences in metabolomics among all the groups.

## 5. Conclusions

Jinlida granules can improve glycemic control and glycemic variability in patients with newly diagnosed T2D.

## Data Availability

The original data sets of this study are available on request to the corresponding author.

## Conflicts of Interest

The authors declare that the study was conducted without any potential conflict of interest.

## Authors' Contributions

Jiemin Pan and Yuejie Xu contributed equally to this work.

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## Supplementary Materials

Traditional Chinese Medicine Symptom Grading and Quantitative Standard *(Supplementary Materials)*

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*Review Article*

# Impact of Incretin-Based Therapies on Adipokines and Adiponectin

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Adipokines are a family of hormones and cytokines with both pro- and anti-inflammatory effects released into the circulation to exert their hormonal effects. Adipokines are closely involved in most metabolic pathways and play an important modulatory role in lipid and carbohydrate homeostasis as they are involved in the pathophysiology of most metabolic disorders. Incretin-based therapy is a newly introduced class of antidiabetic drugs that restores euglycemia through several cellular processes; however, its effect on adipokines expression/secretion is not fully understood. In this review, we propose that incretin-based therapy may function through adipokine modulation that may result in pharmacologic properties beyond their direct antidiabetic effects, resulting in better management of diabetes and diabetes-related complications.

## 1. Introduction

Diabetes mellitus (DM) is the most prevalent metabolic disorder globally [1]. This chronic metabolic disease results in dysregulation of metabolic pathways towards injurious pathways such as the hexosamine pathway, fatty acid beta-oxidation and oxidative stress, polyol pathways, and glycation end products [2]. This metabolic dysregulation contributes to the development of cardiovascular complications and diabetic microvascular complications [3–5]. In diabetes, the physiological balance of many cellular modulators such as adipokines is disturbed, and this may be an important underlying cause for the further development of diabetes-related complications [6]. Adipokines are a large family of inflammatory cytokines originating from adipocytes and fatty tissues that modulate metabolic pathways [7, 8] associated with obesity, metabolic syndrome, insulin resistance, and DM [9–11]. As a result, normalizing adipokine expression/circulatory levels of these bioactive molecules contributes to normalizing body metabolism and preventing diabetes-related complications [12].

Incretin-based therapy is a newly introduced class of medication that has hypoglycemic effects through several pathways [13–17]. They have multiple pharmacological effects on many intracellular mechanisms and tissues, but their role in modulating adipokines and adipocyte-derived cytokines is unclear. If incretin-based therapy were to function through adipokine modulation, this might result in

pharmacologic properties beyond their direct antidiabetic effects that may result in better management of diabetes and diabetes-related complications. Thus, this review has provided an updated analysis on the possible impact of incretin-based antidiabetic drugs on adipocyte-derived peptides.

## 2. Incretin-Based Antidiabetic Drugs

Incretins are a group of intestinal metabolic hormones including glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP), which affect their hypoglycemic effects through several pathways, including glucagon release inhibition, insulin secretion, delayed gastric emptying, appetite suppression, reducing intestinal nutrients absorption, improving lipid metabolism, and promoting pancreatic $\beta$-cells' function [13–17] (Figure 1). These peptides act through specific receptors such as the GLP-1 receptor (GLP-1R), which are members of G-protein coupled receptors mainly located on pancreatic $\beta$-cells [15, 18]. Their activation is followed by increased production of cAMP (cyclic adenosine monophosphate), cellular depolarization, and intracellular calcium augmentation leading to glucose-dependent insulin release from pancreatic $\beta$-cells [15, 18].

Two main classes of antidiabetic agents have been developed based on incretin hormones; GLP-1 receptor agonists (RA), and dipeptidyl peptidase-4 inhibitors (DPP-4i) (Table 1) [13, 19]. GLP-1ra modulates their hypoglycemic effects by direct binding to the GLP-1R, while DPP-4i inhibits the breakdown of endogenous GLP-1 [13, 19]. GLP-1 is naturally metabolized by a protease called dipeptidyl peptidase-4 (DPP-4) [20, 21]. DPP-4 inhibitors and GLP-1RA both result in hypoglycemic effects, although they show differences in bodyweight reduction and the risk of adverse effects [21, 22] (Table 1).

## 3. Adipokines

Adipose tissue has a prominent role in maintaining metabolic balance in the human body [24]. It is primarily recognized as an energy store [24], but increasingly it has been shown to have biological activities through synthesizing active biomolecules such as adipokines and adiponectines and releasing them into the circulation [24]. Adipokines and adiponectines are the two main families of inflammatory cytokines produced and released by adipocytes [7, 8]. After discovering leptin in 1994, hundreds of these peptides have been detected and isolated [25, 26]. These adipocyte-derived biomolecules are closely involved in glycemic control since they may impair or enhance normal signal transduction of insulin in peripheral tissues [26–28]. While adipokines commonly impair insulin sensitivity through inflammation, adiponectines improve it via their anti-inflammatory effects [8, 28]. Although there are some overlapping effects between adipokines and adiponectines, adipokines are classified as insulin-sensitizers (i.e., visfatin, ASP (Acylation-stimulating protein), apelin, adiponectines, and FGF-21 (fibroblast growth factor-21)) and adiponectines as insulin-antagonizers (i.e., TNF-$\alpha$ (fibroblast growth factor-21) (produced mainly by macrophages and lymphocytes, but also by other cell types, including adipocytes), IL-6 (Interleukin 6), IL-2, and resistin) [29, 30].

## 4. Incretins and Adipocytokines

Incretin-based drugs have been shown to affect biological peptides such as adipokines and adiponectines [31, 32]. In the following sections, we will detail what is known of the relationships of these antidiabetic agents with the most important adipokines.

## 5. Leptin

Leptin, or satiety hormone, is a peptide mainly secreted by adipocytes and enterocytes that affect energy balance by control of appetite and feeding [33]. The first discovered adipokine indicated that adipose tissue was not passive storage, but rather an endocrine organ [34]. Leptin acts as an essential signal for the brain to control feeding, and loss of its signal is translated to increased food intake and obesity [33], providing a link between energy intake and expenditure to control glycogenesis, lipogenesis, and fat storage; therefore, preventing lipid accumulation, obesity, and downstream-related complications [34]. This balance is lost in obesity and insulin resistance since cellular sensitivity to circulating leptin is diminished in target receptors (i.e., in the arcuate and ventromedial nuclei, as well as other parts of the hypothalamus and dopaminergic neurons of the ventral tegmental area (VTA)) [34].

There are data suggesting incretin-based therapy has close interactions with leptin expression, secretion, or activities [35, 36], interconnected via vagal afferent neurons (VANs) to control feeding and glucose homeostasis [37]. GLP-1 directly induces centric nuclei involved in leptin secretion and feeding behavior [38]. Anini and Brubaker showed that leptin highly induced GLP-1 secretion in a dose-dependent manner in fetal rat intestinal cells, the mouse L cell line (GLUTag), and the human L cell line (NCI-H716) [39]. They found that mice fed with a high fat diet had hyperleptinemia and leptin resistance that treatment with GLP-1 reversed [39]. Tomasik et al. in 2020 reported that the circulating level of leptin was associated with the GLP-1 serum level [40]. They observed that liraglutide significantly reduced serum leptin levels in prediabetic schizophrenia-spectrum disorder patients [40]. Goldsmith et al. in 2015 provided similar evidence indicating GLP-1 administration reduced serum leptin levels in mice [41].

Frøssing et al. 2018 reported that 26 weeks of liraglutide therapy reduced leptin levels in women with polycystic ovary syndrome [42]. Shi and coworkers 2017 showed that exenatide decreased leptin level in type 2 diabetic patients [43]. Lepsen and colleagues 2015 suggested that GLP-1 and leptin cooperate in the weight maintenance and weight loss effects of GLP-1, probably mediated by a decrease in free circulating leptin in obese individuals [44]. This study emphasizes the role of GLP-1 on leptin secretion and suggests that they are both important in lipid metabolism [44]. In another study, Farr and coworkers found that 17 days of GLP-1 therapy reduced serum leptin levels in patients with type 2

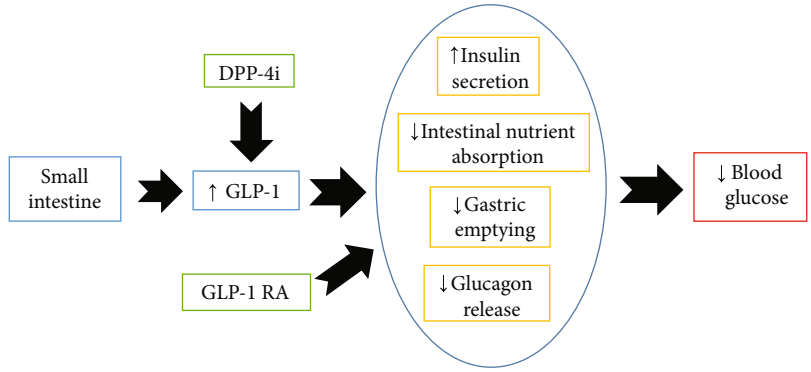


Figure 1: Schematic mechanism of action of incretin-based medications.

