Evidence-Based Complementary and Alternative Medicine

Traditional Chinese Medicine in Ischemia-Reperfusion Injury

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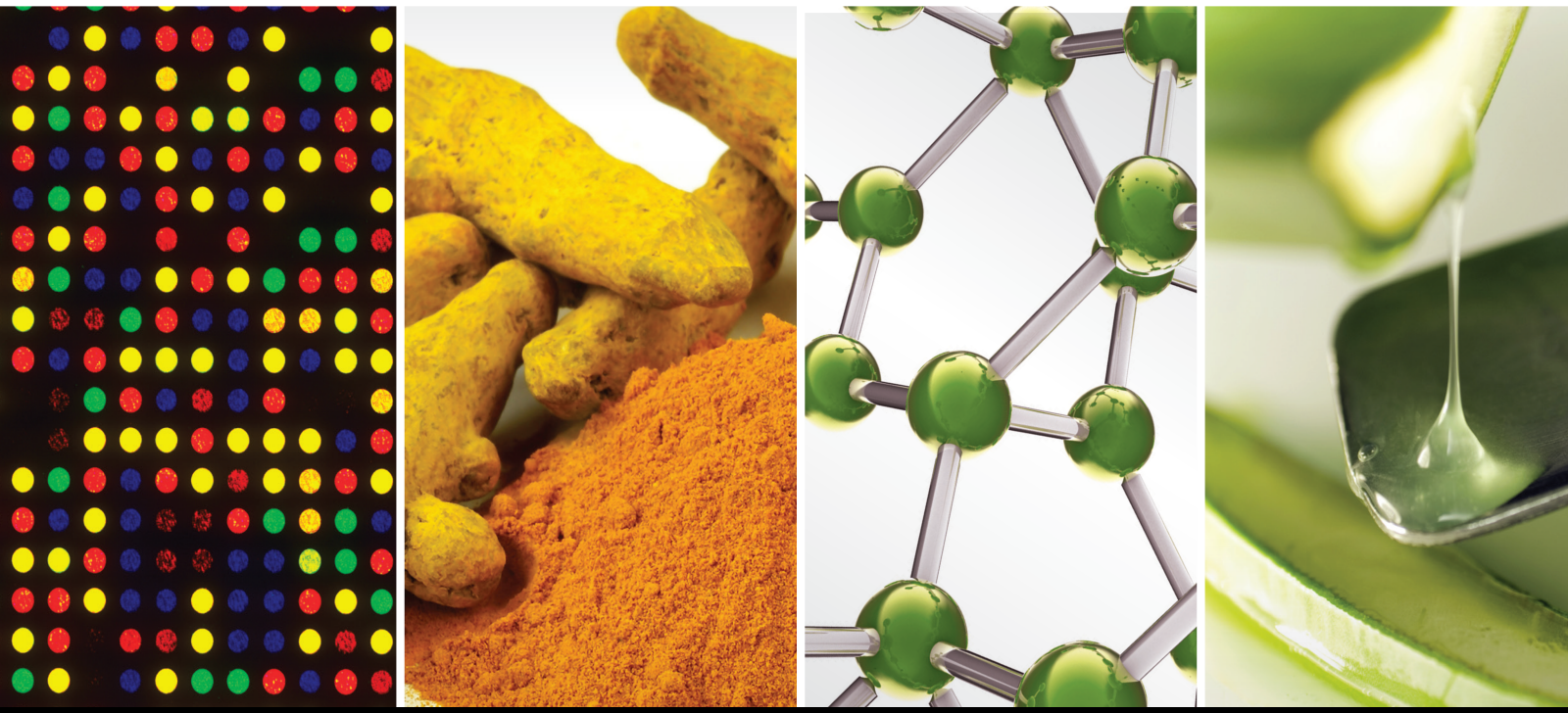

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Contents

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Research Article

Research Trends, Hot Spots, and Prospects for Traditional Chinese Medicine in the Field of Ischemia-Reperfusion Injury

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Ischemia-reperfusion (I/R) injury is one of the most common phenomena in ischemic disease or processes that causes progressive disability or even death. It has a major impact on global public health. Traditional Chinese medicine (TCM) has a long history of application in ischemic diseases and has significant clinical effect. Numerous studies have shown that the formulas or single herbs in TCM have specific roles in regulating oxidative stress, anti-inflammatory, inhibiting cell apoptosis, etc., in I/R injury. We used bibliometrics to quantitatively analyze the global output of publications on TCM in the field of I/R injury published in the period 2001–2021 to identify research hotspots and prospects. We included 446 related documents published in the Web of Science during 2001–2021. Visualization analysis revealed that the number of publications related to TCM in the field of I/R injury has increased year by year, reaching a peak in 2020. China is the country with the largest number of publications. Keywords and literature analyses demonstrated that neuroregeneration is likely one of the research hotspots and future directions of research in the field. Taken together, our findings suggest that although the inherent limitations of bibliometrics may affect the accuracy of the literature-based prediction of research hotspots, the results obtained from the included publications can provide a reference for the study of TCM in the field of I/R injury.

1. Introduction

Ischemia-reperfusion (I/R) refers to a pathological state in which the body organs such as the heart [1–3], kidney [4–6], and brain are reperfused and reoxygenated after the blood supply is restricted. Tissue damage caused by ischemia is the main cause of fatal diseases, such as stroke and myocardial infarction caused by coronary atherosclerosis [7]. During the rescue and treatment of ischemic diseases, medical scientists gradually discovered that the main factor that causes damage to tissues is not the ischemia itself but when the blood supply (reperfusion) is suddenly restored after a period of ischemia damage [8, 9]. In traumatic shock, surgery, organ transplantation, burns, frostbite, thrombosis, and other blood circulation disorders, post-ischemia-reperfusion injury will occur. After the blood supply is restored, excessive free radicals attack the cells in the tissues that have regained blood supply. In the ischemic tissues, the synthesis ability of antioxidant enzymes that can scavenge free radicals is hindered, which intensifies the damage of free radicals to the reperfusion tissue after ischemia. Meanwhile, inflammation, oxidative stress, and the accumulation of harmful substances could cause multiple organ damage and microcirculation disorders. This has been proven in many organs [10–13]. However, the current clinical consensus for the treatment of ischemia such as cerebral ischemia is still to use thrombolytic therapy to restore blood supply as soon as possible and reduce the degree of tissue ischemia. Therefore, I/R injuries cannot be avoided. Yet there is a clinical lack of drugs to alleviate ischemia-reperfusion injury. For more than three thousand years, traditional Chinese medicine (TCM) has been widely used in Asian countries, especially China, Japan, and Korea,

for I/R injuries. In China, many old and authoritative medical books, such as "*Treatise on Cold Damage* (*Shang Han Lun*)," "*Synopsis of the Golden Chamber* (*Jin Kui Yao Lue*)," "*Important Prescriptions Worth a Thousand Gold for Emergency* (*Qian Jin Fang*)," and "*Correction on Errors in Medical Works* (*Yi Lin Gai Cuo*)," recorded many classical formulas, including Chaihu Jia Longgu Muli Tang, Guizhi Fuling Wan, Huanglian Jiedu Tang, Longdan Xiegan Tang, Shengmai San, and Buyang Huanwu Tang because of the remarkable curative effect of TCM in the treatment of I/R, it has received extensive attention from researchers during its development. Therefore, the research results in the field of TCM for the treatment of I/R have been fruitful. Our research team has been committed to research on TCM. In recent years, we have focused on the treatment of I/R with TCM. We are eager to describe the development of TCM for I/R scientifically, objectively, and quantitatively, so that we can better understand how TCM treats I/R and carry out more in-depth and extensive research. We found that bibliometric analysis can help us achieve this goal. Bibliometric is a widely used method by researchers for quantitative analysis of a specific research field to provide an overview of the research trends encountered in that field. After using mathematical and statistical methods, it generates a broad picture of the field, offering an inner structure pattern. It can reveal the quality, thematic and citation landscape of the literature in the field [14]. Through cocitation analysis and citation burst detection, topic clusters, influential contributors, productive institutions, etc., it can offer an accurate description that provides an insight that is beyond the superficial. Meanwhile, benefiting from the development of visualization software, such as VOSviewer and CiteSpace, which is more objective and rigorous and can provide the co-occurrence network diagram.

In this study, we retrieved and collected the research literature on TCM in I/R injury from the Web of Science Core Collection and used VOSviewer and CiteSpace to analyze the literature. The aim of this bibliometric study was to provide a comprehensive overview and inner structure research of TCM in I/R injury. Our work evaluated the efficacy of TCM for any kind of I/R injury. Moreover, innovative approaches such as document cocitation network analysis, research clusters identification and analysis, and reference citation bursts detection were performed to offer insights into the research topics and trends evaluation over time from different perspectives. Our analyses will shed new light for investigators, which is helpful for them to plan and manage their scientific work in future research.

2. Materials and Methodology

2.1. Materials. Literature data of this bibliometrics research were retrieved from the Web of Science Core Collection. Web of Science Core Collection contains several important index types, including Science Citation Index Expanded (SCIE), Social Science Citation Index (SSCI), and Emerging Sources Citation Index (ESCI). In order to make a systematic analysis of I/R injury, we chose citing articles and reviews to make a visual analysis. Search for two terms was carried out, one was "traditional Chinese medicine or Chinese medicinal herbs or traditional Chinese drugs or TCM" and the other was "ischemia-reperfusion injury or ischemia/reperfusion or ischemia reperfusion or ischemia-reperfusion or Ischemia-reperfusion or Ischemic-reperfusion or ODG/R." The document type was set as article or review, the language as English, and the time span as 2001–2021. The search was completed on May 7, 2021.

After checking, the 612 documents were retrieved from the core collections of the Web of Science. We screened and inspected it with an excel table. Through analyzing the titles, abstracts, and even some full-texts, we removed the documents whose content had nothing to do with the treatment of I/R injury with traditional Chinese medicine. Finally, the remaining 446 documents were used for further bibliometric analysis.

2.2. Methodology. Bibliometric analysis and network visualization were performed with VOSviewer (version 1.6.14, developed by Nees Jan van Eck and Ludo Waltman, the Netherlands) and CiteSpace (version 5.6.R4, developed by Chen Chaomei, England). Microsoft Excel 2010 was used to perform the distribution of publication years. Gunn map (http://lert.co.nz/map/) online world map was used to make the distribution of countries and regions.

3. Results

3.1. Distribution of Publications by Year and Organ. We got a total of 612 documents, excluding the duplicates and irrelevant ones, leaving 446. The number of documents in each year is shown in Figure 1. As shown in the figure, the literature on I/R injury from 2001 to 2020 has an overall upward trend, reaching the highest in 2020 with a total of 73 articles published. The TCM on I/R injury is attracting more and more attention from investigators, indicating that it will gradually become a research hotspot even into the future. We have performed a statistical analysis of the I/R injury research of different organs involved in the literature. Except for 10 reviews that cannot be clearly divided, the statistics on the number of documents on each organ are shown in Figure 2. It is clear that the research of I/R injury is mainly cerebral I/R injury, followed by heart and myocardial I/R injury.

3.2. Countries and Regions. According to statistical analysis, a total of 446 documents have been published by research groups from 26 countries and regions. We used the total number of citations of documents in each country and region to make a graph that is convenient for viewing, as shown in Figure 3. In addition, we have counted the top 10 countries and regions with the most published documents, as shown in Table 1. It can be seen from the chart that Chinese research on the treatment of I/R injury with TCM is far ahead in terms of the number of publications and the number of citations. It is worth noting that although the second-ranked United States only published 27 documents between 2001 and 2021, its citations reached 462, with an average citation rate of 5.84%, which is higher than the Chinese figure.

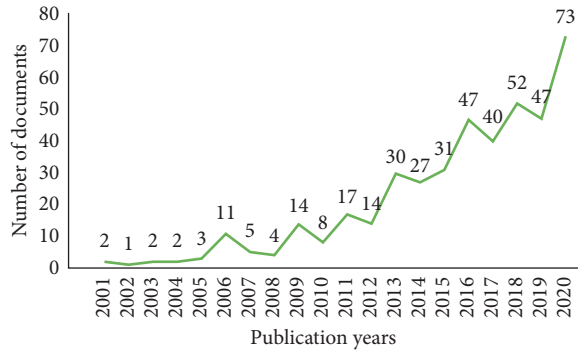
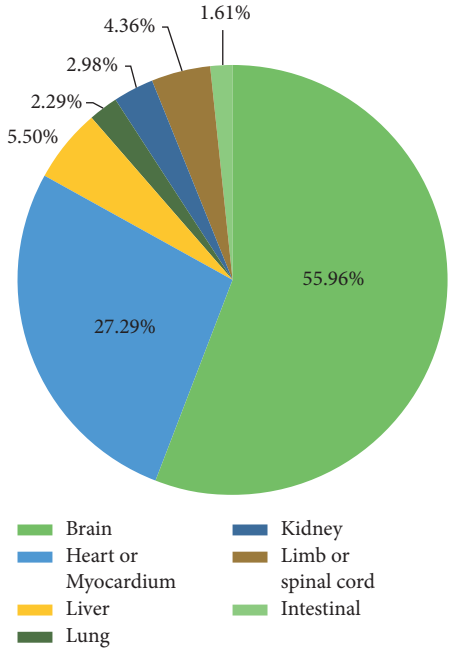


Figure 1: Distribution of publication years from 2001 to 2020.



Organs	Documents
Brain	244
Heart or Myocardium	119
Liver	24
Lung	10
Kidney	13
Limb or spinal cord	19
Intestinal	7

Figure 2: Number of documents of I/R injury in different organs.

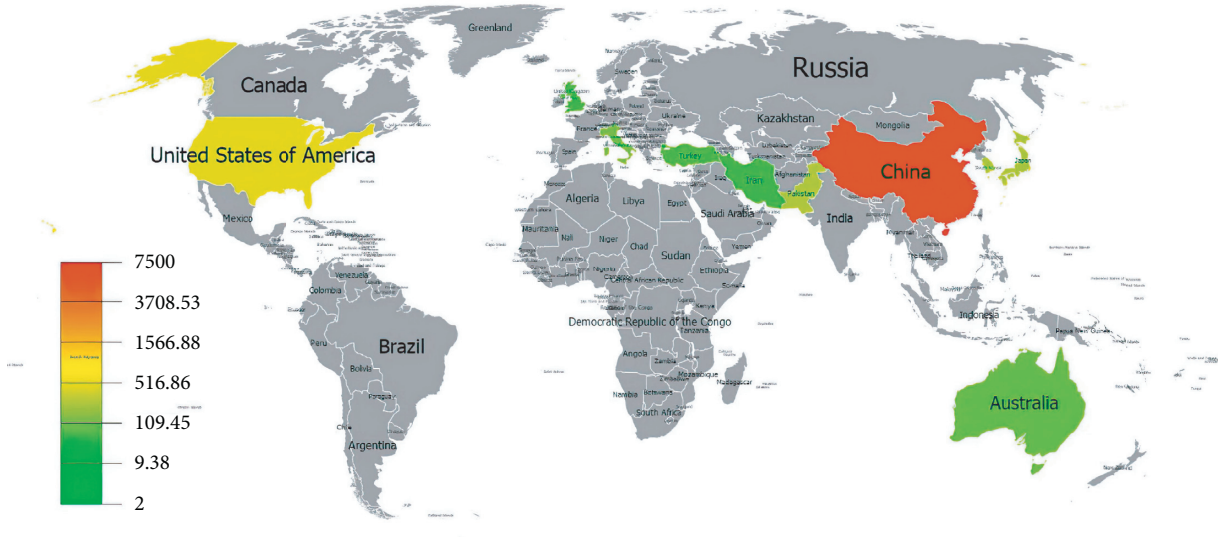


Figure 3: Spatial distribution of the citations of global publications.

Table 1: Top 10 most productive countries/regions with publications on TCM in I/R injury.

Rank	Country/region	Documents	Citations	Total link strength
1st	China	424	7463	52
2nd	USA	27	462	37
3rd	South Korea	9	234	3
4th	Japan	7	228	5
5th	Australia	5	90	6
6th	Italy	4	115	14
7th	England	4	52	4
8th	Pakistan	2	254	4
9th	Iran	2	56	10
10th	Turkey	2	64	10

3.3. Organizations. According to VOSviewer analysis, 446 documents were published by 478 different organizations. After excluding 105 disjointed organizations, the network diagram of the remaining 373 organizations is shown in Figure 4. At the same time, according to the number of output documents, we also list the top ten institutions, as shown in Table 2. From the chart, the top ten institutions are all from China, which shows that China's research on the treatment of I/R injury with TCM is dominant. Among them, Beijing University of Chinese Medicine is the most prolific institution ($n = 26$), and China Pharmaceutical University is the most cited institution ($n = 494$). In addition, the average publication year of Beijing University of TCM is 2017, and the corresponding node color is orange, indicating that Beijing University of TCM has the highest document output rate in 2017.

3.4. Journals. By analyzing the source of publications, it helps to find core journals. Based on data analysis, documents related to TCM in I/R injury published from 2001 to 2021 were distributed in 141 different journals. Excluding 30 journals unrelated to other journals, the relationship network diagram of the remaining 111 journals is shown in Figure 5. Meanwhile, the top ten journals and their 2020 impact factors are shown in Table 3. It can be seen from Table 3 that the *Journal of Ethnopharmacology* has the most publications on TCM in I/R injury, including 61 documents. The 2020 impact factor of the *Journal of Ethnopharmacology* is 3.69. Although *Pharmacological Research* has an impact factor of 5.893 in 2020, it has just recorded 8 documents. So, judging from the number of publications and impact factor of journals, the *Journal of Ethnopharmacology* may be the most influential journal on the treatment of I/R injury with TCM.