Table 1: Approved forms of incretin-based medications.

| | Approved forms | Mechanisms of action | Ref. |
|---|---|---|---|
| GLP-1 RA | Exenatide (exendin-4), albiglutide, liraglutide, lixisenatide, semaglutide, dulaglutide | Agonists of intrinsic incretin receptors | [13, 19] |
| DPP-4i | Sitagliptin, saxagliptin, vildagliptin, linagliptin | Prevent incretin inactivation by inhibition of DPP-4 enzyme | [21, 23] |

diabetes mellitus (T2DM) [45]. They concluded that GLP-1 is involved in the body's energy balance via metabolic hormones like leptin and ghrelin [45]. Similarly, Li et al. 2017 reported that 6 months of sitagliptin therapy reduced leptin plasma level in obese diabetic patients [46]. Moreover, a recent meta-analysis of randomized controlled trials reported that GLP-1RA has inhibitory effects on leptin levels [47]. Overall, it can be seen that GLP-1 therapy affects leptin levels, and some of its metabolic effects are likely mediated by leptin. However, there is no direct evidence to support this, and further clarification studies are required to elucidate the exact molecular interactions between them.

## 6. Ghrelin

Ghrelin, or hunger hormone, is another adipokine peptide that is produced mainly by endocrine cells of the gastrointestinal tract, especially stomach cells [48]. It was discovered as the endogenous ligand of the GHSR (growth hormone secretagogue receptor), but later investigations showed that it is a potent metabolic hormone involved in the control of energy balance, food intake, body weight, adiposity, glucose metabolism, and feeding behaviours [48]. Deregulated levels of ghrelin are involved in the pathophysiology of obesity, adiposity, hyperinsulinemia, insulin resistance, and DM [49, 50].

There are confirmed physiological interactions between endogenous GLP-1 and ghrelin hormone [51]. While GLP-1 is released following feeding, ghrelin levels are increased before food intake, suggesting that ghrelin induces intestinal L-cells to release GLP-1 to prepare the body for incoming food [51]. Gagnon et al. 2015 showed that in the presence of exendin-4, stimulatory impacts of ghrelin on insulin release were completely inhibited in C57BL/6 mice, indicating that the GLP-1R is required for the gluco-homeostatic effects of ghrelin [52]. Another study by Ronveaux and coworkers 2015 suggested that GLP-1 interacts with ghrelin peptide through vagal afferent neurons to promote metabolic pathways [37]. In addition, Lindqvist et al. 2017 showed that ghrelin had regulatory roles on both expression and secretion of GLP-1 in mice [53]. Thus, it seems that endogenous GLP-1 and ghrelin are integrally involved in modulating their metabolic effects. This is further suggested by the work of Babenko et al. 2019, who found that 24 weeks of GLP-1 therapy reduced serum levels of ghrelin in obese T2DM patients [54]. Recently, Skuratovskaia and coworkers in an in silico study found a positive correlation between GLP-1 and ghrelin in patients after LSG (Laparoscopic sleeve gastrectomy) surgery [55].

## 7. Visfatin

Visfatin is a potent adipokine first isolated in 1994 from human lymphocytes as pre-B cell colony enhancing factor (PBEF) [56]. It is expressed in many organs and tissues such as bone marrow, chondrocytes, hepatic cells, muscle, brain, kidney, spleen, testis, and lung, but preferentially in visceral adipose tissue and macrophages [56]. Visfatin acts as a potent proinflammatory cytokine with immunomodulatory effects that is highly expressed in many inflammatory diseases like rheumatoid arthritis, pneumonia, or irritable bowel syndrome [57–59]. In addition, it has complex molecular interactions with metabolic pathways and body homeostasis [60]. Visfatin indirectly modulates metabolic pathways via cellular mediators such as poly (ADP-ribose) polymerase (PARPs), sirtuins (SIRTs), CD38, and CD157 [61, 62]. Visfatin may increase beta cell proliferation, improve insulin sensitivity, enhance glucose uptake, and induce lipogenesis [63].

Visfatin likely binds to the IRs with a similar affinity as insulin and mimics its activities resulting in a prominent role in glucose metabolism [64, 65]. Visfatin has potent modulatory effects on genes involved in lipid homeostasis, such as fatty acid synthase, lipoxygenase, and lipoprotein lipase [60, 66].

Increasing evidence suggests that visfatin acts as a key mediator of the incretin effects [67–69]. Data indicates that the insulinotropic, lipogenic, and glucose-homeostatic properties of incretins are mediated by visfatin [67–69] and that visfatin has modulatory roles on the pleiotropic effects of diabetes-dependent peptides [70].

The effects of incretin-based therapy on visfatin expression/secretion are controversial. Some evidence indicates that GLP-1 suppresses visfatin release [71, 72]. Bala et al. 2011 reported in 100 healthy participants that both insulin and GLP-1 decreased postprandial visfatin levels [71], suggesting that there is a GLP-1/visfatin axis responsible for the rapid suppression of visfatin release upon oral glucose uptake [71]. In addition, Abdelwahed and coworkers 2018 demonstrated that exendin-4 decreases visfatin expression in brain tissue, and this effect mediates the neuroprotective and cognitive enhancer activities of GLP-1 [72]. A recent study by Jin et al. suggested that liraglutide reduced visfatin levels in high fat diet rats [73], and that liraglutide suppressed inflammatory effects of adipokines as well as visfatin leading to greater insulin sensitivity [73]. Likewise, Li et al. 2017 showed that sitagliptin reduces visfatin levels in obese patients with T2DM [46].

Conversely, there is evidence suggesting that GLP-1 increases visfatin levels and upregulates it [74]. For example, Liu et al. 2013 demonstrated in an in vivo study that GLP-1 administration induces visfatin expression via a PKA (Protein kinase A)-dependent pathway in 3T3-L1 adipocytes [74]. They observed that this effect was suppressed by using a PKA inhibitor of H89 [74]. A clinical study in 2014 showed the liraglutide therapy-induced visfatin protein expression level in T2DM patients [75], and another report in 2016 indicated that higher levels of GLP-1 were associated with increased visfatin in maternal and cord blood of participants [76]. Overall, debate remains about the effects of incretin antidiabetic drugs on visfatin that require clarification.

## 8. Resistin

Resistin or adipose tissue-specific secretory factor is a small cysteine-rich peptide that is under the influence of different proinflammatory stimuli, inducing its expression/release and is mainly expressed and released in humans by macrophages (in rodents, it is expressed by adipocytes), and plays an important endocrine role in inflammatory disorders [77]. It binds to the endotoxin receptor TLR4 (Toll-like receptor 4) and an isoform of decorin (a proteoglycan) known as $\Delta$DCN [78, 79]. Resistin release is inhibited by thiazolidinediones [78]. Although several biologic activities such as proinflammatory, proangiogenic, and antiapoptotic properties have been related to resistin, its physiologic importance is still not well understood [80]. However, it is accepted that circulatory levels of resistin are important in energy homeostasis [79, 81]. Its levels commonly increase in obesity and DM [82], and it impairs insulin signaling and induces insulin resistance, while blocking it increases glucose uptake and insulin sensitivity [82]. Resistin is able to induce and promote inflammatory pathways involved in different complications such as hypercholesterolemia, asthma, chronic kidney disease, cirrhosis, atherosclerosis, hepatosteatosis, as well as glucose/lipid intolerance, and insulin resistance [77]. Transgenic animals lacking resistin expression were shown to have reduced levels of postprandial blood glucose due to inhibited hepatic gluconeogenesis [83]. Also, exogenous resistin or its overexpression has been associated with adipose tissue inflammation, increased lipolysis and serum-free fatty acid levels, DAG (Diacylglycerol) accumulation in skeletal muscles, hyperinsulinemia, insulin resistance, and glucose intolerance [84–86].

Evidence suggests that GLP-1 levels in plasma interact with resistin peptide [87] with a positive correlation between resistin and incretin hormones in human blood being reported by Niwa et al. in 2016 [76]. In addition, a recent clinical study showed a positive relation between GLP-1 and resistin in diabetes [87].

Resistin may interact with incretins, although with contradictory results as inhibitory or stimulatory effects have been reported [88]. Kim and coworkers in 2007 reported that GIP increases resistin release from 3T3-L1 adipocytes via a p38 MAPK (p38 mitogen-activated protein kinase) and SAPK/JNK (stress-activated protein kinase/Jun amino-terminal kinase) dependent pathways [68]. They concluded that the addition of resistin to differentiated 3T3-L1 adipocytes mimicked the metabolic impacts of incretins, while resistin was recognized as an insulin antagonizer [68]. Díaz-Soto and colleagues in 2014 reported that a short period of liraglutide increased resistin level in T2DM patients [75]. This evidence suggests that incretin-based drugs may increase resistin levels in short-time administration.

There is evidence supporting incretin-based drugs reducing resistin levels so improving insulin signaling [73]. Jin and coworkers in 2020 demonstrated that liraglutide reversed HFD induced hyper-resistinemia in rats [73]. Quan et al. in 2017 found that exenatide therapy significantly decreased resistin level in obese newly diagnosed diabetic patients [89]. Li et al. in 2015 reported that liraglutide downregulated resistin peptide in patients with T2DM [90]. A meta-analysis in 2015 reported that GLP-1 therapy may reduce the risk of atherosclerosis by lowering the level of resistin and its accompanying inflammation [91]. Similarly, a more recent meta-analysis of randomized controlled trials found that GLP-1RAs decrease resistin levels in diabetes [47]. These data highly suggest that incretin-based drugs exert inhibitory effects on resistin expression/secretion though the inconsistency with apparent short-term administration requires clarification.

## 9. Apelin

Apelin or ligand for G-protein-coupled receptor APJ is a neuroendocrine peptide that is extensively expressed in adipocytes and by a number of other tissues such as kidneys, neurons, vessels, myocardium, gonads, lung, liver, pancreatic islets,

Table 2: current knowledge on effects of incretin-based antidiabetic drugs on adipokines and adipocyte-derived cytokines.

| Adipocyte-derived cytokine | Effects of incretin-based drugs | Ref. |
|---|---|---|
| Leptin | Increase active levels of leptin and potentiate its metabolic effects | [38–44] |
| Ghrelin | Unknown clear interactions may potentiate metabolic impacts of ghrelin | [37, 52–55] |
| Visfatin | Induce visfatin expression/secretion | [74, 75] |
| | Reduce active levels of visfatin | [46, 71–73] |
| Resistin | Decrease resistin activities | [47, 73, 89–91] |
| | Increase resistin and related impacts | [68, 75] |
| Apelin | Induce apelin release | [103] |
| Adiponectin | Increase active levels of adiponectines to enhance insulin sensitivity | [32, 43, 90, 115, 116] |

gastrointestinal tract, and adrenal glands [92]. It has several active isoforms as apelin-36, apelin-17, and apelin-13, each having different biologic functions such as blood pressure control, angiogenesis, feeding behavior, cardiac contractility, cell proliferation, apoptosis, and stress responses [93]. Apelin is derived from mature adipocytes, and it has been recognized primarily as an adipokine and is closely involved in metabolism and energy homeostasis [92]. Apelin synthesis is directly stimulated and dependent on insulin, which explains why obese individuals have higher levels of apelin [94]. Its exact physiologic function is not understood though many studies have reported that plasma level of apelin is increased in obesity with beneficial antiobesity and antidiabetes effects and therefore may have utility in metabolic disorders [95–97]. It was also suggested that apelin has insulinotropic properties [98] with increased glucose uptake through several pathways such as AMPK (AMP-activated protein kinase) and eNOS (Endothelial nitric-oxide synthase) dependent [99], PI3K/Akt (Phosphatidyl inositol 3-kinase/protein kinase B) dependent Glut-4 (Glucose transporter 4) expression/localization [100], and decreased cAMP (Cyclic adenosine monophosphate) [98, 101, 102].

There is little evidence for the dual interactions between incretin-based therapies and apelin peptide [103]. In comparison, apelin may show stronger hypoglycemic effects than incretins [104]. It was shown that apelin induces GLP-1 release in a dose-dependent manner in both in vitro (STC-1 cells) and in vivo (adult rats) models [105], while incretin therapy may induce apelin secretion [103]. Fan et al. in 2015 showed that vildagliptin increased apelin levels in T2DM patients [103], and they concluded that 12 weeks of vildagliptin therapy normalized glycemic indices and improved insulin sensitivity due to the rise in apelin levels [103]. A more recent study showed that incretin therapy might have some further interactions with apelin [106], but more studies are needed.

## 10. Adiponectin

Adiponectin is an anti-inflammatory adipokine synthesized mainly by adipocytes (but also in other tissues as brain, placenta, and muscles) that regulates a number of metabolic pathways involved in glucose and lipid homeostasis [107]. This endocrine peptide plays important modulatory roles in metabolic complications as obesity, DM, metabolic syndrome, atherosclerosis, and nonalcoholic fatty liver disorder (NAFLD) [108, 109]. It has been shown that adiponectin increases insulin sensitivity probably by control of fatty acid oxidation and preventing gluco/lipotoxicity, Glut-4 localization, and suppression of hepatic gluconeogenesis [110–112]. Adiponectin plasma levels are decreased in obesity and by a sedentary lifestyle and increased after aerobic exercise, caloric restriction, and weight loss [113, 114].

Evidence suggests that incretin-based antidiabetes agents have modulatory effects on adiponectin levels [32, 90, 115]. Most data support that incretins increase adiponectin levels. Hosaka et al. in 2009 showed exendin-4 induced adiponectin expression and accompanied with improved inflammatory status and higher insulin sensitivity in 3T3-L1 adipocytes [32]. Bunck and coworkers in 2010 showed exenatide increased adiponectin levels accompanied with improved insulin sensitivity in patients with T2DM [115]. Similarly, Li et al. in 2015 found that liraglutide upregulated adiponectin in T2DM patients [90]. Likewise, Shi et al. in 2017 were also found that exenatide therapy increased adiponectin levels in patients with T2DM [43]. A clinical trial in 2015 confirmed that vildagliptin increased adiponectin in patients with DM [116]. These studies suggest that incretin-based drugs have positive effects on adiponectin synthesize/release, and these effects may explain in part their insulin-sensitizing impact.

## 11. Conclusion

This review has shown that adipokines and adiponectines are modulated by incretin-based pharmacotherapy (Table 2) and may be in part responsible for their pharmacological effects improving glycemic control and improving the overall metabolic profile. However, the effect on adipokines is not fully understood nor fully investigated with no evidence either way on the incretin effects on FGF-2 and ASP, for instance. However, there is evidence for incretins to induce leptin secretion, potentiate metabolic effects of ghrelin peptide to normalize glucose homeostasis, and there are dual interactions between incretins and apelin peptide. The effect on resistin is unclear, but positive effects on visfatin and adiponectin are reported. Overall, incretin therapy appears to have a positive effect on adipokine and adiponectines; however, more studies need to be done to clarify the molecular pathways, pharmacological and physiological effects.

## Data Availability

No primary data is associated with this review article.

## Conflicts of Interest

The authors declare that they have no conflict of interest in this study.

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*Research Article*

# Impact of PCSK9 Immunization on Glycemic Indices in Diabetic Rats

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*Background and Aim*. The impact of Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition on glycemic indices in diabetes mellitus remains far from clear. We explored the effects of PCSK9 inhibition on glycemic indices in the diabetes rat model. *Methods*. To prepare the anti-PCSK9 vaccine, a peptide construct called Immunogenic Fused PCSK9-Tetanus (IFPT) was linked to the surface of nanoliposome carriers. Healthy rats received four subcutaneous injections of the vaccine at biweekly intervals. Two weeks after the last vaccination, anti-PCSK9 antibody titers, PCSK9 targeting, and inhibition of PCSK9–low-density lipoprotein receptor (LDLR) interaction were evaluated. After verification of antibody generation, the immunized rats were intraperitoneally treated with a single dose (45 mg/kg) of streptozotocin (STZ) to induce diabetes mellitus. The levels of fasting blood glucose (FBG) were measured, and the oral glucose tolerance test (OGTT) as well as the insulin tolerance test (ITT) were carried out to assess glycemic status. At the end of the study, the total cholesterol, low-density lipoprotein cholesterol (LDL-C), triglyceride, and high-density lipoprotein cholesterol concentrations were assayed. Histopathology examination of the liver and pancreas was also performed using the hematoxylin-eosin staining method. *Results*. The prepared nanoliposomal vaccine could strongly induce anti-PCSK9 antibodies in the vaccinated rats. Within one week following the STZ injection, the FBG level was lower in the vaccinated group vs. diabetic control group (49% ($-171.7 \pm 35$ mg/dL, $p < 0.001$)). In the OGTT, the injected rats showed improved glucose tolerance as reflected by the reduction of blood glucose levels over 180 min, compared with the diabetic controls. Moreover, the ITT demonstrated that, after the insulin injection, blood glucose concentration declined by 49.3% in the vaccinated group vs. diabetic control group. Expectedly, the vaccinated rats exhibited lower (-26.65%, $p = 0.03$) plasma LDL-C levels compared with the diabetic controls. Histopathology examination of pancreas tissue demonstrated that the pancreatic islets of the vaccinated rats had a slight decline in the population of $\beta$-cells and few $\alpha$-cells. Normal liver histology was also observed in the vaccinated rats. *Conclusion*. PCSK9 inhibition through the liposomal IFPT vaccine can improve the glucose and insulin tolerance impairments as well as the lipid profile in diabetes.

## 1. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a plasma protein that is majorly generated and released by the liver cells. The liver-secreted PCSK9 is principally known for its role in the regulation of low-density lipoprotein (LDL) receptor (LDLR) on the surface of hepatocytes and, thereby, hemostasis of LDL cholesterol (LDL-C) in the bloodstream [1]. The plasma circulating PCSK9 controls the hepatic LDLR *via* posttranslational modification through targeting the extracellular domain of LDLR, epidermal growth factor-like repeat A (EGF-A), and subjecting it to the lysosomal digestion [2]. Indeed, EGF-A is a responsible domain for the LDLR recycling to the cell surface [3–5], and PCSK9 binding impedes the regular comeback of the LDLR to the cellular membrane and facilitates its digestion in lysosome compartments, leading to reduced liver clearance of the plasma LDL-C [6, 7].