3.5. Authors. A total of 2621 authors were joined in 446 documents. According to the author's literature number and the number of citations, we can get the core authors in the field of TCM in I/R injury. The evaluation criteria of core authors included the number of published documents and total citations. Table 4 lists the specific parameters, and the author relationship is shown in Figure 6. Zhu Yan and Yu Boyang both have 10 documents, while the number of citations of Yu Boyang ($n = 215$) is higher than that of Zhu Yan ($n = 196$). In addition, from the visualization map in Figure 4, we can observe that the two authors are not related. After comparison, it is found that Zhu Yan has 3 documents related to the brain and 7 documents related to the heart and myocardium, while Yu and Boyang have 7 documents related to the brain and 3 documents related to the heart and myocardium. It can be concluded that Zhu Yan and his team are mainly dedicated to the study of heart and myocardial I/R injuries, and Yu Boyang and their team are mainly dedicated to the study of cerebral I/R injuries. In addition, there are many emerging research teams involved in the emergence of I/R injuries, indicating that I/R injuries are still a hot spot.

3.6. Keywords. Keywords are the core vocabulary of an article and appear frequently. Searching and analyzing based on keywords is a quick and direct way to understand this article. There are a total of 2311 keywords in the 446 documents. After selecting the first 200 words, excluding words such as "ischemia-reperfusion injury," which would obviously be high-frequency words, the remaining words network visualization map shows the co-occurrence relations as shown in Figure 7. The size of the circle indicates the occurrence of keywords. In addition, we used Table 5 to list the top 10 keywords. As shown in Table 5, the high-frequency keywords are apoptosis, oxidative stress, and stroke, indicating that the molecular mechanisms of stroke in I/R injury treated with TCM are a hotspot. At the same time, we used CiteSpace to make a burst map, as shown in Figure 8, strength represents the intensity of the burst, and the red bar represents the duration of the hot spot. Among them, the intensity value of nerve regeneration is the highest, but the burst time is shorter, and it was only a research hotspot in 2013–2014. According to the figure, in the past three years, the protection of ischemic stroke, cerebral infarction, and other cardiovascular and cerebrovascular diseases has been the current research hotspot of TMC in the treatment of I/R injury.

3.7. Citations. According to the literature citation analysis of documents, which reflects the number of citation times of the literature, the following observations are drawn: Among the 446 documents, "*Pinocembrin: A Novel Natural Compound with Versatile Pharmacological and Biological Activities*"

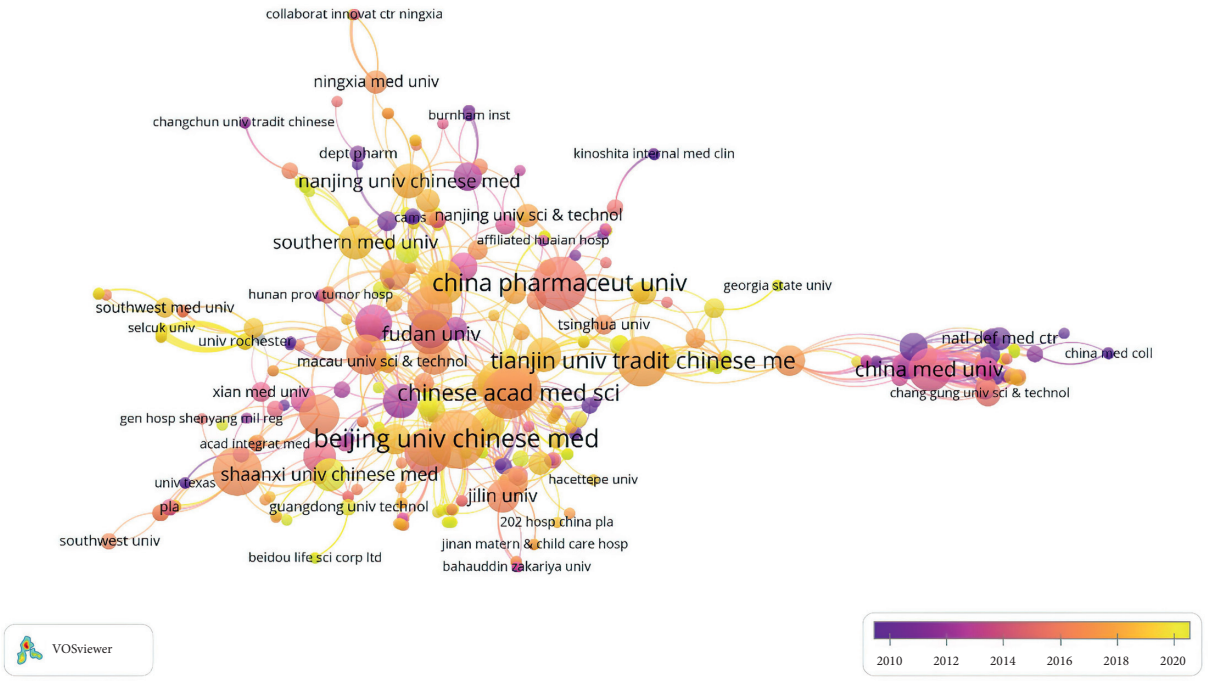


Figure 4: Visualization of organized documents data.

Table 2: Top 10 most productive organizations.

Rank	Organizations	Documents	Citations	Total link strength
1st	Beijing University of Chinese Medicine	26	310	35
2nd	China Pharmaceutical University	22	494	17
3rd	Chinese Academy of Medical Sciences	19	310	57
4th	Tianjin University of Traditional Chinese Medicine	19	270	28
5th	Fourth Military Medical University	18	242	23
6th	Capital Medical University	16	412	32
7th	Peking Union Medical College	15	265	48
8th	China Academy of Chinese Medical Sciences	15	175	32
9th	Shanghai University of Traditional Chinese Medicine	15	232	21
10th	Zhejiang University	15	167	21

ranked first, published by Rasul and Azhar in 2013 and cited 142 times. However, in our visual analysis process, we found that this document does not form a network with other articles. That is to say, although this document has the highest number of citations, other documents and this document are not mutually cited, so we eliminated it. Table 6 lists the top 10 documents with the remaining number of citations. The mutual network diagram of the remaining documents is shown in Figure 9. It can be seen from the chart that the "*Neuroprotective role of Z-ligustilide against forebrain ischemic injury in ICR mice*" published by Kuang X in 2006 is the most cited document on the net. The second and third places are "*Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats*" published by Kao TK in 2006 and "*Tetramethylpyrazine reduces ischemic brain injury in rats*" published by Liao SL in 2004. Obviously, these three articles are documents related to TCM and cerebral I/R injury and all have a certain relationship with neuroprotection, which further confirms that the treatment of cerebral I/R injury by TCM through neuroprotection is the main concern.

4. Discussions

During the preceding two decades covered by this research, the number of annual publications increased gradually and reached a peak in 2020. The curve indicated that TCM on I/R injury has drawn more and more attention, and it will continue to be a research hotspot. Moreover, according to the data analysis of document types, we found that most of them were original articles, and only a few were reviews. The reason behind this phenomenon is that there is a continuing requirement for novel investigation at this stage.

Taken together, 2311 keywords were retrieved from all of the documents. The statistical analysis of keywords suggests that we need to regard the statistical results of word frequency of keywords as a net structure, as shown in Figure 7. According to the statistical results of key molecules, apoptosis is the most common form of cell death, indicating that apoptosis has received more attention from researchers. Furthermore, the number of studies on other mechanisms (including oxidative stress, inflammation, and

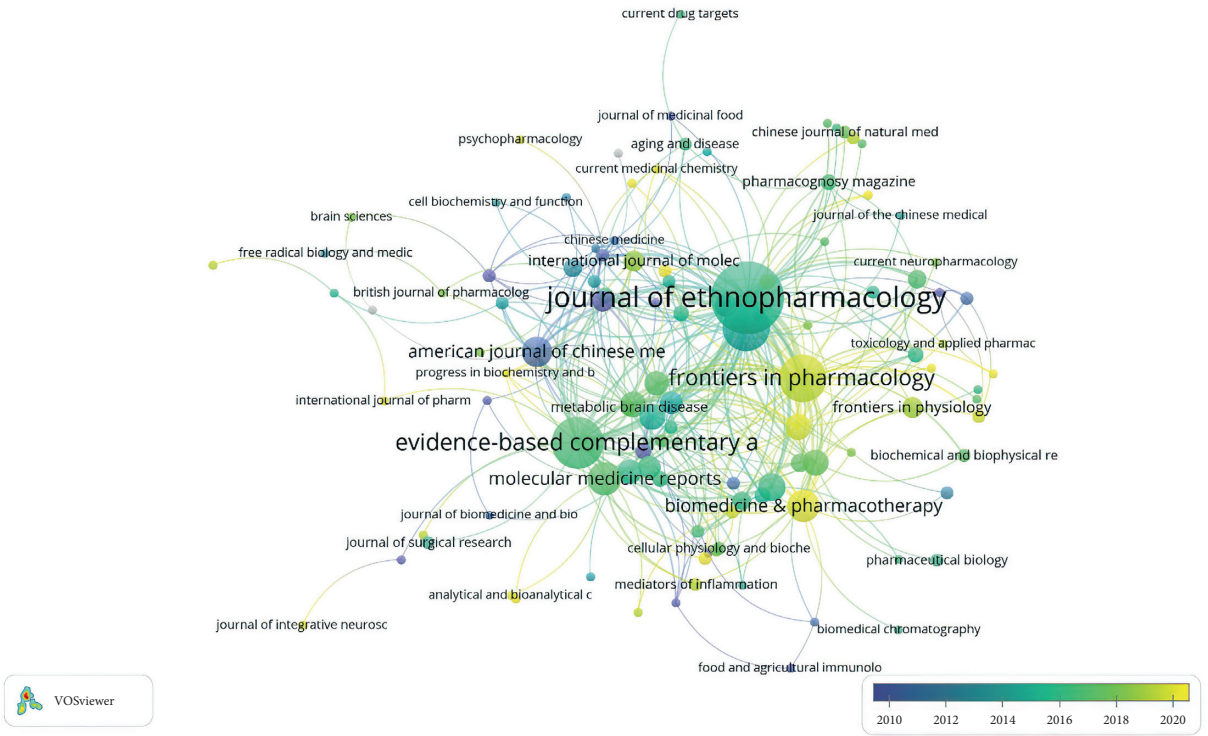


Figure 5: Visualization of journals documents data.

Table 3: Top 10 most productive journals.

Rank	Journals	Documents	2020 impact factor
1st	Journal of Ethnopharmacology	61	3.69
2nd	Evidence-Based Complementary and Alternative Medicine	31	1.813
3rd	Frontiers in Pharmacology	26	4.225
4th	Neural Regeneration Research	26	3.171
5th	Molecular Medicine Reports	13	2.1
6th	Biomedicine and Pharmacotherapy	12	4.545
7th	Am J Chinese Med	11	3.498
8th	BMC Complementary and Alternative Medicine	9	2.833
9th	Pharmacological Research	8	5.893
10th	PLOS One	8	2.74

Table 4: Top 10 most productive authors.

Rank	Authors	Documents	Citations
1st	Yu Boyang	10	215
2nd	Zhu Yan	10	196
3rd	Wang Yong	9	96
4th	Zhang Qian	8	112
5th	Kou Junping	8	174
6th	Zheng Guoqing	7	117
7th	Li Chun	6	76
8th	Wang Wei	6	80
9th	Fan Guanwei	6	129
10th	Li Fang	6	58

neuroprotection) and stroke ranked high is greater than those on other types, and the total link strength is also high. From the perspective of bibliometrics, this phenomenon shows that these molecules or diseases are frequently studied as research subjects. In addition, we listed the major molecular mechanisms of some classic single herbs and formulas in Table 7. These results can be used as a reference when investigators select their starting point in TCM on I/R injury.

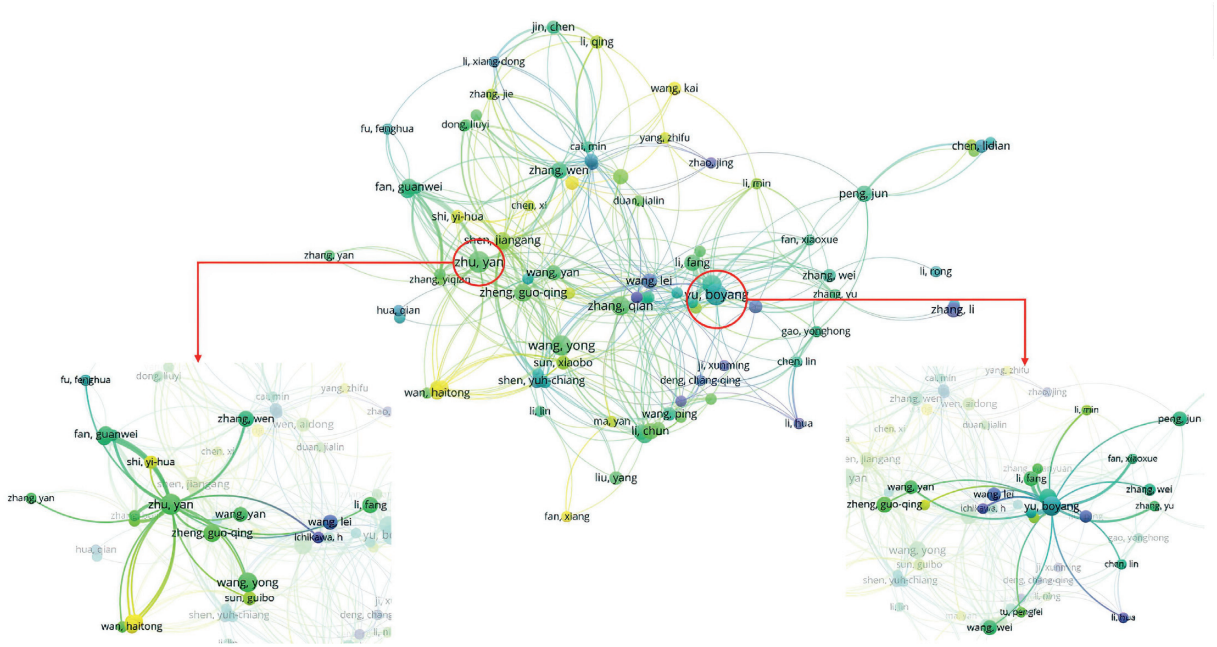


Figure 6: Visualization of authors documents data.

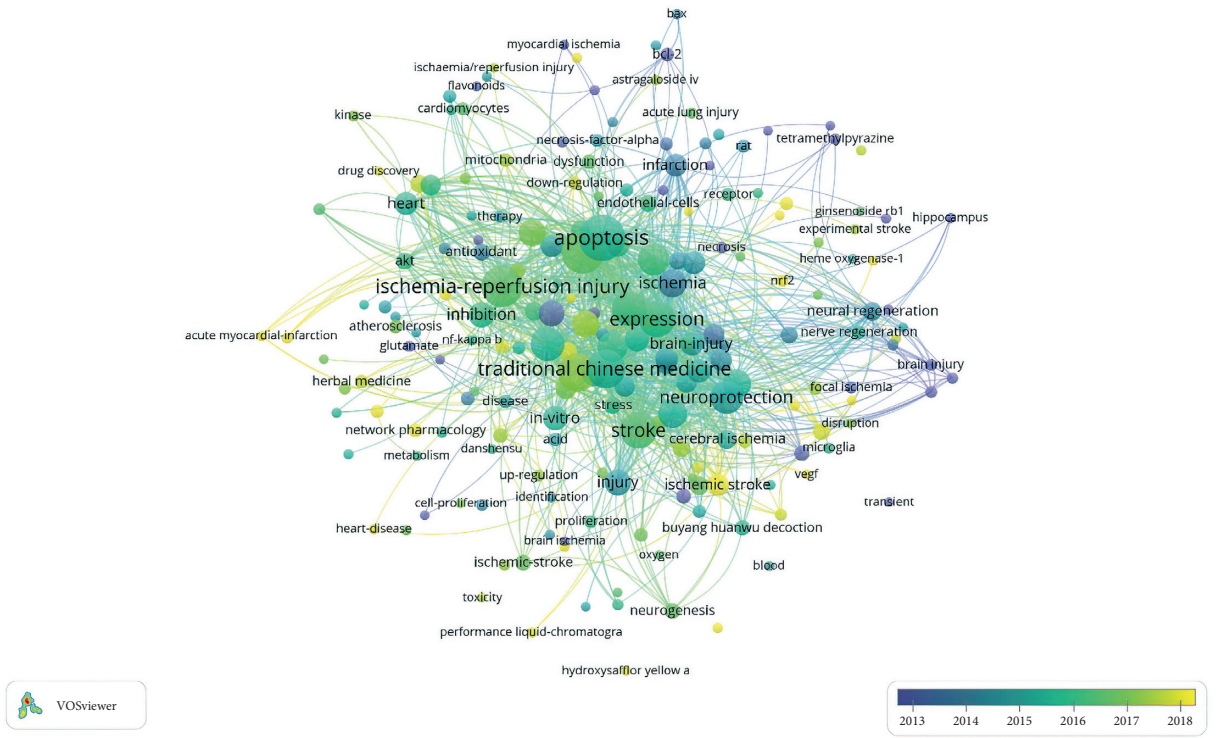


Figure 7: Visualization of keywords data.

By analyzing the distribution of publication sources, judging from the number of publications and the impact factor of journals, *The Journal of Ethnopharmacology* might be the most influential journal. As we know, *the Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, animals, and microorganisms and their biological and pharmacological effects. This journal is very friendly to research articles on ethnic medicine including prescription. Ranked second is *Evidence-Based Complementary and Alternative Medicine*, the referred journal is an international, peer-reviewed journal that seeks to apply scientific rigor to the study of complementary and alternative medicine modalities, particularly traditional Asian healing systems. Precisely, TCM has been used for a long time in

Table 5: Top 10 most occurring words.