Notably, patients with diabetes mellitus (DM) suffer from atherogenic dyslipidemia identified by increased LDL-C, hypertriglyceridemia, and decreased antiatherogenic high-density lipoprotein (HDL) particles [8, 9]. LDL-C has been found as a therapeutic target in diabetic dyslipidemia, and LDL-reducing approaches have been shown to reduce cardiovascular (CV) events in individuals with diabetes [10]. Statins are the main LDL-lowering agents, which act through suppression of cholesterol biosynthesis [11] plus a multitude of pleiotropic effects [12–18]. Although statin therapy shows strong effectiveness in ameliorating the CV endpoint events [19–21], many meta-analyses of randomized controlled trials reveal a documented link of the statin use to the elevated chance of new-onset DM [22–26].

PCSK9 inhibitors, mainly monoclonal antibodies (mAbs), provide a strong LDL-reducing approach that, in combination with statins at maximally tolerated doses, can decrease LDL-C approximately by 73% [27, 28] and decrease CV outcomes [29–31]. Although data from various clinical trials firmly show an appreciable mitigating effect of the PCSK9 inhibitor evolocumab on hyperlipidemia in T2DM patients [32], there are still some concerns regarding the correlation between PCSK9 inhibitors and DM complications [33–36]. Therefore, it is essential to evaluate the impact of a newly developed PCSK9 inhibitor on glycemic indices and the progression of diabetes.

To address this, preclinical and clinical studies in diabetic models and individuals are inevitable. Our previous study showed that a nanoliposomal vaccine targeting PCSK9 could significantly induce the generation of antibodies inhibiting the plasma PCSK9 and thereby reducing the plasma LDL-C in an experimental model of atherosclerosis [37–40]. To understand the effect of anti-PCSK9 therapy on glycemic indices, we evaluated the preventive impact of the nanoliposomal anti-PCSK9 vaccine in rats with streptozotocin- (STZ-) induced diabetes.

## 2. Methods

*2.1. Nanoliposomal Vaccine Preparation and Characterization.* Nanoliposomal vaccine embracing an immunogenic peptide conjugated to the surface of liposome nanoparticles was constructed according to the previously described method [41]. In brief, the lipid film hydration method was employed to provide liposome nanoparticles. A peptide construct containing PCSK9 and tetanus epitopes termed immunogenic fused PCSK9-tetanus (IFPT) peptide was attached to the surface of the prepared nanoparticles using the postinsertion method. The linkage efficiency and peptide content of the prepared liposomal IFPT (L-IFPT) formulation were measured by HPLC (high-performance liquid chromatography) analysis (Knauer; Berlin, Germany). Physical properties of the peptide-linked liposomes, including particle size, charge, and homogeneity, were assessed by the dynamic light scattering (DLS) approach on a Zetasizer (Nano-ZS, Malvern, UK). The verified L-IFPT formulation was adsorbed to 0.4% alum adjuvant (Sigma-Aldrich) at the 1 : 1 ($v : v$) ratio and used for in vivo study on STZ-induced diabetic rats.

*2.2. The Animal.* A total of 24 male Wistar Albino rats ($179 \pm 5.5$ g) were provided by the Laboratory Animal Research Center of the Faculty of Medicine Mashhad University of Medical Sciences, Mashhad, Iran. All animal handling procedures were conducted strictly based on the animal welfare guidelines approved by the Institutional Ethics Committee and Research Advisory Committee. Rats were weighed weekly and at the end of the experiment, located in an air-conditioned space at a room temperature of $22 \pm 2^{\circ}$C with 12 : 12 h light/dark cycle, and fed a standard rodent diet and water *ad libitum*. Upon starting the experiment and prior to STZ-induced diabetes, rats were administered either with vaccine formulation or saline buffer. The tail vein blood collection was carried out two weeks following the last immunization for the titration of the plasma anti-PCSK9 antibody, and after STZ injection for the FBG measurement. At the end of the experiment, rat euthanasia was performed by intravenous injection (30 mg/kg) of thiopental sodium [42, 43], and blood was collected *via* heart puncture to check out the plasma lipid profile. Pancreas and liver tissues were isolated to determine their weight and cell destruction.

*2.3. The Vaccination Scheme.* One week before the study, the rats were domesticated to be randomly subjected into two groups, a vaccine-treated group ($n = 8$, $208 \pm 6.14$ g) and a nontreated group ($n = 16$, $207 \pm 15.4$ g). The vaccine group rats were biweekly immunized 4 times subcutaneously (s.c.) with a 200 $\mu$L L-IFPTA formulation containing 20 $\mu$g peptide, while nontreated group rats simultaneously received saline buffer. The time point of the first immunization is referred to as week 0 (W0). Three boosters were then implicated at W2, W4, and W6 (Figure 1). The blood was withdrawn at the time point W6 (Figure 1), and the plasma samples were prepared and used for antibody titer analysis. The vaccination schedule, including the dose and the duration, was planned based on our previous study [41].

*2.4. Evaluating the Efficacy of Nanoliposomal Anti-PCSK9 Vaccine in Rats.* To determine the efficacy of the liposomal vaccine in rats, the plasma anti-PCSK9 antibody titer, the

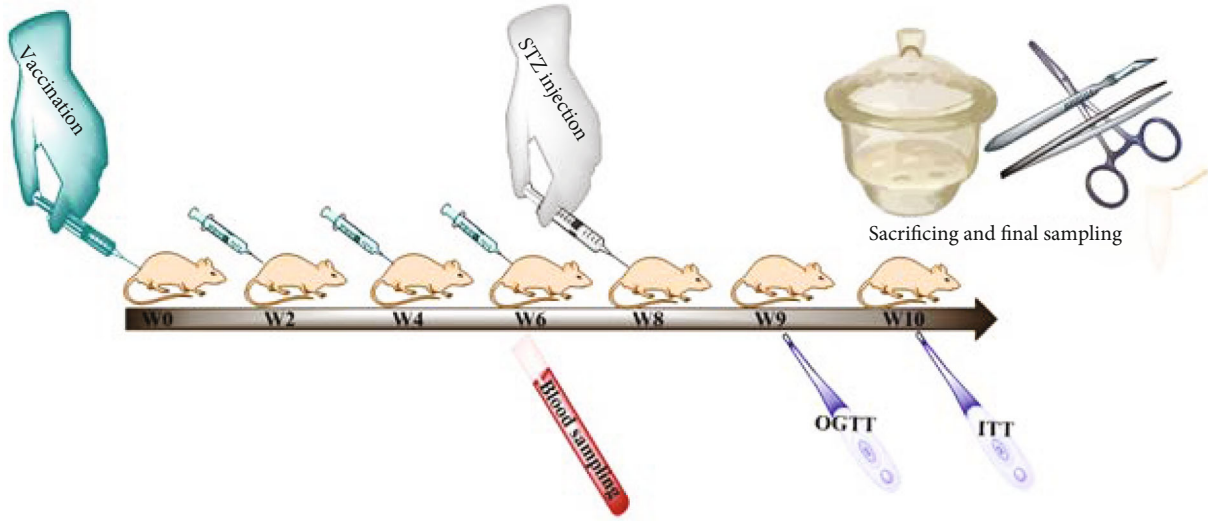


Figure 1: Schematic of animal interventions during the study.

plasma PCSK9 concentration, antibody-targeted PCSK9, and antibody-inhibited PCSK9/LDLR interaction were analyzed as explained in our recent study [41].

Concisely, the liposomal vaccine-induced anti-PCSK9 antibody was evaluated by the ELISA method using serially diluted plasma ($1:4\times1:400$). A microwell plate reader (Sunrise, Tecan, Switzerland) was employed to detect the optical density (OD) at 450 nm. The dilution factor attributed to 50% of the maximal optical density ($OD_{max}/2$) was measured to define the antibody titer [41]. To quantify the level of the free plasma PCSK9 in vaccinated mice, a PCSK9 ELISA kit (CircuLex™, Cy-8078, MBL, Woburn, MA) was employed as instructed by the manufacturer. This PCSK9 ELISA kit was also used to assay the interaction of vaccine-produced antibodies with PCSK9 and, thereby, determine inhibition of the rat plasma PCSK9 by the generated antibodies [41]. To find the functionality of the induced antibodies, the ability of the vaccinated rat's plasma for inhibition of the PCSK9-LDLR interaction *in vitro* was assessed by a PCSK9-LDLR *in vitro* binding assay kit (CircuLex™, Cy-8150, MBL, Woburn, MA). The higher ELISA OD shows a higher amount of PCSK9-LDLR interaction, in which in the presence of anti-PCSK9 antibodies, such interaction is impeded and, as a result, detected ELISA OD is reduced [41].