Rank	Keywords	Occurrences	Total link strength
1st	Apoptosis	114	835
2nd	Oxidative stress	95	681
3rd	Stroke	71	497
4th	Expression	69	509
5th	Activation	60	435
6th	Neuroprotection	57	460
7th	Inflammation	53	364
8th	Mechanisms	47	345
9th	Brain	45	336
10th	Artery occlusion	43	341

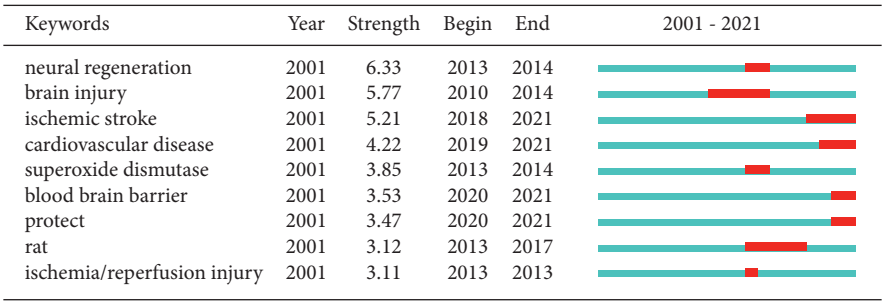


Figure 8: Top 9 keywords with the strongest citation bursts.

Table 6: Top 10 most cited documents.

Rank	Title (year)	Citations
1st	Neuroprotective role of Z-ligustilide against forebrain ischemic injury in ICR mice (2006)	130
2nd	Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats (2006)	120
3rd	Tetramethylpyrazine reduces ischemic brain injury in rats (2004)	103
4th	Ameliorating effects of traditional Chinese medicine preparation, Chinese materia medica and active compounds on ischemia/reperfusion-induced cerebral microcirculatory disturbances and neuron damage (2015)	96
5th	Neuroprotective effect of morroniside on focal cerebral ischemia in rats (2010)	79
6th	Pharmacological actions and therapeutic applications of salvia miltiorrhiza depside salt and its active components (2012)	70
7th	Geniposide prevents hypoxia/reoxygenation-induced apoptosis in H9c2 cells: improvement of mitochondrial dysfunction and activation of GLP-1R and the PI3K/AKT signaling pathway (2016)	68
8th	Anti-aging implications of astragalus membranaceus (huangqi): a well-known Chinese tonic (2017)	67
9th	Total saponins of *Panax notoginseng* modulate the expression of caspases and attenuate apoptosis in rats following focal cerebral ischemia-reperfusion (2009)	66
10th	Neuroprotective herbs for stroke therapy in traditional eastern medicine (2005)	64

Asia, with a very strong history of traditional application and distinctive features, which is very much in line with the taste of the journal. Tied for third is *Neural Regeneration Research* and *Frontiers in Pharmacology*. *Neural Regeneration Research* mainly focuses on neurological research. Compared with *the Journal of Ethnopharmacology* and *Evidence-Based Complementary and Alternative Medicine*, it is more targeted for research related to stroke and other neurological injury diseases, while *Frontiers in Pharmacology* is more comprehensive and includes the latest research in pharmacology. Grasping and paying attention to the core journals in this field will not only help us quickly understand the research progress in this field. On the other hand, we can also consider submitting articles to these journals for our own research results in this field.

As we know, TCM has its own unique theory. In the theory of TCM, I/R injury is attributed to *Qi* and blood deficiency, blood stasis syndrome, toxic heat (Chinese name: *Redu*) accumulation, and wind moving within the liver (Chinese name: *Ganfeng Neidong*). According to our quantitative analysis, as shown in Figure 10, by sorting out the types of TCM, the research on the treatment of I/R injury with TCM mainly focuses on the following categories:

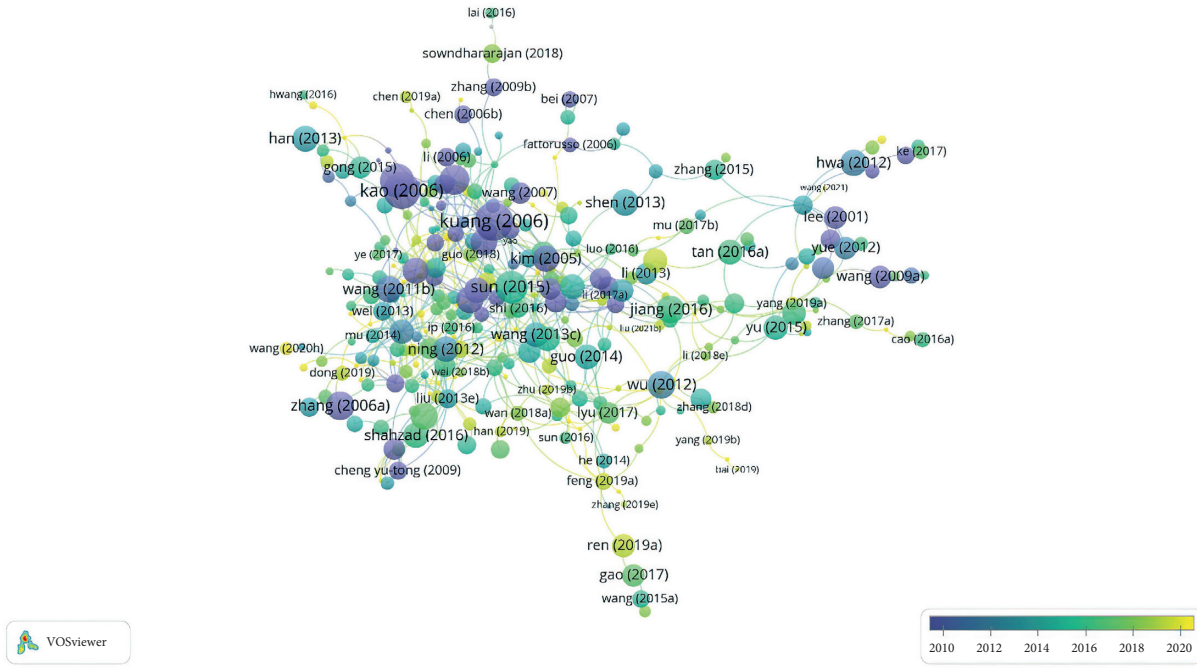


Figure 9: Visualization of citations data.

Table 7: Examples of typical mechanisms of TCM against I/R injury.

Mechanism	TCM	Model	Specific mechanism	Ref.
Antioxidant stress	Xueshuantong injection	MCAO	Nrf2, HO-1, and NQO1↑, activate the Nrf2-VEGF pathway to promote angiogenesis and antioxidant effects	[15]
	Salvia	MCAO	By scavenging free radical activity	[16]
Anti-inflammatory	Guizhi-Fuling capsules	MCAO	IL-1β and TNF-α↓, IL-10 and IL-10R↑	[17]
	Qingkailing	OGD/R	TNF-α,COX-2, iNOS, and p-p38↓	[18]
	Tongxinluo	MCAO	Effectively protects ischemia-reperfusion injury through the Cx43/calpain II/bax/caspase-3 pathway and reduces cell death	[19]
	Tongxinluo	MCAO	Mediated by activating the PI3K/Akt pathway	[20]
Antiapoptosis	Emodin	OGD/R	Induces the expression of Bcl-2 and GLT-1 through the ERK-1/2 signaling pathway, inhibits neuronal apoptosis and reactive oxygen generation, and reduces glutamate toxicity	[21]
	Qingda granule	OGD/R	lncRNA GAS5, bax, caspase-3↓, miR-137, and Bcl-2 ↓	[22]
Neuroprotection/neuroregeneration	Shouwu Yizhi decoction	MCAO	By upregulating the expression of miR210 induced by VEGFA, the notch pathway is activated to promote angiogenesis	[23]
	Huatuo Zaizao pill	MCAO	BDNF↑, improves the neurogenesis level of cerebral ischemic animals	[24]

(1) Tonic Chinese medicine. As we all know, *Panax ginseng* C. A. Mey. (Chinese name: *Renshen*), *Astragalus* membranaceus (Chinese name: *Huangqi*), *Salvia miltiorrhiza* Bge. (Chinese name: *Danshen*), etc., are classic and effective tonics in Chinese medicine. Liang et al. found that Danshen can improve liver I/R injury through antioxidation, promote microcirculation, and reduce Kupffer cell activation [25]. In a rat MCAO model treated with *Astragalus* injection, it was found that *Astragalus* injection can downregulate the expression of the JNK3 gene after cerebral I/R injury in rats, inhibit neuronal apoptosis, reduce infarct volume, and improve neurobehavioral function [26]. Researchers used the H9C2 cardiomyocyte hypoxia/glucose reoxygenation model to explore the effect of ginseng polysaccharides on myocardial I/R injury. The results showed that ginseng polysaccharides can maintain the function of myocardial mitochondria, thereby inhibiting myocardial ischemia. Cell apoptosis caused by reperfusion also increases the expression of glucocorticoid receptors and estrogen receptors, which in turn mediates the activation of the RISK pathway and the endothelial nitric oxide synthase-

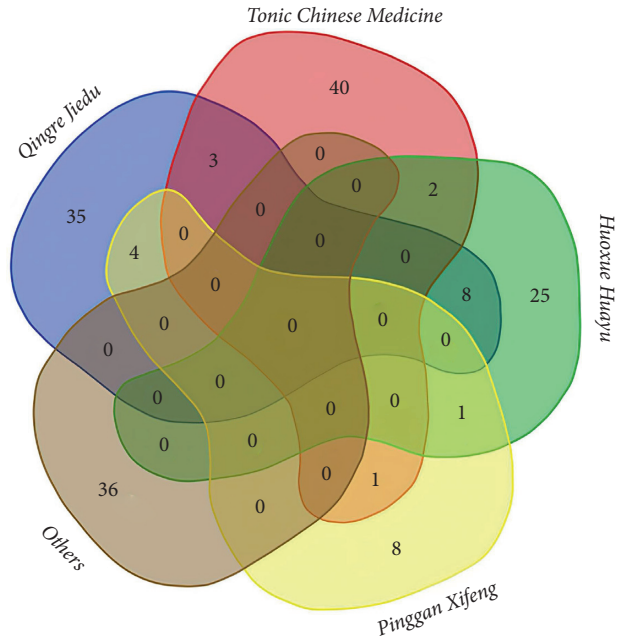


Figure 10: Classification of five major types of Chinese medicine.

dependent mechanism to resist reperfusion injury [27].

(2) Activating blood and removing blood stasis (Chinese name: *Huoxue Huayu*). TCM for promoting blood circulation and removing blood stasis is widely used in vascular diseases, almost all prescriptions contained. Studies have found that this type of Chinese medicine has miraculous effects. *Panax notoginseng* (Burk.) Chen (Chinese name: *Sanqi*), as a typical TCM for promoting blood circulation and removing blood stasis, studies found that *Panax notoginseng* saponins can directly downregulate the overexpression of proinflammatory factors like interleukin-1β and tumor necrosis factor-α, and at the same time upregulate the expression of anti-inflammatory factors like interleukin-10 in the core area of cerebral infarction, thereby preventing permanent nerve damage in rats after MCAO. At the same time, the researchers also combined with the SH-SY5Y cell model of OGD/R injury and found that notoginseng total saponins can also provide neuroprotection by inhibiting the overexpression of *NgR1*, *RhoA*, and *ROCK* [28, 29].

(3) Antipyretic and antidote (Chinese name: *Qingre Jiedu*). As early as the Tang Dynasty, Sun Simiao's "*Qianjin Yifang*" was recorded on the treatment of strokes with heat clearing and detoxifying methods. Modern studies have shown that the treatment of antipyretics and antidotes has significant advantages over the control group in terms of improving neurological function and anti-inflammatory effects. In addition, studies showed that geniposide may inhibit H/R-induced cardiomyocyte apoptosis by activating *GLP-1R* and *PI3K/AKT* signaling pathways and reversing mitochondrial dysfunction [30].

(4) To calm the liver and relieve wind (Chinese name: *Pinggan Xifeng*). The famous document "*Su wen*" recorded that "*all winds are dizzy, all belong to the liver.*" In the theory of TCM, many endovascular diseases such as stroke and hypertension are caused by the hyperactivity of *Ganyang shangkang*. Studies showed that the phenolic substances in Gastrodia elata Bl. (Chinese name: *Tianma*) can stimulate the endogenous antioxidant response of astrocytes and neurons and promote reactive astrocytes to provide antioxidants and nerves to neurons, thereby reducing neuronal damage [31].

There are many forms of TCM for the treatment of I/R injuries. Clinically, it is mainly used for single herbs and prescriptions. To learn more about the most researched single herbs and prescriptions, we listed the top ten most frequently prescribed single herbs in Table 8. According to the statistics, *Danshen* and *Buyang Huanwu Decotion* ranked first in single herbs and prescriptions, respectively. As we know, *Danshen* is a classical medicine for activating blood and removing blood stasis and belongs to the *Huoxue Huayu* category. *Buyang Huanwu Decotion* is recorded in "*Correction on Errors in Medical Works* (*Yi Lin Gai Cuo*)"in the *Qing* Dynasty. It has a miraculous effect on the clinical sequelae of cerebrovascular accidents, as well as hemiplegia and paraplegia caused by other reasons. *Buyang Huanwu Decotion* is mainly composed of *Huangqi*, *Chuangxiong*, *Honghua*, etc., *Huoxue Huayu* category medicines. This concept is consistent with the current clinical concept of restoring blood supply as soon as possible for ischemic diseases. We further disassembled the prescription and analyzed it together with the single herbs and divided it according to the theory of TCM. As shown in Table 9, the *Qingre Jiedu* is the most widely used, followed by *tonic*

Table 8: Top ten single herb in the literature.

Sort	Chinese name	Latin name	Documents
1	Danshen	*Salviae miltiorrhizae* radix et rhizoma	21
2	Renshen	*Ginseng* radix et rhizoma	16
3	Huangqin	*Scutellariae* radix	12
4	Honghua	*Carthami* flos	9
5	Chuanxiaong	*Chuanxiong* rhizoma	7
5	Gegen	*Puerariae lobatae* radix	7
5	Sanqi	*Notoginseng* radix et rhizoma	7
6	Wuweizi	*Schisandrae chinensis* fructus	5
7	Danggui	*Angelicae sinensis* radix	4
7	Huangqi	*Astragali* radix	4

Table 9: Top ten prescriptions in literature.

Sort	Compound name	Compound components	Documents
1	Buyang Huanwu decoction	Huangqi, Danggui, Chishao, Dilong, Chuanxiong, Honghua, Taoren	15
2	Tongxinluo	Renshen, Shuizhi, Quanxie, Chishao, Chantui, Tubiechong, Wugong, Tanxiang, Jiangxiang, Ruxiang, Suanzaoren, Bingpian	13
3	Danhong injection	Danshen, Honghua	8
4	Huanglian Jiedu decoction	Huanglian, Huangqin, Huangbo, Zhizi	6
5	Shenfu injection	Hongshen, Heishunpian	4
5	Shengmai san	Renshen, Maidong, Wuweizi	4
6	Taohong Siwu soup	Danggui, Shudi, Chuanxiong, Baishao, Taoren, Honghua, Niuhuang, Shuiniujiao, Shexiang, Zhenzhu, Zhusha	3
6	Angong Niuhuang wan	Xionghuang, Huanglian, Huangqin, Zhizi, Yujin, Bingian	3
6	Gualou Guizhi soup	Gualou, Guizhi, Baishao, Gancao, Shengjiang, Dazao	3
6	Yiqi Fumai injection	Hongshen, Maidong, Wuweizi	3

Chinese medicine. The abovementioned results indicate that the first consideration for TCM in the treatment of ischemic diseases is also to restore blood supply. However, while supplying blood, the use of *Qingre Jiedu* medicines can reduce the toxic and side effects caused by reperfusion. In addition, *tonic Chinese medicine* could comprehensively strengthen the body's resistance through multiple channels and multiple targets working together to achieve curative effects. This is also the reason for the remarkable curative effect and charm of TCM.

5. Conclusions

This study is the first bibliometric study using visual analysis software to analyze publications of TCM in the treatment of I/R injury. Through system statistics and summary, we found TCM has a significant effect on I/R injury. Furthermore, this research field has received widespread attention from researchers around the world. However, how to scientifically and rationally evaluate the efficacy of TCM in the treatment of I/R injury, clarify its specific mechanism, and how to deeply explore the advantages of TCM in the treatment of I/R injury are the key issues that need to be strengthened in the future. Overall, this study provides insight into the trends and characteristics of TCM in the treatment of I/R injury, and should provide a helpful reference for further in-depth research.

Abbreviations

I/R: Ischemia-reperfusion
TCM: Traditional Chinese medicine.

Data Availability

The 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.

Authors' Contributions

Yu Sun, Yuan Cai, and Man Xiao contributed equally to this work. YS and YC designed and wrote the manuscript and revised the manuscript. MX and MHH were in charge of statistical analysis. GQS, BBS, and HL participated in visual analysis. YMP and PHL participated in study design and data collection. All the authors revised and approved the final manuscript.

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Supplementary Materials

Graphical abstract to describe the frame and elaborate the theme of the article Figure S1. (*Supplementary Materials*)

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Review Article

Ischemia-Reperfusion Injury in Peripheral Artery Disease and Traditional Chinese Medicine Treatment

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Peripheral artery disease (PAD) is a serious public health issue, characterized by circulation disorder of the lower extreme that reduces the physical activity of the lower extremity muscle. The artery narrowed by atherosclerotic lesions initiates limb ischemia. In the progression of treatment, reperfusion injury is still inevitable. Ischemia-reperfusion injury induced by PAD is responsible for hypoxia and nutrient deficiency. PAD triggers hindlimb ischemia and reperfusion (I/R) cycles through various mechanisms, mainly including mitochondrial dysfunction and inflammation. Alternatively, mitochondrial dysfunction plays a central role. The I/R injury may cause cells' injury and even death. However, the mechanism of I/R injury and the way of cell damage or death are still unclear. We review the pathophysiology of I/R injury, which is majorly about mitochondrial dysfunction. Then, we focus on the cell damage and death during I/R injury. Further comprehension of the progress of I/R will help identify biomarkers for diagnosis and therapeutic targets to PAD. In addition, traditional Chinese medicine has played an important role in the treatment of I/R injury, and we will make a brief introduction.