*2.5. STZ-Induced T1DM.* 14 days following the last vaccination (W8, when the antibody titer was at the peak level, based on our previous finding [41]), both vaccine-treated and nontreated groups were subjected to a diabetes experiment to evaluate the antidiabetic effects of the anti-PCSK9 vaccine. Therefore, T1DM condition was induced in overnight-fasted (12 h) rats by a single intraperitoneal injection of STZ (45 mg/kg, Sigma-Aldrich) freshly dissolved in citrate-buffered saline (0.1 M, pH 4.5) [44]. The rats in the nonvaccinated group were randomized into two groups: the normal control (NC) group ($n=8$; unvaccinated and received citrate buffer) and the diabetic control (DC) group ($n=8$; unvaccinated and received STZ). The vaccine-treated group ($n=8$) received STZ and was assigned as the vaccinated STZ-injected (VS) group. The first week after STZ injection, rats in the DC group had FBG concentrations $>250$ mg/dL that confirmed the T1DM model [44].

*2.6. Oral Glucose Tolerance Test (OGTT).* To assess the glucose tolerance ability of each rat, an OGTT was conducted on rats that were fasted overnight with a glucose dose of 2 g/kg at W9. Briefly, glucose solution was orally given, and blood glucose concentrations were checked by a glucometer (EasyGluco, South Korea) at time point 0 min (before glucose load) and 30, 60, 90, 120, 150, and 180 min after the oral glucose load [45]. The resultant data were expressed as an integrated area under the curve for glucose ($AUC_{glucose}$), which was calculated by trapezoid rule using GraphPad Prism version 7.04.

*2.7. Insulin Tolerance Test (ITT).* An insulin tolerance test was performed to determine the measure of peripheral utilization of glucose. At W10, insulin (0.8 U/kg) was intraperitoneally administered to overnight-fasted rats. Blood glucose was measured at time point 0 min (before the insulin injection) and 15, 30, 45, 60, 75, 90, and 120 min after the insulin injection [46]. The results were expressed as $AUC_{glucose}$.

*2.8. Lipid Profile Analysis.* The plasma levels of LDL-C, HDL cholesterol (HDL-C), triglyceride (TG), and total cholesterol (TC) were assessed at the end of the study (W10) with commercial kits (BioSystems) as instructed by the manufacturer.

*2.9. Histopathology Examination.* At last, rats were euthanized, and organ samples were collected. Immediately upon removal, small pieces of the isolated pancreas and liver tissues were cut and immersion fixed in 10% buffered formalin. The formalin-embedded tissues were gradually dehydrated, embedded in paraffin, cut into 5 $\mu$m sections, deparaffinized, and eventually stained using the hematoxylin and eosin

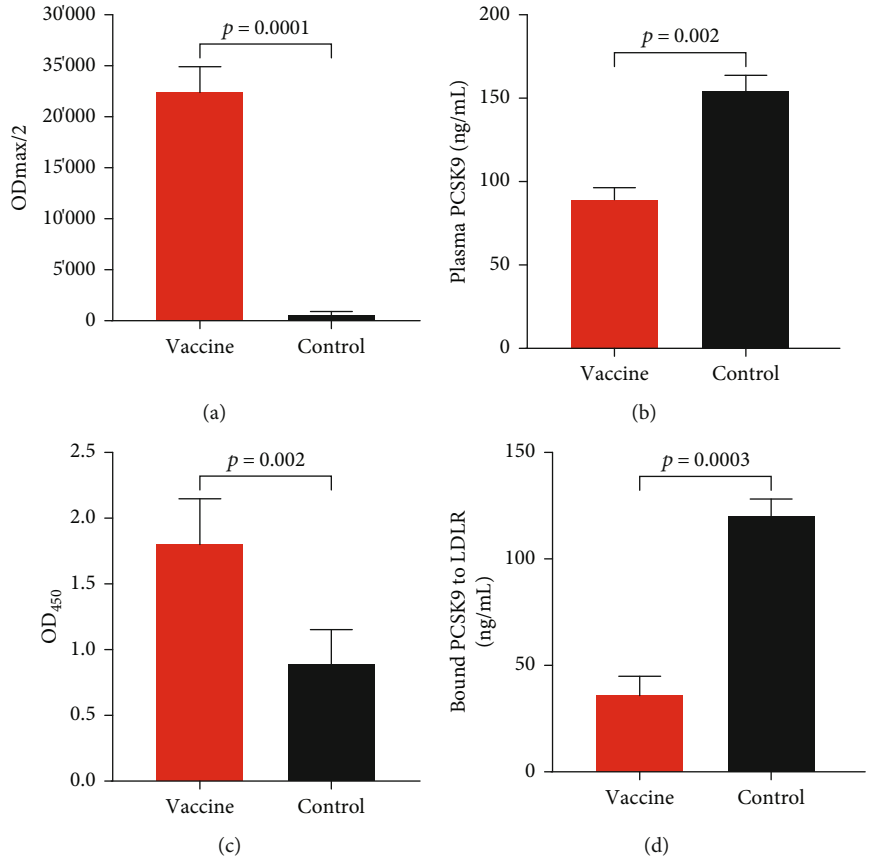


FIGURE 2: The function of liposomal anti-PCSK9 vaccine in rats upon 4 vaccinations. (a) Anti-PCSK9 peptide IgG titers ($OD_{max}/2$) in vaccinated and control rats ($n = 8$/group). (b) Concentrations of the plasma PCSK9 in vaccinated and control rats were 89 ± 7 ng/mL and 154 ± 10 ng/mL, respectively ($n = 3$ replicates of the pooled samples of 8 rats per group). (c) Direct detection of vaccine-generated anti-PCSK9 antibodies targeting plasma PCSK9. Increased $OD_{450}$ is indicative for evaluating the direct binding of anti-PCSK9 antibodies to plasma PCSK9 from vaccinated and control rats ($n = 3$ replicates of the pooled samples of 8 rats per group). (d) *In vitro* evaluation of PCSK9/LDLR interaction. Plasma sample of vaccine rats contained vaccine-generated anti-PCSK9 antibodies that could decrease PCSK9 interaction to LDLR by 30% when compared with the plasma sample of control rats ($n = 3$ replicates of the pooled samples of 8 rats per group). Bars show mean values, error bars show ±SD.

(H&E) method. The histology of H&E-stained sections was checked out by an expert pathologist, using light microscopy supplied with a digital camera under a magnification of 400x.

*2.10. Statistical Analysis.* GraphPad Prism (version 7.04) and IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, NY, USA) were used for statistical analysis. The results were analyzed using the one-way ANOVA and Bonferroni post hoc multiple comparison test to evaluate the significance of the differences between the animal groups. Values were expressed as the mean ± SD or the mean ± SEM, lower-upper 95% confidence interval of the mean. Results with $p < 0.05$ were considered as statistically significant.

## 3. Results

*3.1. Nanoliposomal Formulation.* The empty nanoliposomes and the IFPT-linked nanoliposomes were found to have a size range from 150 nm to 180 nm in diameter, in which the polydispersity index was <0.2, revealing the preparation of nanovesicles with high homogeneity. Analysis of surface charge also showed that the prepared formulations had negative zeta potential. As revealed by HPLC analysis, 96% of IFPT peptides added at the beginning were linked to liposome nanoparticles.

*3.2. Efficacy of Liposomal Anti-PCSK9 Vaccine in Rats.* The L-IFPTA vaccine could induce a high-titer IgG antibody against the PCSK9 peptide in rats upon 4 vaccinations in biweekly intervals (Figure 2(a)). Vaccine-generated anti-PCSK9 antibodies showed specific targeting of the plasma PCSK9 in the vaccinated rats. As demonstrated in Figure 2(b), the plasma concentrations of the free PCSK9 in the vaccine group (89 ± 7 ng/mL) were significantly ($p = 0.002$) lower than that in the control group (154 ± 10 ng/mL). The plasma PCSK9 concentration was significantly reduced by 57.8% in the vaccinated rats when compared with the control rats. As revealed by the PCSK9 inhibition assay using the ELISA method, the plasma of vaccinated rats could emerge as a noticeably higher $OD_{450}$ signal than that of the control rats (Figure 2(c)),

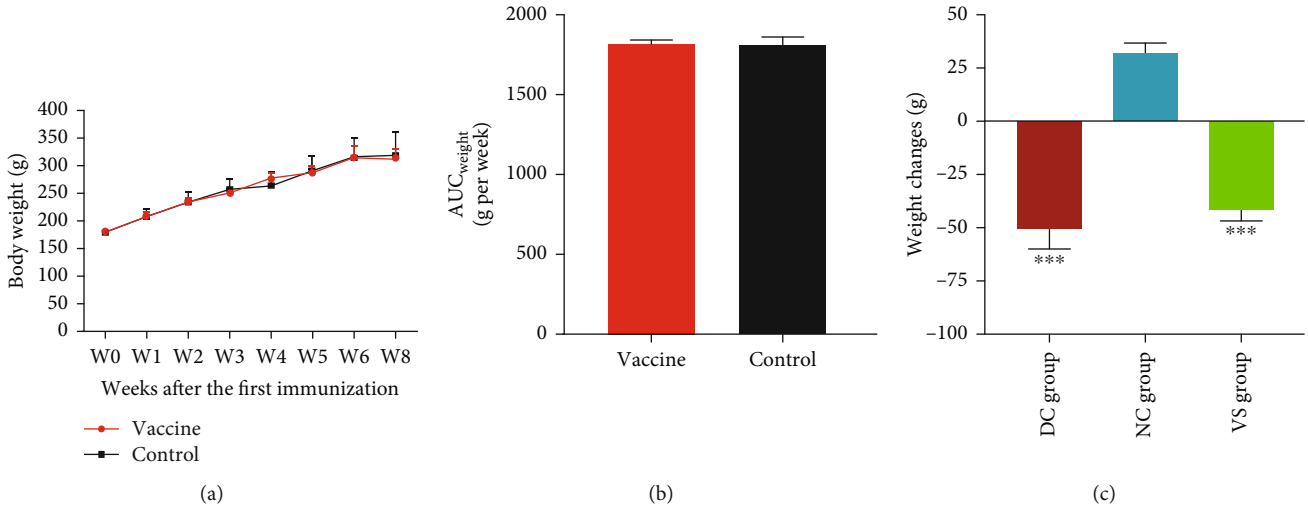


FIGURE 3: The body weight gain (a) and corresponding areas under the weight curve ($AUC_{weight}$) (b) in the vaccine-treated and nontreated control groups during the vaccination period, from week 0 (W0) to week 8 (W8). Data are expressed as the mean ± SD. (c) The body weight changes two weeks after STZ injection in the vaccinated STZ-injected (VS), diabetic control (DC), and the normal control (NC) rats, from week 8 to week 10. Data are expressed as the mean ± SEM. $^{***}p < 0.001$ compared to the NC group.

showing a direct and specific targeting of the plasma PCSK9 by liposomal vaccine-induced anti-PCSK9 antibody in rats. Additionally, the induced antibodies could markedly inhibit *in vitro* binding of PCSK9 to LDLR, showing the functionality of the induced antibodies. Of note, it was found that in the attendance of the vaccinated rat's plasma, *in vitro* interaction of murine PCSK9 and LDLR was significantly hindered by 30%, compared with the plasma sample of the control group (Figure 2(d)). In sum, the L-IFPTA vaccine could induce specific and functional antibodies that inhibit PCSK9-LDLR interaction through specific targeting of the plasma PCSK9 in rats.

*3.3. Body Weight Change.* The body weight changes during the vaccination period in the vaccine-treated (V) and nontreated control (C) groups were calculated by initial weight (W0) subtraction from the final weight of the animal at the time point of STZ injection (W8). The body weight was significantly raised in the V group (129.5 ± 8.87 g weight gain, $p < 0.001$) and the C group (138.5 ± 12.75 g weight gain, $p < 0.001$) (Figure 3(a)). Likewise, the integrated areas under the weight curve ($AUC_{weight}$) over the vaccination period were not statistically different ($p > 0.05$) in the V group (1812 ± 22.75 g) and the C group (1806 ± 48 g) (Figure 3(b)). After STZ injection, body weight gain significantly failed in the VS and DC groups but not in the NC group. Hence, during the two weeks after STZ injection, the body weights of the VS and DC groups had significantly dropped by -16.17 ± 6.3% (−50.56 ± 21 g, $p = 0.009$) and −14.35 ± 4% (−41.35 ± 10 g, $p = 0.01$), whereas the NC group showed a significant body weight gain by 10 ± 2.4% (31.85 ± 9.8 g, $p = 0.02$) (Figure 3(c)).

*3.4. Liposomal Anti-PCSK9 Vaccine Reduces FBG Levels.* Within one week following STZ injection, the FBG measurement indicated that the DC rats suffered a significant ($p < 0.0001$) hyperglycemia (351.7 ± 23.58 mg/dL, 95% CI: 301-402 mg/dL) compared to the NC group (87 ± 2 mg/dL, 95% CI: 82-91 mg/dL), verifying STZ-induced diabetes mellitus. Interestingly, there was no significant increase in the FBG levels in the VS group (180 ± 36 mg/dL, 95% CI: 94-266 mg/dL) in comparison to the NC group. The FBG level was 49% (−171.7 ± 35 mg/dL, $p < 0.001$) lower in the VS group versus the DC group (Figure 4(a) and Table 1).

*3.5. Liposomal Anti-PCSK9 Vaccine Improves Glucose Sensitivity.* To evaluate glucose sensitivity in the vaccinated STZ-injected rats (VS), OGTT was performed one week after STZ injection (W8). Oral glucose administration (2 g/kg) in the DC rats showed a significant elevation in the blood glucose levels (after 60 min) and exhibited that exogenous glucose administration significantly impaired glucose tolerance compared to the NC rats. The VS rats had significantly improved glucose tolerance ability compared to the DC rats. Further, the VS rats recorded a significant reduction in the level of blood glucose over a period of 180 min compared to the DC rats (Figure 4(b)). The integrated area under the glucose curve ($AUC_{glucose}$) over 180 min of the DC rats was significantly ($p < 0.0001$) higher than that of the NC rats. The measurement of AUC values demonstrated that blood glucose levels were significantly ($p = 0.007$) decreased by 34.5% in the VS rats compared to the DC rats (Figure 4(c)). In the VS rats, glucose levels after 60 min started a markedly decreasing trend to reach baseline levels at time 180, while in the DC group, a consistent level of glucose was indicated between 60 and 120 min. Although glucose levels had slowly dropped after 120 min in the DC rats, it did not reach baseline levels at 180 min (Figure 4(b)).

*3.6. Liposomal Anti-PCSK9 Vaccine Improves Insulin Sensitivity.* To measure the insulin sensitivity, an insulin challenge (0.8 U/kg, i.p.) was performed four days after the OGTT. Blood glucose concentration and $AUC_{glucose}$ in the DC group were significantly ($p < 0.0001$) higher at different