1. Introduction

Peripheral artery disease (PAD) is defined as the obstructing or narrowing of the arteries of low extremities due to atherosclerotic plaque, subsequently restricting or blocking blood flow to the affected lower extreme. The PAD is characterized by the reduced oxygen and energy delivery to lower limbs, resulting in exertional leg pain that limits the ability of walking, which would be resolved through rest. If limb ischemia is severe, it may cause pain on rest or amputation [1]. Since atherosclerosis is a systemic disease, a portion of patients with PAD will have heart or cerebrovascular disease [2]. The most risk factors of PAD are consistency with myocardial infarction and stroke, which indicated that PAD is an independent risk factor [3, 4]. Patients with short-distance claudication or severe ischemia undergo revascularization to restore blood, which prevents limb pain at rest and limb amputation. Nevertheless, PAD is still a serious health hazard problem with significant morbidity and mortality. Further understanding of physiopathology needs further research to improve therapeutic strategies.

Ischemia-reperfusion (I/R) is characterized as the reduction of blood supply to the tissue or organ, which subsequently leads to vascular restoration and concomitant reoxygenation of downstream tissue [5]. The restriction of oxygen supply leads to insufficient metabolic supply, causing tissue hypoxia. Contrary to expectations, restoration of blood and oxygen is associated with aggravation of injury and promotion of inflammation. The pathophysiology of I/R injury is various (Figure 1). Mitochondrial dysfunction can reduce energy supply and oxidative stress, and inflammation

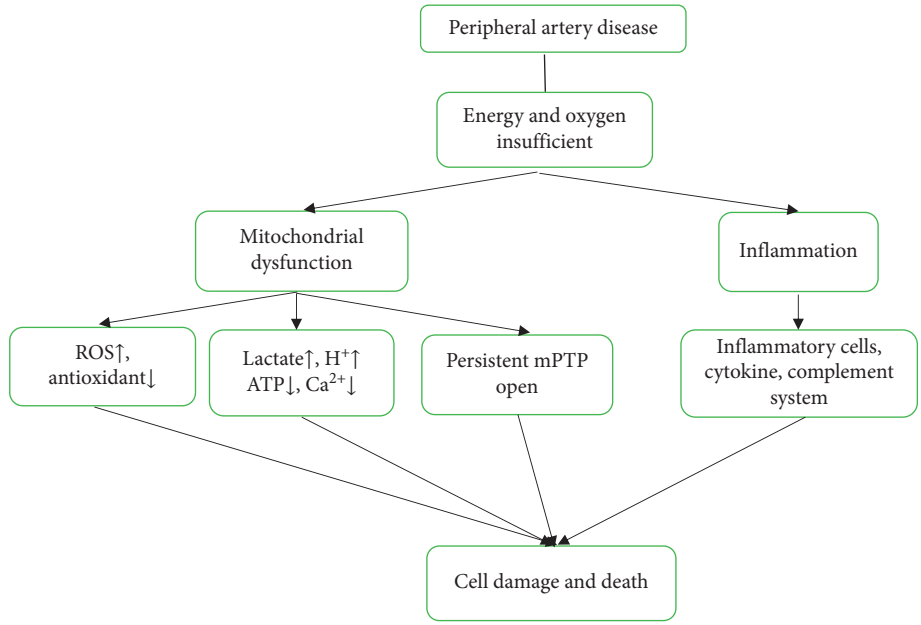

Figure 1: The pathogenesis of PAD. PAD is characterized by reduced oxygen and energy delivery to lower limbs, and undergoing revascularization would restore blood. However, this surgery would induce I/R injury. The mechanisms of I/R injury are multifactor, mainly consisting of mitochondrial dysfunction and inflammation.

may result in intermittent claudication, limb pain at rest, and amputation. Reactive oxygen species (ROS) derived radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (-OH), hypochlorous acid (HOCI), and nitric oxide-derived peroxynitrite. ROS are potent oxidizing property, causing cell membrane damage by lipid peroxidation, which is responsible for local and systemic damage caused by I/R injury. In addition, inflammation plays an important role in I/R injury. Depending on the degree and the duration of ischemia of the affected organ, it can trigger remote complications such as the heart and kidney [6, 7].

Traditional Chinese medicine (TCM) has been used in the treatment of various diseases for more than 2000 years. Several studies have shown that TCM can be used in the treatment of ischemia and I/R injury through different mechanisms, including regulation of energy metabolism, inhibition of antioxidants, and reduction of inflammatory cytokines. This review covers the main mechanisms of skeletal I/R injury, and the application of TCM in I/R therapy is introduced, which may provide a theoretical basis and novel idea for dealing with I/R injury of PAD.

2. Mitochondrial Dysfunction

Mitochondria participate in multiple physiological functions, including energy metabolism, Ca^{2+} signal, cell differentiation, and apoptosis [8–10]. During recent years, many studies have described the mitochondrial functions in I/R injury. The reduction of blood supply causes insufficiency of oxygen and affects the function of the electron transport chain. Skeletal muscle is energy dependent, mainly provided by mitochondrial metabolism. With increased ATP turnover, the skeletal muscle transforms from rest to activity, and the substrates of energy production can be oxidized [11]. The I/R injury of the lower limb affects the local muscle environment through various processes, resulting in the reduction of muscle function . The sensibilities of different muscles are discrepant due to their antioxidant capacities [12, 13]. PAD-induced I/R injury causes myopathic and neuropathic changes [14], which may also impair the function of mitochondria [15, 16].

ATP is mainly produced by the oxidative phosphorlation process in resting myocyte. Sestrin2 functions as a scaffold protein, which interacts with OXPHOS components to keep mitochondrial integrity under I/R stress [17]. The producing substrates of energy include phosphate compounds, glucose, glycogen, and lipids in mitochondria. In addition, mitochondria are the major source of ROS. Both complexes I and III of the mitochondrial respiratory chain can produce ROS. The main reactive species are superoxide anion and nitric oxide (NO), subsequently forming secondary reactive species, such as H_2O_2 and peroxynitrite [18]. Therefore, the mitochondria play an important part in the skeletal muscle fiber physiology, both in energetic metabolism for energy supply and cell signaling.

Ischemia in the lower extremity restricts the nutrient and oxygen supply, leading to a mass of ionic and metabolic changes. Since oxygen is lacking, mitochondrial OXPHOS can be affected, and the potential of the mitochondrial membrane decreased [19]. The activities of the electron transport chain (complexes I, II, and IV) are changed during ischemia [20, 21], which lead to reduced synthesis of ATP and elevation in concentrations of inorganic phosphate and adenine nucleotide [22, 23]. In the progression of ischemia, ATP is catabolized into xanthine and hypoxanthine; subsequently, the substrates conduce to ROS production during

the progression of reperfusion [22, 24]. For continuously providing energy, anaerobic metabolism and phosphocreatine pathways are activated to generate ATP. ATP, phosphocreatine, and glycogen exhaust within 7 hours, which correlates with skeletal muscle death [23, 25–28]. These changes of metabolism cause accumulation of H^+, nicotinamide adenine dinucleotide (NAD), and lactate, which make extra- and intracellular acidified. [6, 29–32]. Then, the Na^+/H^+ exchanger is activated to recover H^+. Various ionic exchangers of the sarcolemma are restrained by low ATP, including Na^+-K^+-ATPases and Ca^{2+}-ATPases. And Na^+-Ca^{2+} antiporters are reversed to recover the Na^+ concentration, subsequently resulting in the accumulated concentration of Ca^{2+} [6, 33]. The accumulation of Ca^{2+} causes damage of cell integrity by degrading lysozymes, proteases, and nucleases and induces inflammation and cell death through necrosis and apoptosis [34, 35].

ROS are one of the main factors responsible for local and systemic damage in the progress of I/R injury. ROS include O_2^-, H_2O_2, -OH, HOCI, and NO-derived peroxynitrite. During the progress of ischemia, ROS are mainly produced by complexes I and III of the mitochondrial respiratory chain [32, 36, 37]. Several enzymes play an important role in the production of ROS, such as the xanthine oxidase (XO) system, NADPH oxidase (NOX) system, and nitric oxide synthase (NOS) system.

XO system includes XO and xanthine dehydrogenase (XDH). XO plays a primary role in ROS production, which localizes in macrovascular endothelial cells of the skeletal muscle. In the ischemic state, XDH is converted into XO because of the low ATP level, and XO may induce the production of ROS during the conversion of hypoxanthine to uric acid. NOX enzymes promote the production of superoxide and hydrogen peroxide, and NOS uncoupling leads to the generation of ROS; both processes may induce I/R injury. These ROS damage membranes, including those of the mitochondria. Damaged mitochondrial membranes lead to the release of caspases and activation of apoptosis. In addition, hypoxia increased the activity of NOS forming NO, which reacts with superoxide to give peroxynitrite that damages nucleic acids and lipids. Meanwhile, defense systems can reduce ROS-induced damage, which include catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione, coenzyme Q, and vitamin E. However, in the PAD muscle, matrix SOD has been demonstrated to be deficient, which is the initial line of ROS defense in mitochondria.

Sufficient oxygen supply in the reperfusion progression is the primary reason of myocyte death via generating excessive quantities of ROS. Production of ROS in mitochondria is a self-amplified process. This process is hard to eliminate as the antioxidant defenses are also changed by ischemia. Meanwhile, ischemia and reperfusion can further affect the activity of mitochondrial complexes I, II, III, and IV, which affects the membrane channel and increases cytosolic Ca^{2+} concentration [24, 37, 38]. Elevated Ca^{2+} concentration stimulates proteases and phospholipases, which affect membrane receptors, ion channels, and enzymes, leading to cell membrane degradation and decreasing cell survival rate [39, 40]. Furthermore, osmotically active molecules accumulate and recover in cells generating an osmotic gradient within intra- and extracellular environments, which causes cells to water uptake, swelling, and break up [23]. It was reported that improving mitochondrial quality control is critical to improve the effectiveness of current treatments in PAD such as exercise [41]. Therefore, alleviation of oxidative stress may be a useful strategy to deal with I/R injury. And inhibition of the XO system, NOX system, or NOS system may be a feasible method. In addition, increasing endogenous antioxidants can directly regulate ROS, which may alleviate I/R injury.

In order to maintain normal physiological function, mitochondria are constantly changing dynamically, which is called mitochondrial dynamics. Mitochondria dynamics involves mitochondrial fusion, fission, and autophagy, which plays an important role in maintaining cellular physiological function and hemostasis. Studies indicated that mitochondrial dynamics changed during I/R injury. Mitochondrial fusion helps mitigate stress by mixing partially damaged mitochondria. And fission is needful to create new mitochondria. However, during overloaded cellular stress in some diseases, including I/R injury, fission may facilitate apoptosis [42].

The mitochondrial permeability transition pore (mPTP) is located in the inner mitochondrial membrane. The mPTP is a nonselective multiprotein channel, which can be regulated by various cell factors, such as ROS, ATP, inorganic phosphate, pH, Ca^{2+}, and membrane potential. The biochemical changes during I/R injury can turn up the mPTP. The persistent mPTP opening deregulates the release of matrix Ca^{2+}, restricts OXPHOS, swells the matrix, and eventually ruptures the outer membrane by the release of apoptotic proteins and cell death. Meanwhile, the opening of mPTP can promote the production of ROS.

3. Inflammation

I/R injury is associated with the activation of inflammation and immune system. The characteristic of reperfusion injury is immune responses, including natural antibody recognition of neoantigens and activation of the complement system. I/R induced by PAD occurs in a sterile environment, which has been termed sterile inflammation. Sterile inflammtion shares similar response to those ivoked by microorganism.. The sterile immune response, through pattern recognition molecules such as toll-like receptors (TLRs), activates immune cells. Ligand binding to TLRs activates downstream signaling pathways, subsequently inducing the generation of proinflammatory cytokines and chemokines [43]. During I/R, with the cell damage and death, endogenous molecules can activate these receptors. And ligands are termed damage-associated molecular patterns (DAMPs). DAMPs are normally located in intracellular, they will release to extracellular at the time of tissue damage [44, 45]. The function of DAMPs is that they activate immune response, restrict harmful immune response, and promote tissue integrity [46, 47]. TLR4 is one of the famous pattern recognition receptors, which mediates inflammation through its activation by lipopolysaccharide.

Oxidative stress can enhance the activation of TLR4 [48]. Deletion of TLR4 is hyporesponsive to lipopolysaccharide [49]. Antagonists for TLR4 or regulators which reduce TLR4 expression may be a useful treatment.

During I/R, accumulation of inflammatory cells has been found. These inflammatory cells include monocytes, dendritic cells, and granulocytes [50–53]. The role of inflammatory cells is not fully studied. They may activate inflammation and accelerate tissue injury or restrict the recovery of injury [54].

The benefited function of inflammatory cells depends on their production. For example, dendritic cells may produce inflammatory cytokine interleukin-10 (IL-10) [55, 56]. They can downregulate the expression of tumor necrosis factor-α (TNF-α), IL-6, and ROS. Nearly, all inflammatory cells express NADPH oxidase contributing to format ROS and peroxynitrite. Peroxynitrite may induce oxidative DNA injury and activate nuclear enzyme poly (ADP-ribose) polymerase-1 (RARP-1). Granulocytes are involved in tissue repair. Howerver, if they are accumulated enough, they may lead to uncontrolled inflammation and tissue injury [57]. In addition, I/R injury inducesadaptive immune response, which involves various T lymphocytes. The function of T lymphocytes needs further research in PAD-induced I/R.

The complement system is a biological cascade and promotes clear pathogens from the organism. It acts as an immune surveillance system, which can discriminate healthy host tissue, apoptotic cells, foreign intruders, and cellular debris [58]. In the progress of I/R injury, the complement system is activated. It was confirmed that ischemia upregulates the expression of the antigen on cellar surfaces, which binds to the IgM natural antibody. Natural antibodies are a major component of B1 cells, which produce IgM and IgG [59]. Antigen-antibody complex causes C1 binding, complement activation, and formation of C3a and C3b. Subsequently, C3b activates a complement cascade causing to form a membrane attack complex (MAC). The MAC can stimulate macrophages to release prostaglandin E2, and neutrophils release ROS, IL-1, etc [59–61]. Studies showed that inhibiting the component of the complement could be an effective treatment of I/R injury, but it needs further verification [62–64].

Platelet aggregated excessively and platelet-derived mediators aggravate injury during I/R. Endothelial interactions activate platelets [65]. Subsequently, the platelets transport to the sites of injury. In addition, I/R promotes coagulation [17]. It was reported that several anticoagulants can inhibit clot formation [66, 67], such as tissue factor inhibitor, protein C, and antithrombin heparin. Besides, cytokines are factors that transmit signals between cells and include various and numerous families of polypeptide regulators. They can play a role in immunomodulating. It was verified that IL-1, IL-6, thromboxane A2, and necrosis factor are referred to I/R injury.

In conclusion, inflammation is important progression, which may cause cell damage and repair. It inhibits the activation of the complement system and reduces proinflammatory cytokines; chemokines are a potential therapeutic strategy to reduce tissue damage, induced by I/R injury.

4. Cell Damage and Death in I/R Injury

I/R injury-induced tissue injury includes two portions: ischemia injury and reperfusion injury. When ischemia progresses, metabolites accumulated, and metabolic acidosis occurred. If the blood supply is restored, the increased inflammation and ROS production aggregated injury. If the damage is slight, the function of cells may activate the recovery system to maintain their function and survival. However, if the injury is severe, cells will die through the apoptotic or necrotic pathway [68]. Different ways of cell death through various pathogenesis (Figure 2).

I/R induces cell death via various mechanisms, including necrosis, necroptosis, apoptosis, and autophagy [65]. Necrosis is characterized as cell and organelle swelling [69]; subsequently, the surface membranes ruptured, and intracellular contents spilled out [65]. Necrotic cells induce intensive immune stimulation, which lead to inflammatory cell infiltration and cytokine release. If the cells encounter excessive stress, necrosis occurred [70]. The progression of necrosis induces serious changes in the external environment, which are induced by chemical, biological, or physical injury. Necrosis is usually considered to be random and uncontrolled processes, in which the cell responses to overwhelming stress. Necroptosis is termed to be programmed necrosis [71]. It occurs in pathologic states, especially I/R injury. Necroptosis shares similar features with necrosis. Necroptosis is activated by cell stress or death receptors, such as TNF receptor-1 and Fas receptor. The combination of death receptors and ligands leads to mobilization and activation of a group of receptor-interacting protein kinases (RIKs). RIP1 and RIP3 are members of the receptor-interacting protein kinase (RIPK) family. The formation of the necrotic complex between RIP1 and RIP3 can mediate caspase-independent cell necrosis [72, 73]. Overexpressed RIP3 may induce upexpression of both ROS and Ca^{2+} and enhance NF-κB protein regulation [74]. Low-expressed RIP3 may suppress apoptosis [69, 75]. The activation of RIP3 occurs in TNF-induced necroptosis. There is an association between necroptosis and inflammation in the pathogenesis of I/R injury. So, the research on the association may be useful to understand the mechanism and provide guidance for treatment.

Apoptosis is programmed cell death, characterized as shrinkage of cells and nuclei, with plasma membrane integrity persisted. It is less immunostimulatory than necrosis. The mechanisms of apoptosis include two major pathways: intrinsic and extrinsic pathways. Extrinsic pathway is the death receptor pathway, activated by death ligands and receptors such as TNF-α, tumor necrosis factor-related weak inducer of apoptosis (TWeAK), Fas ligand, tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL), and TL1A [76–79]. These complexes may induce to cleave caspase-3 and subsequently kill cells through proteolysis in injured cells [80]. Intrinsic pathway is a mitochondrial pathway, activated by hypoxia, cellular toxins, and radiation. This progress involves B-cell lymphoma-2 (Bcl-2) protein family members, including Bax and BaK [81, 82]. These prodeath

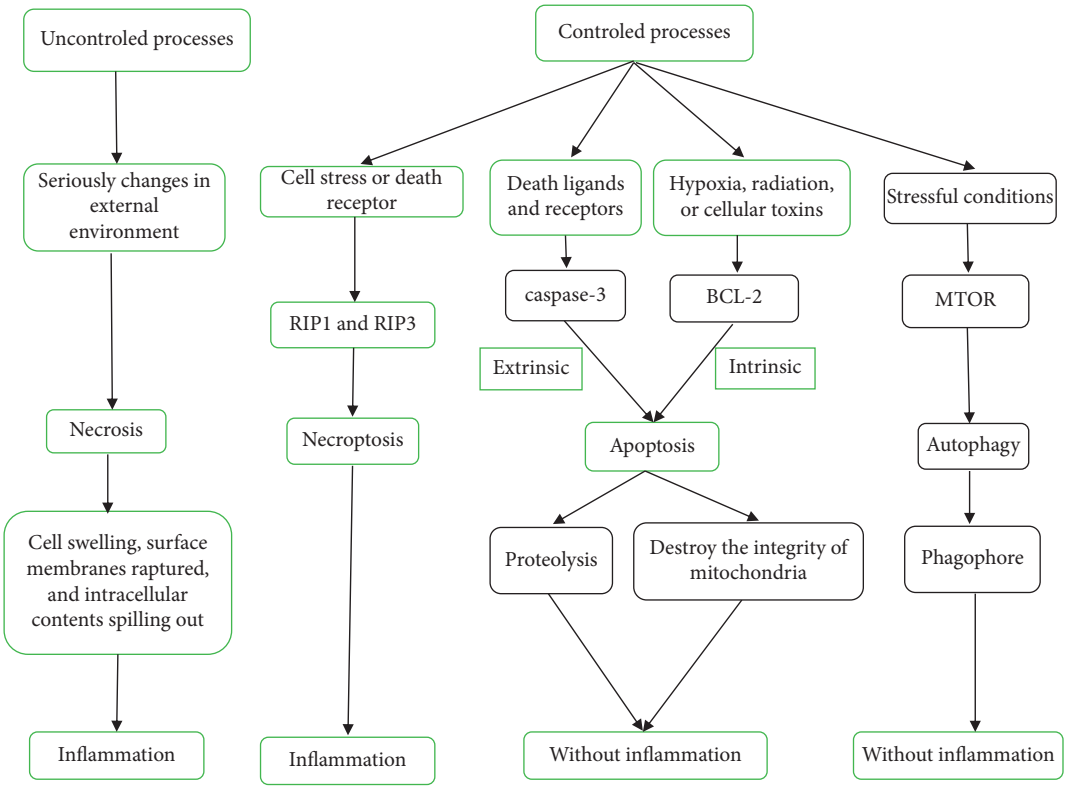

Figure 2: Cell death modalities in I/R injury. Different mechanisms of cell death: necrosis is characterized as cell and organelle swelling; subsequently, the surface membranes ruptured, and intracellular contents spilled out. Necrotic cells induce intensive immune stimulation, which lead to cell infiltration and cytokine release, infiltrate inflammatory cells, and generate cytokines. Necroptosis is defined as programmed necrosis, which shares similar features with necrosis. Apoptosis is programmed cell death, and it is less immunostimulatory than necrosis. The mechanisms of apoptosis include two major pathways: intrinsic and extrinsic pathways. Autophagy is the main way of cells to disposal of protein aggregates and damaged organelles.

proteins transport proapoptotic proteins from the intermembrane to the outer membrane by activating the permeability of the membrane [83]. Subsequently, pro-death proteins bind to the apoptotic protease-activating factor-1 (APAF1) and assemble the apoptosome; then, the complex activates caspase-3 and -9, inducing cellular protein cleavage [69]. Bcl-2 proteins are activated and unregulated and accumulated on mitochondrial membranes of ischemic cells [84–87]. Ischemia needs oxidative stress, evoked by reperfusion, to activate Bcl-2 proteins. Numerous apoptogenic factors are released including cytochrome c, caspase activator Omi, high-temperature-required protein A2 (HtrA2), second mitochondria-derived activator of caspases (Smac), and direct inhibitor of apoptosis protein (IAP) binding protein with low pI (DIABLO), but their roles and whether their inhibitors could be used for I/R injury are unclear.

Autophagy is the main mechanism of cells to disposal of damaged protein aggregates and cellular toxins [69, 88]. It may provide survival mechanism of cells to resistance of stressful conditions, such as infection, hypoxia, and mitochondrial dysfunction. However, if autophagy is out of control, it will lead to death of cells. In the process of autophagy, biological macromolecules and damaged organelles in the cytoplasm will be degraded in membrane vesicles. Autophagy involves cytoplasmic components and ruptured organelles. This process can be activated by I/R injury [89]. The main regulator of autophagy is the mammalian target of rapamycin (mTOR). mTOR will be inactivated in stress or nutrition deficiency. Inactivated mTOR will inhibit the formation of phagophores. The extension of the autophagic vesicle requires the participation of the autophagy-related protein 8/light chain 3 (Atg8/LC3) complex and Atg12-Atg5-Atg16 complex. Autophagy might upregulate the survival rate of cells. The inhibition of autophagy may amplify I/R injury [90–92]. However, if the injury is severe, cellwould be deregulated by autophagy. [93–95]. Autophagy begins with assembly of phagophore. [96, 97]. Vesicular autophagosome is formed by phagophore expansion to fully encase the cell constituents. Autophagy is regulated by mTOR. However, other regulatory mechanisms of autophagy need to be further investigated.

By interrupting the cell death process, cell survival rate can be increased, and the recovery time for lower limb function can be reduced, which may be effective ways to reduce I/R injury.

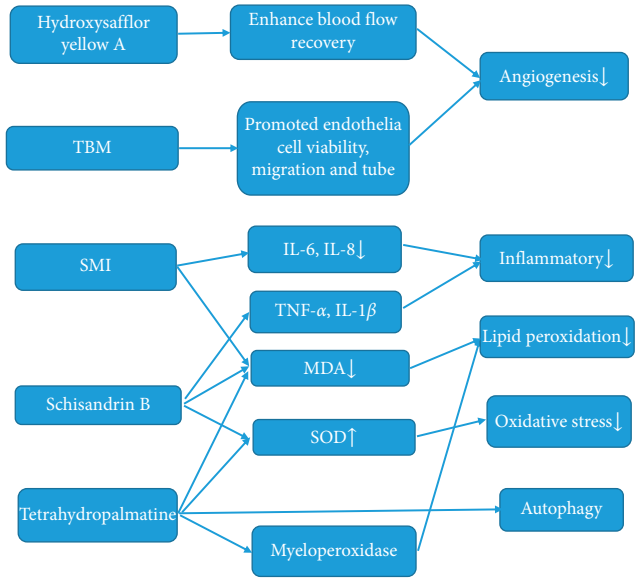

Figure 3: Hydroxysafflor yellow A enhances blood flow recovery and increases capillary and arteriole densities. This indicated that hydroxysafflor yellow A could promote angiogenesis. Tubeimoside-I (TBM) may promote angiogenesis by using the hindlimb ischemia model. If the patient pretreated in Shengmai injection (SMI) before applying automatic gas-filled tourniquet, their IL-6, IL-8, and MDA levels would be significantly decreased. This indicated that SMI may attenuate lipid peroxidation and systemic inflammatory response. Schisandrin B reduces MDA, increases SOD activity, and attenuates plasma inflammatory cytokines. These suggested that schisandrin B reduced I/R injury of the skeletal muscle by attenuating oxidative stress and inflammation. Study on tetrahydropalmatine showed that it may reduce myeloperoxidase and MDA, increase SOD, and inhibit autophagy.

5. Treatment of Traditional Chinese Medicine

TCM has been applied in China and some other Asian nations for more than 2000 years. Several studies have shown that TCM can be used in the treatment of I/R injury. TCM may reduce I/R injury through angiogenesis, antioxidant effect, reducing oxidative stress, inhibiting inflammatory cytokines' release, and so on.

In the mouse hindlimb ischemia model, hydroxysafflor yellow A enhances blood flow recovery and increases capillary and arteriole densities. This indicated that hydroxysafflor yellow A could promote angiogenesis [98]. Another research study found that tubeimoside-I (TBM) promoted endothelial cell viability, migration, and tube formation in human umbilical vein endothelial cells. In the hindlimb ischemia model, TBM may promote angiogenesis [99].

IL-6, IL-8, and plasma malondialdehyde (MDA) were used to indicate lipid peroxidation and systemic inflammatory response. If the patient pretreated with Shengmai injection (SMI) before applying automatic gas-filled tourniquet, their IL-6, IL-8, and MDA levels would be significantly decreased. This indicated that SMI may attenuate lipid peroxidation and systemic inflammatory response [100]. Schisandrin B may ameliorate ischemia histological changes of the skeletal muscle. In addition, schisandrin B reduces MDA, increases SOD activity, and attenuates plasma inflammatory cytokines. These suggested that schisandrin B reduced I/R injury of the skeletal muscle by attenuation oxidative stress and inflammation [101]. Study on tetrahydropalmatine showed that it may reduce myeloperoxidase and MDA, increase SOD, and inhibit autophagy (Figure 3).

I/R injury results from the complex pathophysiology process, which links to multiple mechanisms; any treatment targeting single link is insufficient to resolve this disease. Current studies provide abundant evidence on the mechanisms of TCM in I/R injury. However, most studies focus on single compound, extracted from Chinese herbs. In fact, most TCMs are used together to form a formula. TCM formula has advantages that may affect multiple targets, which may enhance efficacy and attenuate toxicity. The interactions between different components need further research, which may effectively explore the network of TCM formula. This may be another important research direction. In addition, TCM will be pretreated by decocting or other methods before use, which is an important part of TCM treatment. Nevertheless, its effect on TCM is reported scarcely.

6. Conclusion

I/R injury is an important clinical problem in PAD; it is still a critical challenge for doctors. Mitochondria play a central role in I/R injury on account of cell signaling, oxidative stress, energy production, and cell damage. The cell death pathways rely on the degree of injury and the microenvironment. However, the mechanisms of I/R injury are complex and include various aspects. An enhanced understanding of the pathophysiology and cell death pathways is critical for new therapies. In addition, TCM has been used to treat diseases for a long time. Recent research has verified the potential utility of TCM for the treatment of I/R injury. However, the mechanisms and combination of TCMs need further research.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zenghui Liang and Wentao Zhang contributed equally to this work.

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Research Article

Protective Effect of Joa-Gui Em through the Improvement of the NLRP3 and TLR4/NF-κb Signaling by Ischemia/Reperfusion-Induced Acute Renal Failure Rats

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Joa-gui em (左歸飮, JGE) is known to be effective for treating kidney-yin deficient syndrome. However, there is a lack of objective pharmacological research on improving kidney function. This study was designed to evaluate whether JGE improves renal function and related mechanisms in rats with acute renal injury induced by ischemia/reperfusion (I/R). The acute renal failure (ARF) group was subjected to reperfusion after inserting a clip into the renal artery for 45 min. The ARF + JGE (100 or 200 mg/kg/day) groups were orally administered for four days after their I/R surgery, respectively. JGE treatment suppressed the increase in kidney size in the ARF animal model and alleviated the polyuria symptoms. In addition, to confirm the effect of improving the kidney function of JGE, lactate dehydrogenase levels, blood urea nitrogen/creatinine ratio, and creatinine clearance were measured. As a result, it decreased in the ARF group but significantly improved in the JGE group. Also, as a result of examining the morphological aspects of renal tissue, it was shown that JGE improved renal fibrosis caused by ARF. Meanwhile, it was confirmed that JGE reduced inflammation through the nucleotide-binding oligomerization domain-like receptor pyrin domain containing-3 (NLRP3) and toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB) signaling pathways, which are the major causes of acute ischemic kidney injury, thereby improving renal function disorder. The JGE has a protective effect by improving the NLRP3 and TLR4/NF-κB signaling pathway in rats with acute renal dysfunction induced by I/R injury.

1. Introduction

The kidney is essential in maintaining homeostasis by regulating fluid volume through the excretion and reabsorption of water. Ischemic kidney injury due to ischemia/reperfusion (I/R) causes various kidney dysfunction, eventually leading to acute renal failure (ARF). Moreover, kidney function is impaired, urine cannot be excreted normally, and the body loses its balance of water and electrolytes. The rapid increase in the levels of serum creatinine (Cr) decreases glomerular filtration in the kidneys [1]. It is widely assumed that ARF generally occurs due to acute tubular necrosis, usually due to ischemic renal injury [2]. ARF animal model has structural remodeling in the renal tubular epithelium [3, 4]. I/R-induced renal impairment significantly affects kidney function because the supply of reperfusion to the kidney causes a significant cellular metabolic disturbance and tissue inflammation [5, 6]. In inflammatory kidney diseases, the nucleotide-binding oligomerization domain-like receptor pyrin domain containing-3 (NLRP3) inflammasome is a multi-protein complex induced by harmful factors in the body and plays an essential role in the inflammatory response [7, 8]. Activation of NLRP3 inflammasomes mediates the activation of caspase-1 and the secretion of inflammatory cytokines, including interleukin-(IL-) 1β and IL-18, resulting in a type of cell death called

pyroptosis [9–11]. Therefore, the NLRP3 inflammasome and proinflammatory cytokines, including IL-1β and IL-18, directly affect the renal tubular epithelium and cause renal dysfunction [12].

Some studies have reported that the NLRP3 inflammasome promotes epithelial-mesenchymal transition (EMT) by the transforming the growth factor-β_1 (TGF-β_1)/Smad signaling [13]. The NLRR3 inflammasome increases TGF-β_1 expression, and TGF-β_1 is associated with EMT and leads to renal fibrosis [14]. EMT of renal tubular cells is defined as the process that contributes to fibrosis through a phenotypic change to myofibroblasts [15]. Furthermore, TGF-β_1 mediates the formation of extracellular matrix proteins and pro-fibrotic factors, including fibronectin, collagen, and matrix metalloproteinases [16]. Like this, the relationship between NLRP3 inflammasomes and kidney disease is important, and it is necessary to confirm the efficacy of drugs to improve the related signaling pathways. Therefore, we tried to examine the efficacy of herbal medicines that have been used in traditional Korean medicine to improve kidney function.

In Korean traditional medicine, there are four categories of renal disease symptoms, including deficiency of kidney yang (腎陽虛), deficiency of kidney-yin (腎陰虛), insufficiency of kidney essence (腎精虛), and insufficiency of kidney gi (腎氣虛). Joa-gui em (左歸飮, JGE) is known to be effective in treating kidney-yin deficient syndrome [17, 18]. However, there is a lack of objective pharmacological research on improving acute renal failure (ARF) model. Therefore, this study was conducted to confirm the effect of JGE on the improvement of renal function and related mechanisms in an I/R-induced ARF animal model.

2. Materials and Methods

2.1. Preparation of Joa-Gui Em. The voucher specimen used in this study (HBI 192–15) was stored in the Hanbang Cardio-Renal Syndrome Research Center of Wonkwang University (Iksan, Korea). The herbal medicines used to extract the JGE were purchased from the Herbal Medicine Cooperative Association (Iksan, Korea). The six herbal medicines that make up JGE are as follows: Rehmannia glutinosa Libosch (80 g), Dioscorea batatas Decne (80 g), Cornus officinalis Siebold (80 g), Lycium chinense Mill (80 g), Poria cocos Wolf (60 g), and Glycyrrhiza uralensis Fisch (40 g). The herbal medicines were soaked in 2 L of distilled water and left at room temperature for 1 hour and then boiled for 2 hours (at 100°C). The boiled herbal decoction was centrifuged for 10 minutes (at 4°C, 3000 rpm) to remove impurities and concentrated in a rotary vacuum evaporator (N-11, Rikakikai, Tokyo, Japan), and then a freeze dryer was used to create a powder. Dried JGE was stored at 4°C until use, and for in vivo experiments, it was diluted in distilled water at an appropriate dose before oral administration and used.

2.2. Animals. Sprague-Dawley male rats (5 weeks old, weight 170–190 g) were purchased from Samtako (Samtako Bio Korea, Osan, Korea) and maintained in a 12 hr light/dark cycle in a thermo-hygrostat (45% humidity). To make an animal model of ARF induced by I/R, anesthesia was performed using sodium pentobarbital (50 mg/kg, intraperitoneal injection) and surgery was performed. Anesthetized rats induced blocking both renal arteries with clips to prevent blood from passing through them for 45 minutes, and control rats underwent sham surgery without clips. Animals recovered in metabolic cage for 4 days. It was divided into 4 groups as follows: Cont, control group; ARF, ARF group; ARF + JGE100, JGE 100 mg/kg/day-treated ARF group; ARF + JGE 200, JGE 200 mg/kg/day-treated ARF group. Rats were administered JGE by oral gavage for 7 days. This study was tested on animals with the approval of the Institutional Animal Care and Use Committee (IACUC) of Wonkwang University (WKU19-46).