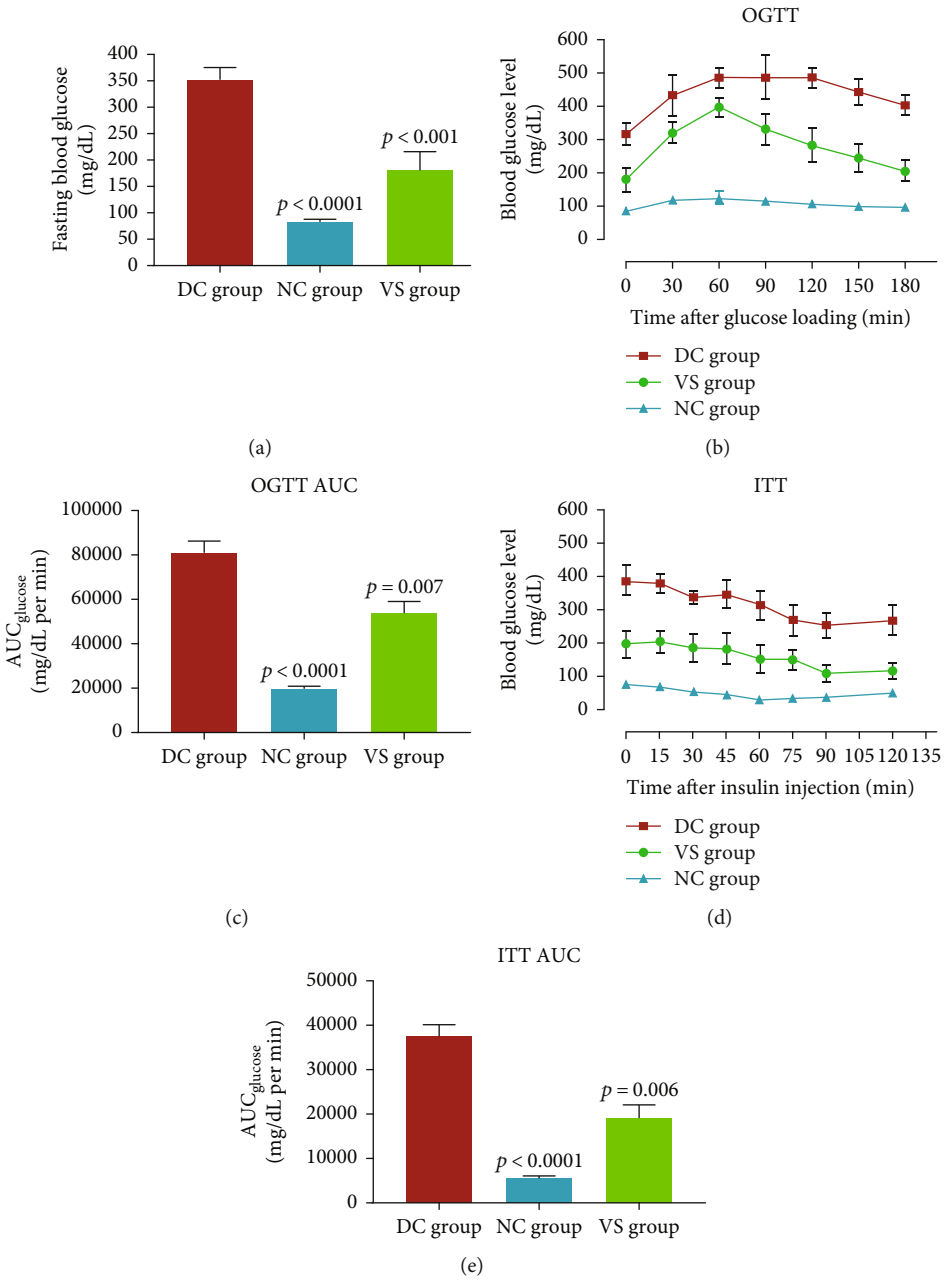


Figure 4: The levels of fasting blood glucose (FBG) in the vaccinated STZ-injected (VS), diabetic control (DC), and normal control (NC) rats during the week after STZ injection. Data are represented as the mean ± SEM. *** indicates $p < 0.0001$ compared to both the VS and NC groups (a). Oral glucose tolerance test (OGTT) (b) and corresponding areas under the glucose curve ($AUC_{glucose}$) over 180 min (c) following feeding (2 g/kg) of the vaccinated STZ-injected (VS), diabetic control (DC), and normal control (NC) rats with oral glucose. Data are represented as the mean ± SD. The mean values of $AUC_{glucose}$ in the VS and DC groups showed a significant difference ($p < 0.0001$) compared to the NC group. When compared to the DC group, the mean values of $AUC_{glucose}$ in the VS group were significantly ($p = 0.007$) different. The insulin tolerance test (ITT) (d) and corresponding areas under the glucose curve ($AUC_{glucose}$) over 120 min (e) following insulin administration (0.8 U/kg) in the vaccinated STZ-injected (VS), diabetic control (DC), and normal control (NC) rats. Data are represented as the mean ± SD. The mean values of $AUC_{glucose}$ in the VS and DC groups showed a significant difference ($p < 0.0001$) compared to the NC group. When compared to the DC group, the mean values of blood glucose levels and $AUC_{glucose}$ in the VS group were significantly ($p = 0.006$) lower.

time points after insulin administration compared to the NC rats. Blood glucose concentration and $AUC_{glucose}$ in the VS rats were significantly ($p = 0.006$) lower during ITT in comparison to the DC rats. Blood glucose concentrations in the VS rats were not significantly higher at 90 and 120 min after the insulin injection compared with the glucose levels at the corresponding time points in the NC rats (Figure 4(d)). As found by comparison of AUC values, blood glucose levels showed a 49.3% decrease in the VS group compared to the DC group (Figure 4(e)).

Table 1: Statistical analysis* of FBG values in the different experimental groups, one week after STZ injection.

| Groups | Mean 1 (mg/dL) | Mean 2 (mg/dL) | Mean difference | SE of difference | 95% CI of difference | $p$ value |
|---|---|---|---|---|---|---|
| DC vs. NC | 351.7 | 87 | 264.8 | 32.6 | 184.5 to 345 | <0.0001 |
| VS vs. NC | 180 | 87 | 93 | 37.8 | -186.4 to 0.2 | 0.0506 |
| VS vs. DC | 180 | 351.7 | -171.7 | 35 | 85 to 258 | <0.0001 |

*Statistical analysis was performed using one-way ANOVA and Tukey-Kramer's*post hoc* multiple comparison test. CI: confidence interval; DC: diabetic control group; FBG: fasting blood glucose; NC: normal control group; SE: standard error; VS: vaccinated STZ-injected group; vs.: versus.

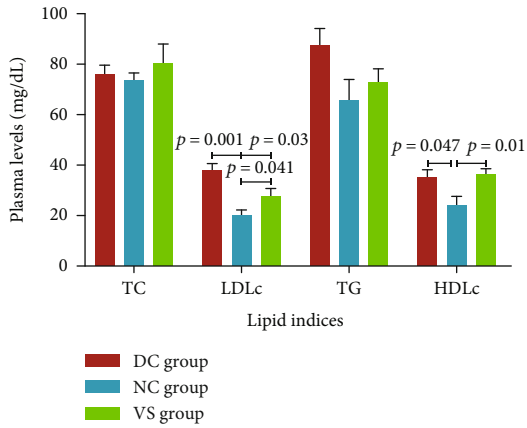


Figure 5: The plasma levels (mg/dL) of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) in the vaccinated STZ-injected rats (the VS group), diabetic control rats (the DC group), and normal control rats (the NC group) at the end of the study. $N = 8$. Data are expressed as the mean ± SEM.

*3.7. Liposomal Anti-PCSK9 Vaccine Reduces the Plasma LDL-C.* At the end of the experiment, the measurement of lipid indices displayed no significant difference in the plasma TC and TG between different groups. No difference was indicated in the plasma HDL-C between the DC and VS groups. A significantly and appreciably raised LDL-C (90%, $p = 0.001$) and HDL-C (45.8%, $p = 0.047$) were found in the DC rats compared to the NC rats. The VS rats showed appreciably lower (-26.65%, $p = 0.03$) plasma LDL-C levels than those in the DC rats. Compared to the NC group, the VS rats exhibited significantly higher plasma levels of LDL-C (39.4%, $p = 0.041$) and HDL (51.56%, $p = 0.01$) (Figure 5).

*3.8. The Relative Weights of the Pancreas and the Liver.* The relative organ weights were measured as (organ weight/body weight) × 100, at the last week of the experiment (W10). Relative pancreas weights were 0.424 ± 0.045, 0.28 ± 0.039, and 0.376 ± 0.017 g/100 g body weight in the VS, DC, and NC rats, respectively. The relative pancreas weight of the VS group was significantly higher (51 ± 14.6%, $p = 0.001$) than that of the DC group, while there was no significant difference when compared to the NC group. However, the relative pancreas weights of the DC group showed a 34 ± 23% ($p = 0.02$) decrease compared to the NC group. In the case of the liver, the relative weights were found to be 3.33 ± 0.42, 3.73 ± 0.25, and 4.01 ± 0.62 g/100 g body weight in the VS, DC, and NC rats, respectively. Statistical analysis demonstrated that the relative liver weights were not significantly different between all experimental groups.

*3.9. Pancreas Histopathology.* The histopathology alterations in pancreas were exhibited after H&E staining in all rats (Figure 6). Microscopic examination of pancreas sections demonstrated the normal morphology and proportion of exocrine acinar architecture and Langerhans islets without evidence of cellular degeneration and necrosis, in the NC rats. Pancreatic islets stained lighter than the surrounding acinar cells. The normal islet cells showed predominantly insulin-producing $\beta$-cells with granular basophilic cytoplasm and few eosinophilic glucagon-producing $\alpha$-cells. The acinar cells including pyramidal cells with apical acidophilic cytoplasm, which stained intensely, were settled in lobules with prominent basal nuclei (Figure 6(a)). In the DC rats, pathological alterations of both exocrine and endocrine compartments were observed. Swollen-acinar cells containing small vacuoles were noted. Interlobular ducts were lined by flattened epithelium. The pancreatic islets demonstrated a remarkably reduced population of basophilic $\beta$-cells and several eosinophilic $\alpha$-cells. The islets contained eosinophilic shapeless deposits, suggestive of cellular necrosis. The endocrine pancreas demonstrated areas of degeneration and necrosis among the endocrine cells comprising the islets of Langerhans (Figure 6(b)). The pancreatic islet of the VS rats showed a slightly diminished population of $\beta$-cells and only a few $\alpha$-cells. Again, cellular degeneration and necrosis were observed within the islets of Langerhans. Atrophic acinar cells were evident and the border between exocrine and endocrine compartments were notably less distinct. Overall, the VS pancreas exhibited a lesser intensity of eosin compared to the NC rats (Figure 6(c)).

*3.10. Liver Histopathology.* The H&E-stained slides of the liver in the NC, DC, and VS rats displayed the normal hepatic histological architecture consisting of hepatic lobules with a normal central vein. Each lobule was made up of radiating sheets, strands of polygonal hepatocytes forming a network around a central vein. Hepatocytes have well-defined cell borders with pink eosinophilic cytoplasm and mostly central single nuclei; inclusions were not found. There were no hemorrhagic areas or fibrosis evident (Figures 6(d)–6(f)).

## 4. Discussion

A direct association between DM and elevated risk of "atherosclerotic CV disease" has been documented [47, 48].