2.3. Renal Function Test. Rats in each group were measured for water intake and quantitative urine collection in separate metabolic cages (24 hr). Urine osmolality (Model 3900, Advanced Instruments Inc., Norwood, MS, USA), and electrolytes (NOVA 4, Biochemical, Waltham, MA, USA) levels were measured using the collected urine. After the experiment was completed, the blood of the experimental animals was taken to measure lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and Cr in the plasma. After the experiment was completed, the experimental animal was blood was taken, and the BUN, Cr, and LDH in the plasma were measured using biochemical analyzer (NX700, FUJIFILM Corporation, Tokyo, Japan). Plasma and urine creatinine clearance (Ccr) were measured with a spectrophotometer using a colorimetric method (Milton Roy, Rochester, NY, USA). Ccr (ml/min/kg) = urine Cr (mg/ml) x urinary volume (UV, ml/kg/min)/plasma Cr (mg/ml).

2.4. Western Blot Analysis. Protein samples (30 μg protein) were electrophoresed and transferred to a nitrocellulose membrane. The membrane was then blocked in 5% bovine serum albumin (with Tris-buffered saline) for 2 hours and then incubated with an appropriate primary antibody. The next day, the secondary antibody was reacted for 1 hour and then visualized using chemiluminescence (EzWestLumi plus, ATTO Technology, NY, USA). Primary antibodies included cryopyrin NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), pro-caspase-1, TGF-β_1, IL-1β, toll-like receptor 4 (TLR4), myeloid differentiation primary response gene 88 (MyD88), nuclear factor kappa B (NF-κB) p65, and β-actin (Santa Cruz Biotechnology Dallas, TX, USA). Protein expression levels were imaged using iBright FL100 image analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Histopathologic Examination. Kidney tissues were isolated from each rat and fixed with 10% paraformaldehyde in phosphate-buffered saline (PBS, 0.01 M) for 24 hr. The kidney tissues were dehydrated with a sequence of ethanol solutions and embedded in paraffin (sectioned on slides,

6 μm). For histopathological comparison, sectioned kidneys were stained with hematoxylin and eosin (H&E), periodic acid shift (PAS), picrosirius red, and Masson's trichrome staining imaged using a EVOS^{TM} light microscope (M5000, Thermo Fisher Scientific, Bothell, WA, USA).

2.6. Statistical Analyses. All results were presented as mean ± SEM. Statistical significance between groups was performed using ± standard error (SE). p value < 0.05 was considered statistically significant. The significant differences between groups were validated by paired t-test. All statistical analyses were conducted using SigmaPlot 10.0.

3. Results

3.1. High-Performance Liquid Chromatography (HPLC) Analysis of JGE. The chemical composition of JGE was analyzed using ultra-high performance liquid chromatography. Fifteen compounds, including eight markers (5-hydroxymethyl furfural [5-HMF], allantoin, betaine, catalpol, cocamidopropyl betaine, glucose, glycyrrhizin, liquiritin, liquirtin apioside, maltotriose, morroniside, phenylalanine, quinic acid, sucrose, tryptophan, and valine) were verified based on the authentic compounds or tentatively identified according to the retention time, exact mass spectrometry (MS), and MS/MS fragments. In particular, major bioactive compounds such as 5-HMF and catalpol for *Rehmanniae Radix Preparata*, allantoin for *Dioscoreae Rhizoma*, betaine for *Lycii Fructus*, morroniside for *Corni Fructus*, glycyrrhizin, liquiritin, and liquiritin apioside for *Glycyrrhizae Radix et Rhizoma* were detected in JGE (Figure 1).

3.2. Effect of JGE on Physical Measurements. I/R injured rats are commonly used as a model for experimental ARF research. The body weights (BWs) of all rats in the ARF group were significantly decreased and were markedly restored by oral administration of JGE200 ($p < 0.05$) (Table 1). The kidney weight/BWs of the I/R injury rats treated with JGE were significantly decreased compared with the non-JGE treated I/R injury rats (Table 1). However, there was no difference between the ARF and JGE-treated groups in terms of heart weight/BW (Table 1).

3.3. Effect of JGE on Urinalysis for Kidney Function. ARF is characterized by a sudden loss of kidney function in concentrating urine. In order to investigate the change in urine and water intake of JGE, the rats of each group were kept in separate metabolic cages for 4 days, and urine samples were collected. UV was significantly increased in the ARF group, which was markedly decreased by oral administration of JGE100 ($p < 0.05$) and JGE200 ($p < 0.01$) on day 4 (Table 2). These results indicated that the ability to concentrate urine was impaired due to the I/R injury. However, JGE treatment did not significantly change urinary osmolality in ARF rats (Table 2). Urinary sodium excretion was decreased by the I/R injury and was markedly restored by the oral administration of JGE200 ($p < 0.05$) on day 4 (Table 2). Urinary potassium excretion was reduced in ARF rats, which was restored significantly by oral administration of JGE100 ($p < 0.05$). Urinary chloride excretion was decreased in ARF rats, which was markedly restored by oral administration of JGE100 ($p < 0.05$).

3.4. Effect of JGE on Renal Functional Parameters. BUN/Cr ratio was significantly increased in ARF rats compared with control rats. BUN/Cr was significantly decreased by the oral administration of JGE in a dose-dependent manner (Table 3). Ccr was significantly lower in the ARF group than in the control group. Oral administration of JGE significantly restored Ccr (Table 3). In addition, we measured the effect of JGE on the plasma levels of LDH in ARF rats. As a result, LDH levels were increased by I/R injury and significantly decreased by the oral administration of JGE100 ($p < 0.01$) and JGE200 ($p < 0.001$) (Table 3).

3.5. Effect of JGE on Histological Changes in Kidney. To determine the protective effect of JGE on renal cortical glomeruli injury, renal morphology was analyzed using H&E and PAS staining. Light microscopic examinations showed glomerular injury and vacuole formation in the renal cortex of rats in the ARF group. However, JGE treatment improved the glomerular injury of the renal cortex (Figure 2(a)). H&E and PAS staining were performed to confirm the protective effect of JGE on the extrarenal medulla and inner medulla damage, and histological analysis was performed, and representative micrographs of the kidneys of each group were obtained. It has been demonstrated that tubular dilatation, tubular epithelial damage, cast formation, and debris accumulation were found in the external and internal water quality of ARF (Figure 2(b)). The renal outer medulla and inner medulla tubules destroyed by I/R injury were treated with JGE to prevent the lesions of the tubules (Figure 2(c)). These results imply that JGE has the effect of improving pathological damage to the kidney in I/R-induced ARF rats.

3.6. Effect of JGE on NLRP3 Inflammasome Expression in Kidney. The NLRP3 inflammasome signaling pathway plays an essential role in renal inflammation. To evaluate the effect of JGE on the NLRP3 inflammasome induced by I/R injury, NLRP3 inflammasome protein expression was decided by western blot analysis. NLRP3 inflammasome signaling factors, NLRP3, IL-1β, pro-caspase-1, and ASC protein expression, increased in response to I/R-induced ARF rats, but decreased by JGE treatment (Figure 3(a). In addition, the NLRP3 inflammasome binds to the NF-κB inflammatory pathway to mediate IL-1β transcription and activation. JGE treatment decreased the protein expression associated with the TLR4/MyD88/NF-κB inflammatory pathway (Figure 3(b)).

3.7. Effect of JGE on Renal Fibrosis. To confirm the protective effect of JGE on renal fibrosis, histological analysis was performed by staining with Masson Trichrome and

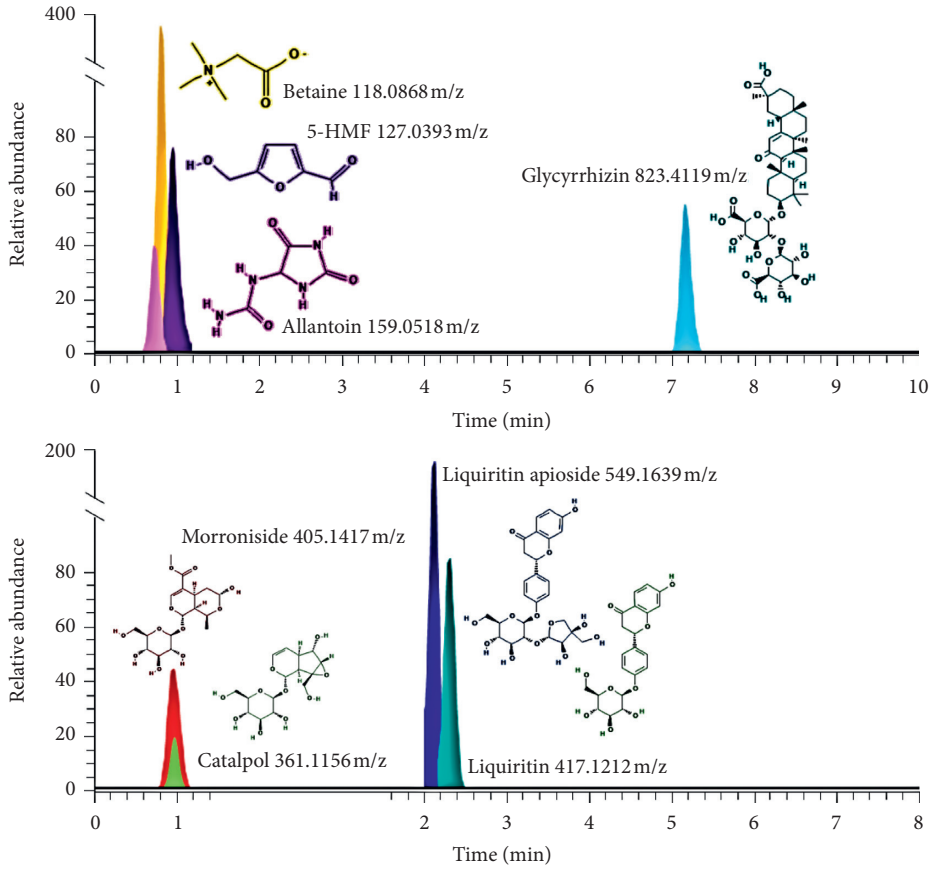

Figure 1: Three-dimensional chromatogram of JGE obtained using HPLC-PDA. Extracted ion chromatograms from the ultra-performance liquid chromatography analysis of charged molecular ions, showing eight bioactive compounds.

Table 1: Effect of JGE on physical measurements.

	Cont	ARF	ARF + JGE100	ARF + JGE200
BW (g)	217.90 ± 2.07	191.63 ± 2.60***	197.27 ± 2.44	202.24 ± 3.13#
KW/BW (g/kg)	8.37 ± 0.04	12.53 ± 0.35***	11.46 ± 0.30#	10.39 ± 0.53##
HW/BW (g/kg)	3.66 ± 0.02	3.49 ± 0.03***	3.55 ± 0.03	3.56 ± 0.04

Cont., control; ARF, ischemia/reperfusion- (I/R-) induced acute renal failure (ARF) group; ARF + JGE100, JGE 100 mg/kg/day; ARF + JGE200, JGE 200 mg/kg/day for 4 days; KW: kidney weight; HW: heart weight; BW: body weight. Values are expressed as mean ± SE. ($n = 10$).*** vs. cont.; # $p < 0.05$, ## $p < 0.01$ vs. ARF.

Table 2: Effect of JGE on urinalysis for kidney function.

	Cont	ARF	ARF + JGE100	ARF + JGE200
UV (ml/day/kg)	45.41 ± 2.06	70.66 ± 3.28***	61.31 ± 2.71#	57.92 ± 1.77##
Uosmol (mOsm)	2503.8 ± 131.8	1420.5 ± 86.8***	1643.1 ± 114.8	1615.8 ± 62.3
$U_{Na}V$ (mmol/L/Kg)	786.75 ± 19.82	485.24 ± 30.09***	566.01 ± 30.92	606.73 ± 39.40#
$U_{K}V$ (mmol/L/Kg)	1556.98 ± 51.95	995.33 ± 64.84***	1302.99 ± 99.76#	1101.53 ± 66.83
$U_{Cl}V$ (mmol/L/Kg)	1324.03 ± 37.16	871.52 ± 46.16***	1044.75 ± 64.87#	968.47 ± 61.45

Cont, control; ARF, ischemia/reperfusion- (I/R-) induced acute renal failure (ARF) group; ARF + JGE100, JGE 100 mg/kg/day; ARF + JGE200, JGE 200 mg/kg/day for 4 days; UV, urinary volume; Uosmol, urinary osmolality. Values are expressed as mean ± SE. ($n = 10$). cont*** vs. cont.; # $p < 0.05$, ## $p < 0.01$ vs. ARF.

picrosirius red. In addition, TGF-β_1 is a major profibrotic cytokine that drives EMT. To investigate the effect of JGE on renal fibrosis induced by I/R injury, TGF-β_1 protein expression was determined (Figure 4(a)). It was known that TGF-β_1 expression is increased in response to NLRP3 inflammasome. As shown in Figure 4(a), I/R enhanced TGF-β_1 protein expression, whereas it was inhibited by JGE treatment. In addition to determine the protective effect of

Table 3: Effect of JGE on renal functional parameters.

	Cont	ARF	ARF + JGE100	ARF + JGE200
BUN/Cr	31.86 ± 1.14	$52.27 \pm 1.73^{***}$	$43.82 \pm 2.13^{\#\#}$	$43.46 \pm 0.88^{\#\#\#}$
Ccr (ml/min/kg)	0.46 ± 0.03	$0.26 \pm 0.02^{***}$	$0.33 \pm 0.03^{\#}$	$0.34 \pm 0.02^{\#\#}$
LDH (U/L)	1612.7 ± 150.7	$2089.2 \pm 136.6^{*}$	$1474.5 \pm 108.6^{\#\#}$	$1104.9 \pm 102.7^{\#\#\#}$

Cont., control; ARF, ischemia/reperfusion- (I/R-) induced acute renal failure (ARF) group; ARF + JGE100, JGE 100 mg/kg/day; ARF + JGE200, JGE 200 mg/kg/day for 4 days; Cr, creatinine; Ccr, creatinine clearance; BUN, blood urea nitrogen; LDH, lactate dehydrogenase. Values are expressed as mean ± SE. ($n = 10$). $^{*}p < 0.05$ $^{***}p < 0.001$ vs. cont.; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. ARF.

JGE on renal fibrosis, renal morphology was analyzed using Masson and PAS staining. The renal fibrosis destroyed by I/R injury was treated with JGE to prevent the lesions of the tubules (Figure 4(b)). Thus, JGE suppressed I/R-induced renal fibrosis in ARF rats.

4. Discussion

JGE was recorded in a traditional Chinese medical book titled "Gyeong-agjeonseo" and comprises six components of herbal medicines: *Rehmanniae Radix Preparat, Dioscoreae Rhizoma, Lycii Fructus, Hoelen, Corni Fructus*, and *Glycyrrhizae Radix Praeparata.* JGE has been used for the treatment of kidney-yin-deficient syndrome [17, 18]. However, there is no evidence that JGE has an effect on I/R-induced ARF. Therefore, this study demonstrated whether JGE improves renal dysfunction of rats with I/R-induced ARF.

In this study, urine volume increased in renal function in I/R-induced ARF rats, while Ccr and excretion of sodium, potassium, and chloride were significantly decreased. These results indicate that ARF induced by I/R caused a defect in the rat with the ability to concentrate urine [19]. ARF is one of the most common glomerular diseases characterized by a marked decrease in glomerular filtration rate, extensive tubular cell necrosis, glomerular damage, and signs of tubular obstruction due to cell debris [20–22]. It is well documented that ARF is characterized by an acute decline in renal function as measured by UV, Ccr, and excretion of sodium, potassium, and chloride [23–25]. In this study, JGE treatment ameliorated renal function, such as Ccr, UV, and the excretion of sodium, potassium, and chloride in I/R-induced ARF rats. These findings suggest that JGE has a potential role in renal dysfunction.

It has been established that tubular dilatation, tubular epithelial damage, cast formation, and debris accumulation were all found in the renal medulla of ARF [9, 26, 27]. To confirm the effect of JGE administration on tubular injury, PAS staining analysis was performed. The present study showed that the cortex, inner medulla, and outer medulla were impaired in rats with ischemic acute kidney injury. In the present study, JGE treatment ameliorated renal injury, including tubular dilatation, tubular epithelial damage, cast formation, and debris accumulation in I/R-induced ARF rats. Furthermore, glomerular injury was also found, and vacuole formation was observed in the cortices of the kidneys. The present data showed that JGE ameliorated glomerular injury by inhibiting vacuole formation in rats with ischemic acute kidney injury.

In a previous study, the elevation of BUN/Cr and levels of LDH in serum was pathognomonic for I/R-induced ARF. This study showed the same results of earlier studies, which reported that the parameters of renal function, including the levels of BUN, Cr, and LDH, are decreased by I/R injury [28]. This study aimed to determine if JGE treatment inhibited renal failure. Therefore, the present study shows that JGE treatment ameliorates renal derangement, as observed by the changes in BUN/Cr and levels of LDH in I/R-induced ARF rats.

In another study of I/R-induced ARF, it was concluded that acute kidney injury causes the NLRP3 inflammasome to be released by directly affecting the renal tubular epithelium, which leads to the activation of caspase-1, causing inflammatory cell infiltration and the activation of cytokines [29–31]. Activated caspase-1 of the NLRP3 inflammasome activates IL-1β and IL-18 [32–34]. In our present study, as a result of Western blot analysis, it was revealed that JGE reduced NLRP3 inflammasome formation in I/R-induced ARF rats. Also, we showed that JGE significantly inhibited the NLRP3 inflammasome in I/R-induced ARF rats of pro-inflammatory cytokines such as NLRP3, ASC, pro-caspase-1, and IL-1β. Therefore, these results suggest that JGE reduces NLRP3 inflammasome formation, thereby reducing the inflammatory response. Furthermore, the NLRP3 inflammasome activates proinflammatory cytokines that are associated with the TLR4/MyD88/NF-κB signaling. Widely expressed in the plasma membrane of immune cells, TLR4 plays a vital role in initiating inflammation, and MyD88 is used by all TLRs and activates NF-κB for induction of inflammatory cytokine genes [35, 36]. The present results showed that JGE treatment significantly suppressed the protein expression of TLR4, MyD88, and NF-κB in I/R-induced ARF rats. Therefore, we speculated that JGE exhibits anti-inflammatory effects by inhibiting the NLRP3 inflammasome and TLR4/MyD88/NF-κB signaling pathway (Figure 5).

Some studies have reported that NLRP3 inflammasomes are required for optimal TGF-β_1 signaling and R-Smad activation [37]. In the absence of NLRP3, TGF-β_1 signaling is interrupted in the renal tubular epithelium, and the expression of TGF-β_1-stimulated genes, which are important for EMT, is reduced [38]. EMT of renal tubular cells is defined as obtaining myofibroblast markers, producing extracellular matrix proteins, and migratory capabilities instead of losing epithelial characteristics, such as tight junction formation and apical-basal polarity [39]. TGF-β_1 is the major profibrotic cytokine that drives the course of EMT [40]. In this study, JGE treatment reduced the protein expression of TGF-β_1 in I/R-induced ARF rats. These results suggest that JGE has a potential role in renal fibrosis.

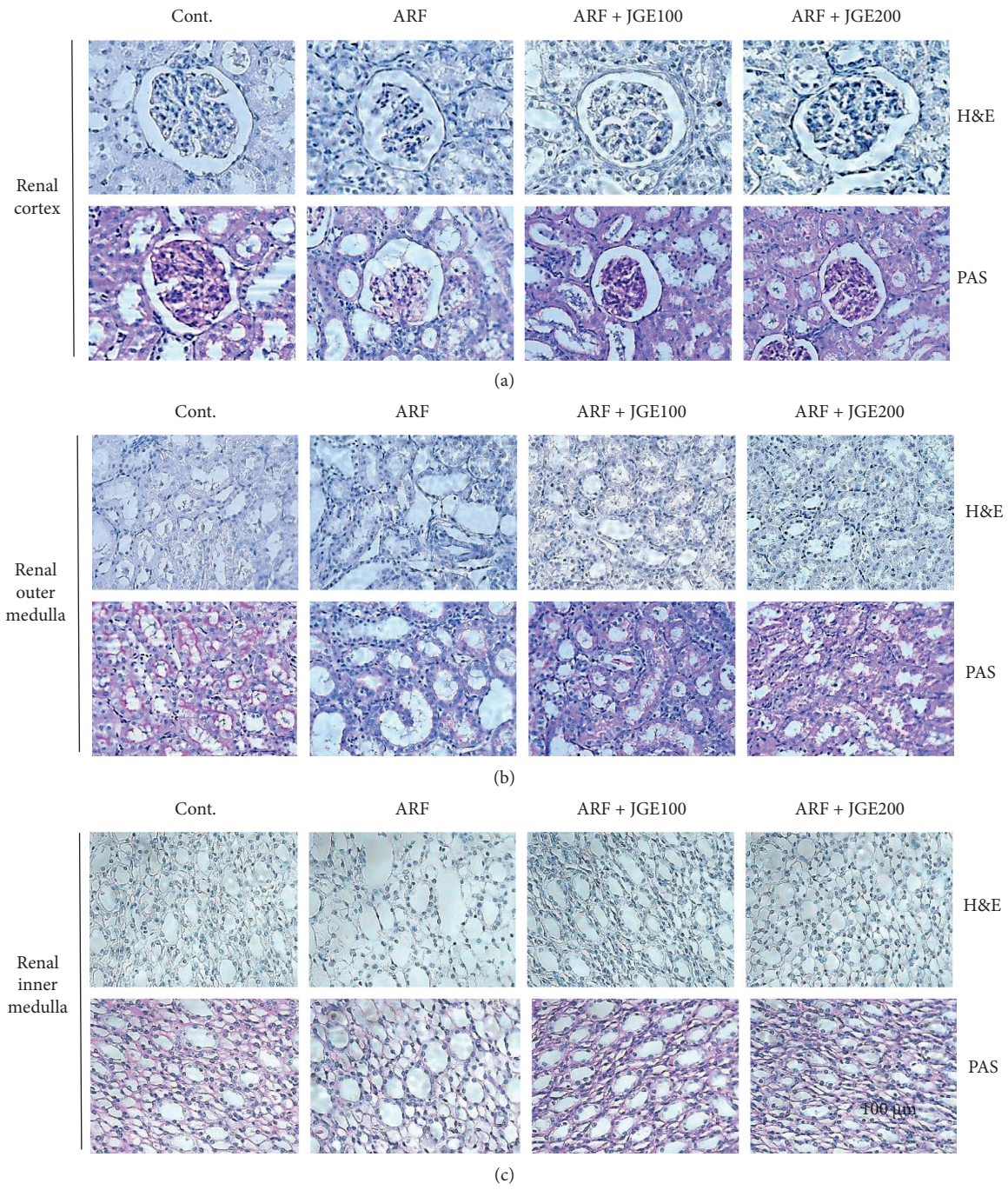

Figure 2: Effect of JGE on the renal cortex (a), outer medulla (b), and inner medulla (c). Sections of the kidney are demonstrated from the control, I/R-induced ARF, and JGE (100 or 200 mg/kg/day)-treated ARF group. Representative microscopic photographs were stained using H&E and PAS (magnification 400×).

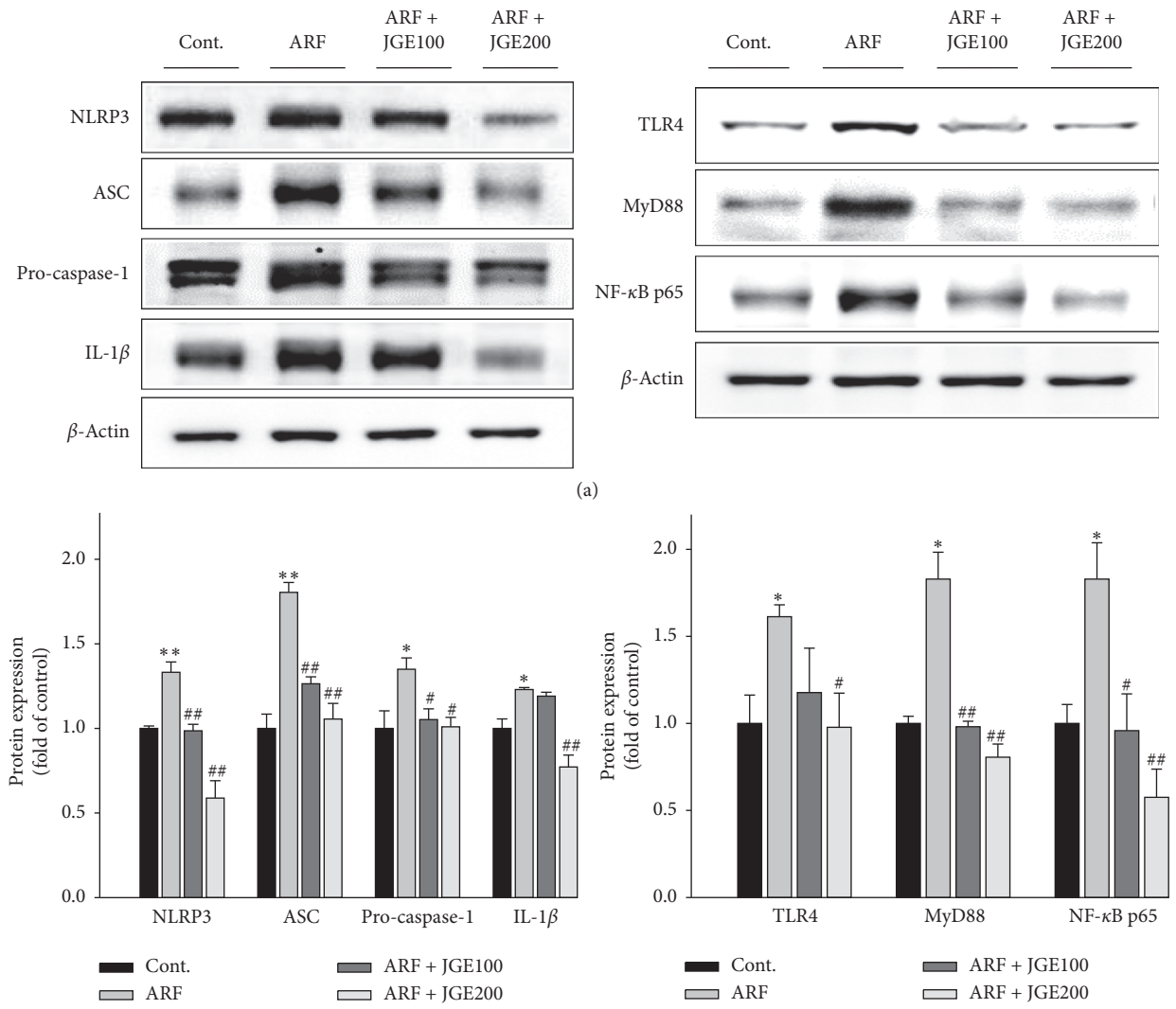

Figure 3: Effect of JGE on proinflammatory cytokine and NLRP3 inflammasome in kidney tissues. Protein expression of NLRP3 inflammasomes, including NLRP3, pro-caspase-1, and ASC (a), and proinflammatory cytokine IL-1β, and the TLR4/MyD88/NF-κB signaling pathway (b) in kidneys were analyzed by western blot analysis. The data shown summarize three independent experiments. Values are expressed as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. ARF.

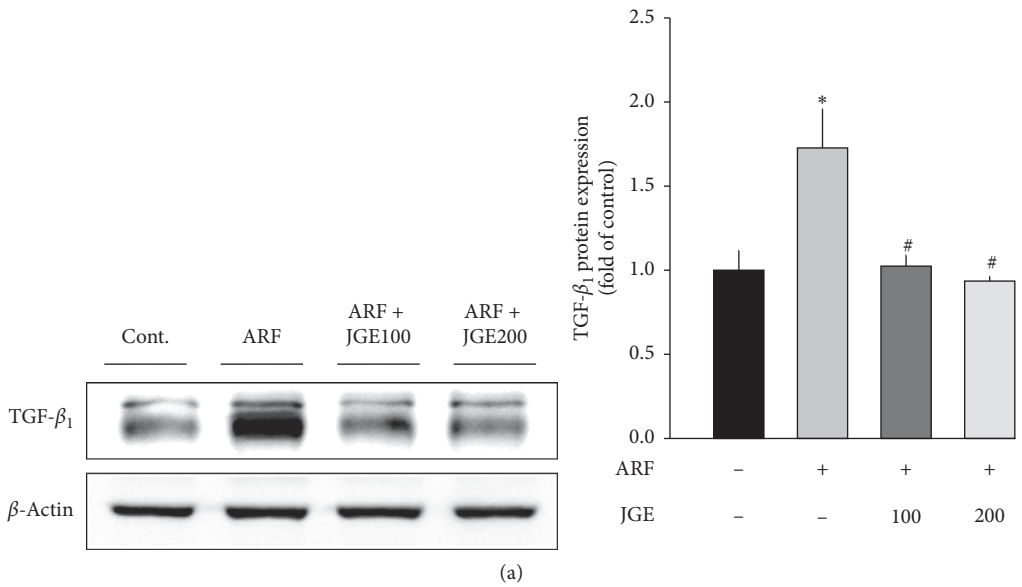

Figure 4: Continued.

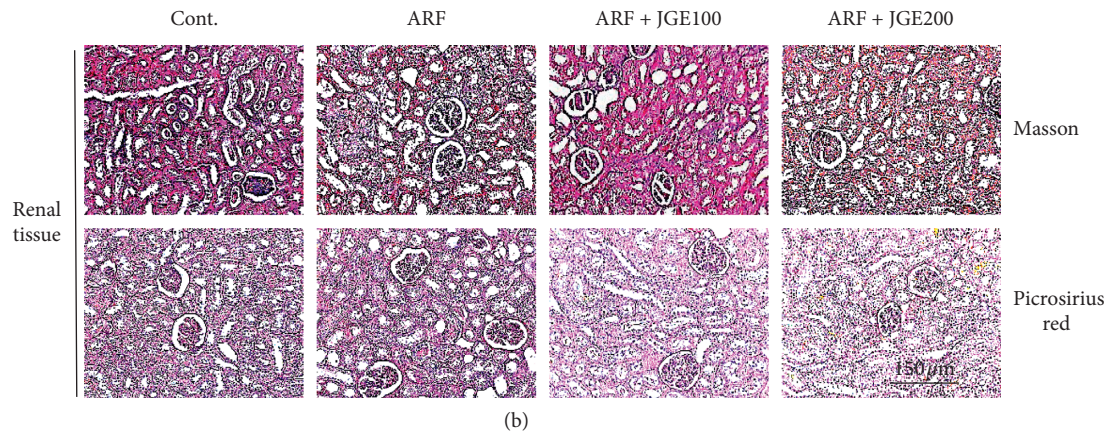

Figure 4: Effect of JGE on renal fibrosis. Protein expression of TGF-ß1 in the kidney tissues was determined by western blot analysis (a). The data shown summarize three independent experiments. Representative microscopic photographs were stained using Masson and PAS (b) (magnification 200 ×). Values are expressed as means ± SE. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. ARF.

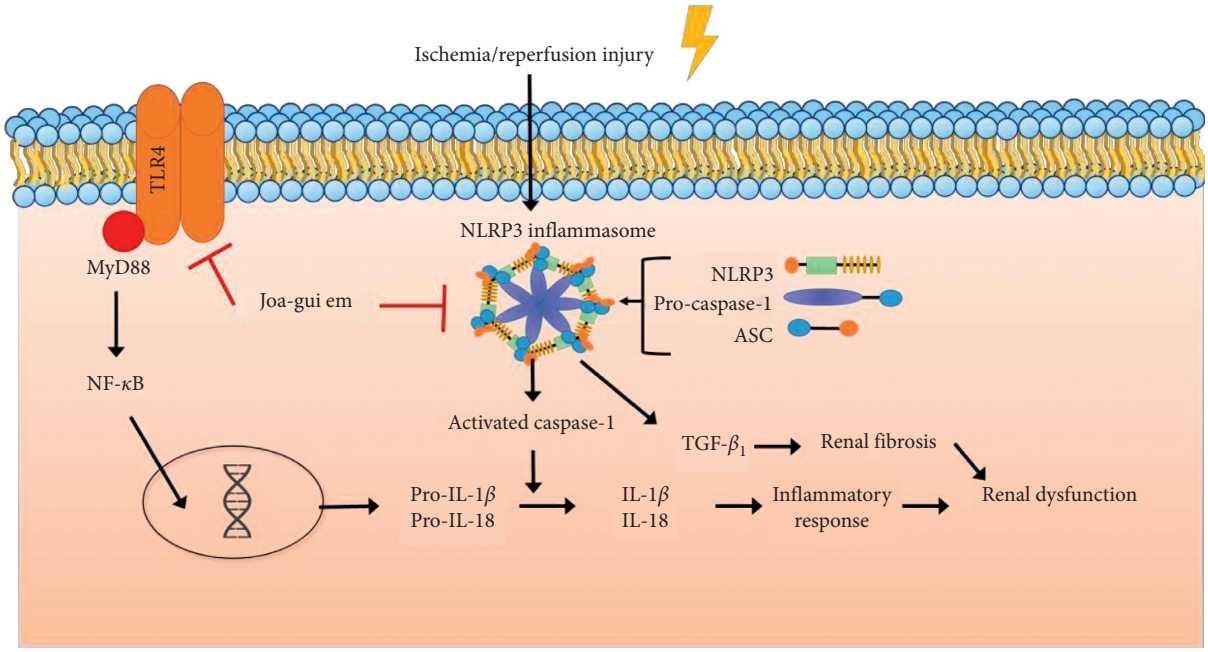

Figure 5: The suggested mechanism of the anti-inflammatory and anti-fibrotic effects of JGE in I/R-induced ARF rats.

5. Conclusion

In conclusion, treatment with JGE has protective effects against renal dysfunction induced by I/R injury via inhibition of the NLRP3 signaling pathway, indicating that JGE should be considered an effective Korean traditional medicine for treating acute kidney injury and renal remodeling.

Abbreviation

ARF: Acute renal failure
BUN: Blood urea nitrogen
Cr: Creatinine
Ccr: Creatinine clearance
H&E: Hematoxylin and eosin
IL-1β: Interleukin-1β
I/R: Ischemia/reperfusion
JGE: Joa-gui em
LDH: Lactate dehydrogenase
MyD88: Myeloid differentiation primary response gene 88
NF-κB: Nuclear factor kappa B
NLRP3: Nucleotide-binding oligomerization domain-like receptor pyrin domain containing-3
PAS: Periodic acid shift
TGF-β1: Transforming the growth factor-β1
TLR4: Toll-like receptor 4

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

All the authors declare that they have no conflicts of interest.

Authors' Contributions

DGK, HYK, and SWN conceived the study and designed the experiments. HYK and JJY performed the experiments. SWN, YJJ, and HMH analyzed the data. SWN and HYK prepared the figures. SWN and HYK revised the figures and wrote the original draft of the manuscript. HSL and DGK revised the manuscript. All the authors reviewed the manuscript.