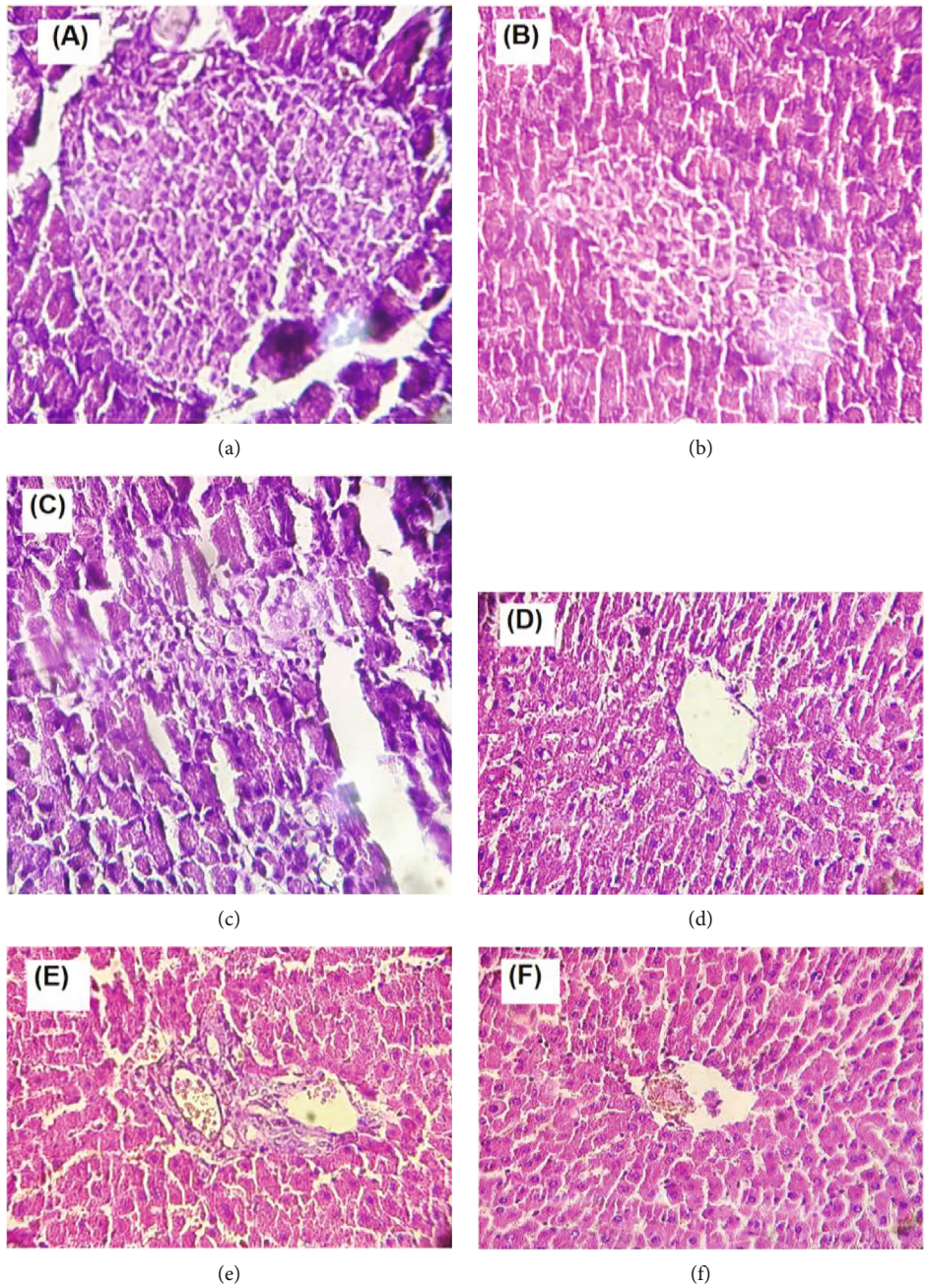


Figure 6: Histopathology of the pancreas in the normal control (NC) group (a), the diabetic control (DC) group (b), and the vaccinated STZ-injected (VS) group (c) at 400x magnification. Histopathology of the liver in the normal control (NC) group (d), the diabetic control (DC) group (e), and the vaccinated STZ-injected (VS) group (f) at 400x magnification.

The most established therapeutic target for managing diabetes-related CV complications is LDL-C [10]. PCSK9 inhibition is a safe and effective LDL-lowering approach. However, experimental and Mendelian randomization investigations demonstrated that genetic variants of PCSK9 manifesting reduced LDL-C levels are accompanied with increased FBG levels and an elevated chance of DM [34–36]. Hence, the safety and efficacy of PCSK9 inhibitors, especially those apart from mAbs, regarding the regulation of glycemic indices in diabetes require further investigations.

Here, we demonstrated for the first time the impact of PCSK9 inhibition *via* the vaccination approach on STZ-induced DM in diabetic rats. Interestingly, the results showed that prophylactic administration of the anti-PCSK9 vaccine can drop LDL-C levels and protect against the progression of STZ-induced diabetes, which was related to a significant improvement of glycemic indices including FBG, OGTT, and ITT, together with lower histopathological changes in the liver and the pancreas tissues. The liposomal anti-PCSK9 vaccine was shown to decrease the plasma

concentrations of functional free PCSK9 in diabetic rats through direct and specific targeting, which was associated with suppression of PCSK9/LDLR interaction, causing an alleviation of plasma LDL-C.

The LDL-lowering effect of the liposomal anti-PCSK9 vaccine has been also found in our other preclinical studies where the preventive [37, 38] and the therapeutic [39, 40] effects in hypercholesterolemic mice as well as the safety of the vaccine in healthy nonhuman primates [49] were evaluated. Such an effect has been similarly reported by the studies using other PCSK9 inhibiting vaccines, including the AFFITOPE®-based anti-PCSK9 vaccine [50, 51], a human recombinant protein-based anti-PCSK9 vaccine [52], and a vaccine comprised of virus-like particles displaying PCSK9 peptides [53]. Interestingly, the just-mentioned vaccine approaches significantly ameliorated hypercholesterolemia in mouse models, such as APOE∗3Leiden.CETP mice used by the AFFiRiS group for developing the AFFITOPE® vaccine [51].

STZ-induced diabetes is a model of DM characterized by hyperlipidemia and $\beta$-cell dysfunction, causing the insulin deficiency and subsequent hyperglycemia and loss of body weight in the experimental animals. In our study, markedly elevated blood glucose, as well as intemperate intake of food and water, were seen in STZ-treated rats (DC group), in comparison to the normal control rats (NC group). STZ-induced hyperglycemia was found to be inhibited in vaccinated rats (VS group). The FBG measurement revealed that the blood concentration of glucose in VS rats was not significantly different from the normal control, which was both markedly lower compared with DC rats. As shown by OGTT analysis, glucose tolerance was significantly impaired in the DC group compared to NC. The anti-PCSK9 vaccine protected VS rats against STZ-induced glucose intolerance and improved glucose sensitivity in the VS group when compared with the DC group. ITT assessment revealed that the insulin sensitivity in STZ-treated rats was profoundly decreased, and the PCSK9 vaccine inhibited such deficiency in VS rats, leading to the enhanced peripheral utilization of glucose *via* the anti-PCSK9 vaccination. Hence, STZ-treated rats on the insulin challenge did not show a significant drop in their blood glucose concentrations, underlying that these diabetic rats lost their peripheral insulin sensitivity and therefore could not use the exogenously administered insulin to decrease glucose concentrations. Such finding shows that the anti-PCSK9 vaccination can protect VS rats against STZ-induced insulin resistance.

Our findings can be supported by the Fourier trial [54] that showed the $HbA_{1c}$ and fasting blood glucose levels are comparable among patients with diabetes, prediabetes, or normoglycaemia treated either with evolocumab or placebo. Moreover, results from a comprehensive analysis of several independent phase 3 clinical trials including subjects without DM show that alirocumab exerts no significant effect on the incidence of DM, or on fasting blood glucose (FBG) and $HbA_{1c}$, in comparison to either ezetimibe or placebo after a 6–18-month follow-up period [55]. However, several Mendelian randomization studies have revealed that loss-of-function mutations in the *PCSK9* gene are correlated with lower LDL-C but higher plasma concentrations of fasting glucose and elevated risk of DM [34–36]. Besides, local deficiency—but not plasma levels—of PCSK9 has been shown to be responsible for overexpression of LDLR in pancreatic cells, which leads to increased intracellular cholesterol amount and $\beta$-cell damage [56]. These results suggest that anti-PCSK9 mAbs, which inhibit PCSK9 merely in the blood circulation, may exert no negative influence on the function of $\beta$-cells, while in the mentioned Mendelian study, the impact of global PCSK9 deficiency was evaluated.

Furthermore, weight loss is a hallmark of DM due to the destruction of structural proteins and muscle damage, which are complications of insulin deficiency. Lack of insulin-induced nutrient uptake promotes hyperphagia, while hyperglycemia induces polyuria and ensuing polydipsia. Although the anti-PCSK9 vaccine could protect the vaccinated rats against STZ-induced hyperglycemia and improved glucose hemostasis to the same extent as the control rats, the loss of body weight gain in the VS group was evident in a similar fashion to the DC group. Such contradictory effects of the vaccination on STZ-induced diabetes can be explained by the exacerbated lipolysis and the elevated lipid peroxidation leading to weight loss in STZ-treated rats [57].

In summary, the aforementioned findings suggest that LDL lowering *via* liposomal vaccine-induced anti-PCSK9 antibodies not only does not exert side effects on glycemic control but also can improve glycemic indices and insulin sensitivity in diabetic animals. The present findings call for additional studies in other experimental models of diabetes to confirm the positive impact of PCSK9 immunization on glycemic indices.

## Data Availability

Data are available from the corresponding author on reasonable request.

## Conflicts of Interest

Maciej Banach works for the speakers' bureau of Amgen, Herbapol, Kogen, KRKA, Polpharma, Mylan/Viatris, Novartis, Novo-Nordisk, Sanofi-Aventis, Teva, and Zentiva; is a consultant to Abbott Vascular, Amgen, Daichii Sankyo, Esperion, FreiaPharmaceuticals, Novartis, Polfarmex, and Sanofi-Aventis; and has received grants from Amgen, Mylan/Viatris, Sanofi, and Valeant. All other authors have no conflict of interest.

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