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Research Article

Pharmacological Mechanisms Underlying the Therapeutic Effects of Danhong Injection on Cerebral Ischemia

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Background. Although Danhong injection (DHI) has been proved to be curative, the mechanism of its action against ischemia stroke (IS) is not clear. Here, we explored the therapeutic basis of DHI by network pharmacology. *Methods*. Putative targets and activity scores for each compound in DHI were obtained from the Traditional Chinese Medicine System Pharmacology Database, Encyclopedia of Traditional Chinese Medicine, and Quantitative Structure Activity Relationships. Next, target proteins of IS were identified on GeneCards and CTD. Overlapping targets of DHI associated with IS were acquired via Venn diagram. GO and KEGG pathway analyses were done using WebGestalt. Cytoscape software was used for PPI network construction and hub nodes screening. Several validation studies were carried out by using AutoDock-Vina, label-free mass spectrometry, and transcriptome RNA-sequencing. *Results*. The 37 active compounds and 66 targets were identified. Of these, 26 compounds and 41 targets belonged to diterpenoid quinones (DQs), which is the predominant category based on chemical structure. The results of enrichments analysis show that 8 DQs target proteins associated with IS were involved in several biological processes and signaling pathway such as apoptotic, cell cycle, cellular response to xenobiotic stimulus process, and the PI3K-Akt signaling. Moreover, 3 nodes in core module involved in PI3K-Akt signaling and 1 hub node were identified by PPI network analysis. Finally, the results of molecular docking and label-free mass spectrometry display good effect on hub node regulation in DHI treatment. *Conclusions*. DQs is the predominant category of DHI and play an important role in antiapoptotic activity mediated by modulating PI3K-Akt signaling. Our findings offer insight into future research and clinical applications in IS therapy.

1. Introduction

Cerebral stroke is the second most leading cause of death and the main cause of disability in worldwide. According to World Health Organization, it led to 6 million deaths in 2016 [1,2]. Ischemic stroke (IS) accounts for nearly 80% of cases and is characterized by occlusion of the cerebral artery, which leads to a temporary lack of glucose and oxygen supply in brain [3,4]. Standard IS therapies involve intravenous injection of recombinant tissue plasminogen activator (t-TPA), antiplatelet therapy, and anticoagulants for patients with atrial fibrillation, or interventions to limit cell damage [5–7]. These single target therapies are limited by the narrow time window of thrombolysis, hemorrhagic tendency, and high cost [8]. For this reason, novel therapeutic strategies are needed.

Danhong injection (DHI) is the most popular Chinese medicine for the treatment of IS and promotes blood circulation and resolves stasis to promote regeneration [9]. DHI is extracted from Radix *Salvia miltiorrhizae* (Danshen, DS) and Flos Carthami Tinctorii (Honghua, HH) [10]. Clinical studies show that DHI is efficacious and safe for IS management [11]. Pharmacological studies show that DHI is neuroprotective in rat models of cerebral ischemic reperfusion injury, possibly by enhancing angiogenesis [12], ameliorating blood-brain barrier (BBB) disruption, relieving brain swelling and hemorrhage [13], attenuating astrocytic dysfunction [14], and reversing neutrophil

infiltration [15]. Although multiple studies have investigated the mechanisms underlying DHI action, its underlying pharmacological mechanisms have not been elucidated at the systematic level.

Network pharmacology characterized by systematization and wholeness and has potential to uncover TCM mechanism via biological networks construction. Chinese herbal medicines act on multitargets via multiple channels, which is very similar to the multipathways and multilevel features of network pharmacology [16]. Here, we used various publicly available bioinformatics resources to investigate the potential pharmacological mechanisms of DHI in IS treatment.

2. Materials and Methods

2.1. Screening for Active DHI Compounds. DS and HH chemical compound data were collected from the Traditional Chinese Medicine Systems Pharmacology (TCMSP, https://tcmspw.com/index.php) database and analysis platform and the Encyclopedia of Traditional Chinese Medicine (ETCM, http://www.tcmip.cn/ETCM/index.php/Home/) database [17,18]. Next, pharmacokinetic properties and comprehensive drug-likeness grading of candidate compounds in DHI were filtered. Frist, the effective compounds screening performed using oral bioavailability (OB) ≥30%, drug-likeness (DL) index ≥0.18, and BBB ≥ −0.3. Next, the second screening was performed using Lipinski Rule of Five. Two-dimensional (2D) structure and canonical smiles of the active compounds were demonstrated using PubChem.

2.2. Identification of Ischemic Stroke Targets and Collection of Putative Target Proteins. IS-associated targets were identified based on comparative toxicogenomics database (CTD, http://ctdbase.org/) [19] and the GeneCards (http://www.genecards.org/) [20], using scores >50% and 30% as cutoffs for higher correlation with IS, respectively. Prediction of proteins related to DHI active compounds was done using quantitative structure activity relationships: TargetNet (QSAR-TargetNet, http://targetnet.scbdd.com) [21] and identified targets transformed to gene symbols on R. The overlapping targets between IS-related targets and active compounds were retained for Venn analysis (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.3. GO and KEGG Enrichment and Network Construction. Go and KEGG enrichment analysis was performed using WEB-based Gene Set Analysis Toolkit (WebGestalk, http://www.webgestalt.org/) [22,23]. The initial parameter setting is shown as follows: species parameter set to "Homo sapiens," filter values parameter set to 0.05, and the term with fewer than 3 detected genes were filtered out. Based on the results of enrichment analysis, the TCM compound regulatory network and the compound-targets-pathway regulatory network were visualized on Cytoscape 3.7.2 software [24].

2.4. PPI Network Construction and Topological Analysis. Overlapping protein targets of DQs associated with IS were considered as initial nodes. Next, the "input nodes and its neighbors" method was used to construct the PPI network using the "bisoGenet" plugin on Cytoscape. The species parameter was set to "Homo sapiens." "Database of Interacting Proteins," "Human Protein Reference Database," "Biological General Repository for Interaction Datasets," "Molecular INTeraction Database," "IntAct molecular interaction database," and "Biomolecular Interaction Network Database" were the main resources for PPI network construction. To extract the core module, we used a method combining degree centrality (DC) and betweenness centrality (BC) values, which was effective in identifying key proteins [25]. DC and BC reflect the influence of corresponding nodes in the full network. The higher the DC and BC, the more significant the node.

2.5. Molecular Docking. 3D structures of key active compounds were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov) and saved in SDF format and subsequently converted to PDB format using OpenBabal 2.3.0. Crystal structures of key receptors were downloaded from protein data bank (PDB, http://http://www.pdb.org) and processed by removing ligand and water motifs and adding hydrogen using the Discovery Studio software. Rotatable bonds were then set in the flexible residues and converted to PDBQT type and resulting grid center site information analyzed using AutoDockTools-1.5.6. Moreover, AutoDock-Vina software was used to calculate binding affinity and find probable binding sites.

2.6. Label-Free Mass Spectrometry Analyses. For evaluating the protein expression changes after DHI treatment in patients with acute ischemic stroke, a total of 6 acute ischemic stroke patients and 6 healthy volunteers without diseases including cerebral injury were enrolled from General Hospital of Northern Theater Command (Trial registration: ClinicalTrials identifier: NCT02176395). The peripheral venous blood samples of healthy volunteers, patients before treatment, and patients after day 14 of DHI treatment were obtained. Firstly, the albumin was removed from the serum using the Abundant Protein Depletion Spin Columns kit (ThermoScientific). Next, the label-free mass spectrometry analyses were carried out on the nLC-Easy1000-Orbitrap Fusion (ThermoScientific). Thirdly, protein identification was performed in the NCBI Database using Mascot2.3. Statistical differences between groups were analyzed using Kruskal–Wallis test for nonparametric values with SPSS23.0.

2.7. GEO Database Validation of DHI Treating IS. To further validate the effects of DHI for the treatment of IS, the genetic samples (series: GSE106680) were obtained from GEO databases (https://www.ncbi.nlm.nih.gov/geo/). In this study, Sprague Dawley (SD) rats were divided into 3 experimental

groups as follows: sham group, vehicle group, and DHI group, each group with 3 samples. To study on protective effects of cerebral ischemia/reperfusion-induced damage, the expression changes of the key receptors after 14-day treatment in cerebral ischemia/reperfusion model were compared among the 3 groups [14]. Statistical differences between groups were analyzed using ANOVA with SPSS23.0.

3. Results

3.1. Identification of Active Compounds and Putative Target Proteins. Details on investigating the pharmacological mechanisms of DHI against IS are shown in Figure 1. DHI consists of Danshen and Honghua. 45 DS and 12 HH components were obtained from the TCMSP database. After screening by pharmacokinetic properties and the Lipinski rule of 5, duplicate removal, and verification on PubChem, 37 active compounds were obtained and the divided into 4 categories: 26 diterpenoid quinones (DQs) (e.g., dehydrotanshinone II A, Danshenol A, and tanshinaldehyde, among others), 3 terpenes (e.g., arucadiol, przewalskin B, and sclareol), 2 flavonoids (e.g., baicalein and carthamidin), and 6 others (e.g., isoimperatorin and Microstegiol) (Figure 2). Based on ETCM database analysis, 21 compounds exhibited a good grade using the ADMET criterion (Table S1).

Higher combining probability indicated a close integration between compounds and targets. A total of 371 putative targets were identified by combined probability score along with the 37 candidate compounds using QSAR-TargetNet. After duplicate removal and name conversion, 66 targets were obtained, 41 of which were putative target proteins of DQs.

Based on this, a compound-target (CT) network was constructed. Figure 3 shows putative targets surrounded by various categories of grouped compounds. Candidate compounds are divided into good, moderate, week, and N/A, marked by purple, yellow, gray, and white borders, respectively. Of these, 20 compounds with good grade belong to DQs, 95% of total. We found that DQs have good pharmacokinetic properties based on the ADMET criterion, and that they may be the main active compounds driving DHI neuroprotective effects (Table S2).

3.2. Potential DHI Targets in Ischemic Stroke Treatment. CTD and GeneCards analyses identified 107872 and 3443 IS-associated gene entries, respectively. With prioritized inference and relevance scores, 436 gene entries were identified from the 2 databases and merged. These targets served as key putative IS-associated proteins (Tables S3-S4). Of the predicted DHI targets, 13 target proteins associated with IS were found, including caspase-9, MAOB, MAOA, NR3C1, CDC25B, RARA, CYP1A2, MCL-1, HSP90AA1, PTGS1, CYP2C19, ABCB1, and RELA. Of these, 8 belonged to the DQs (Figure 4(a)). The putative proteins of DQs' targets associated with IS can uncover the potential functions of DHI treating IS.

3.3. Core Module and Hub Nodes in PPI Network. We obtained a PPI network comprising 1162 nodes and 19211 edges, based on 8 overlapping target proteins belonging to DQs (see Figure 4(c) and 4(d)). Next, a subnetwork comprising 233 nodes and 6148 edges was extracted by top 20th percentile of DC from the PPI network, and then a core module comprising 70 proteins was re-extracted by top 6th percentile of BC from the subnetwork above. We found that 5 of 8 overlapping protein targets of DQs, MCL-1, HSP90AA1, NR3C1, CASP9, and RARA were always in the subnetwork and the core module. Notably, HSP90AA1 was the hub node with highest DC and BC (Tables S5-S6), and 3 of those 5 overlapping protein targets were involved in phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB/Akt) signaling.

3.4. Biological Function of DQs Targeting on IS. To provide further insight into the mechanisms underlying DQs effects on IS at the systematic level, GO and KEGG pathway analyses were done and gene functions depicted based on effect on biological process (BP), cellular component (CC), and molecular function (MF). There were 50 GO terms and 2 KEGG pathways enriched from the 8 overlapping target proteins (Tables S7-S8). The GO-BPs mainly involve in oxygen-containing compound, organic cyclic compound, and apoptotic process, and the 2 KEGG pathways were PI3K-Akt signaling pathway and pathways in cancer. Notably, these two KEGG pathways were the common pathways enriched from the DQs target proteins and DHI target proteins (Figure 4(b) and Table S9). According to KEGG enrichment analysis and PPI network analysis, the PI3K-Akt signaling pathway may be the most important signaling related to the treatment of DHI for IS. Based on these, a compound-targets-pathway (CTP) network was constructed (Figure 5).

3.5. The Affinity between Compounds and Receptors. Binding energy can be calculated to predict affinity between 2 counterparts. Twenty-one active DHI compounds with good pharmacokinetic properties were molecularly docked with 5 key receptors including MCL-1 (PDB ID: 3MK8), HSP90AA1 (2BTH), caspase-9 (2AR9), RARA (5K13), and NR3C1 (6CFN). Binding energy less than 0 indicated that spontaneous combination occurred between 2 molecules. The lower the binding energy is, the stronger the affinity between compounds and targets is. A total of 105 ligand-receptor combinations were computed. Except RARA, most DHI components bind well with key receptors and 93 combinations had affinities of < -7 kcal/mol, accounting for 88.6%, with the strongest binding being with Casp9 (–10.6 kcal/mol) (Figure 6(a)). Molecular docking structures are detailed in Figure 6(b)–6(e).

3.6. Validation on the Targets of DHI for the Treatment of IS. According to the label-free mass spectrometry analyses from our previous trial, compared with healthy volunteers, the expression of HSP90AA1 was upregulated after IS ($P < 0.05$),

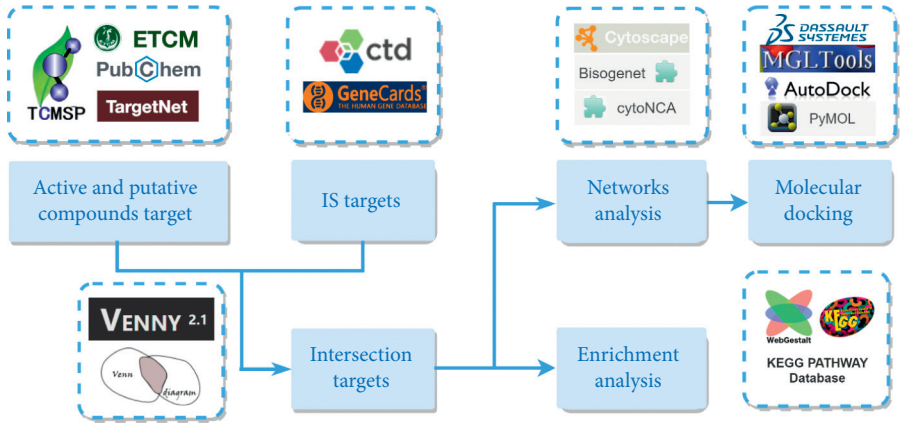


Figure 1: Network pharmacology approach for deciphering pharmacological mechanisms of DHI activity in cerebral ischemia.

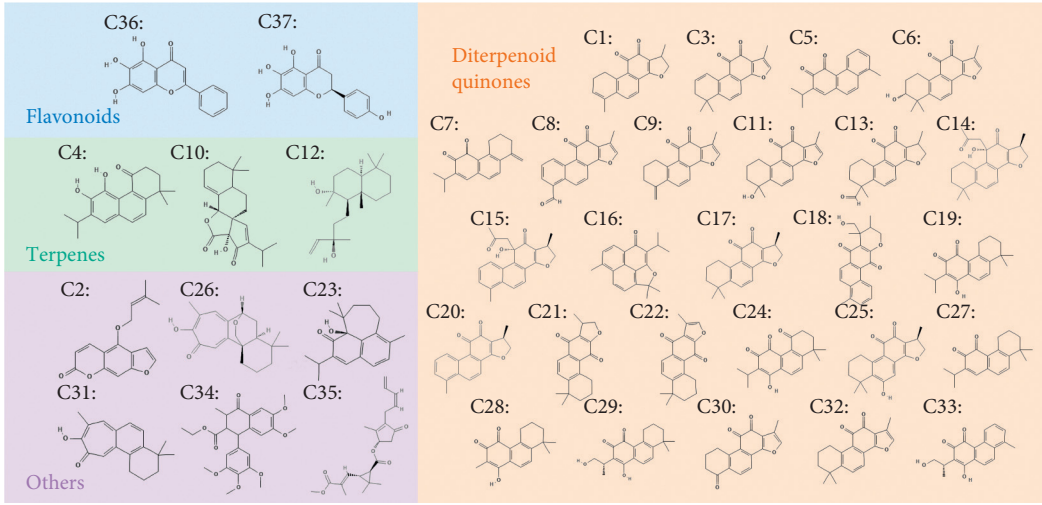


Figure 2: 2-dimensional (2D) molecular structures and classification of 37 DHI candidate compounds. There are 4 classifications, including diterpenoid quinones (26), terpenes (3), flavonoids (2), and others (6).

and after treated with DHI for 14 days, the HSP90AA1 expression was downregulated ($P < 0.05$) (Figure 7(a)). Moreover, according to the expression of mRNA from another independent experiment, compared with the sham group, the expression of Casp9 was downregulated ($P < 0.05$), while MCL-1 showed an upregulation trend in the vehicle. After 14-day treatment of DHI, the expression of Casp9 was upregulated and MCL-1 was downregulated, although there was no significantly statistical difference compared with the vehicle (Figures 7(b) and 7(c)).

4. Discussion

Based on chemical structure analysis and network pharmacology, we find DQs are the major category in DHI compounds. Biological processes of DQs protein associated with IS were involved in regulation of apoptotic, positive regulation of molecular function, and regulation of cell proliferation process, and PI3K-Akt signaling pathway may closely involve in mechanism of action of DQs proteins associated with IS. This find is consistent with the previous studies [26, 27], which confirmed the neuroprotective effect of DHI via the PI3K-Akt pathway, since the specific inhibitor of PI3K-Akt pathway could weaken the neuroprotective effect on brain damage due to ischemic reperfusion in the rats with the middle cerebral artery occlusion.

To make further investigation, we find core module and hub node in PPI network by using DC and BC values in our work. Casp9, Hsp90AA1, and MCL1 were identified from the core module involved in PI3K-Akt signaling. HSP90AA1 was the hub node since highest DC and BC. To verify this, molecular docking simulation was used to predict the binding interaction of compounds in reporter's binding pocket. The results shown that most compounds docked well with all three targets. PKB/Akt can mediate resistance to hypoxia-ischemia through survival and inactivation of apoptosis-associated proteins [28]. HSP90AA1 as the hub node of DHI for the treatment of IS, which could protect Akt kinase activity from dephosphorylation [29]. The active Akt subsequently regulate various targets, including caspase-9

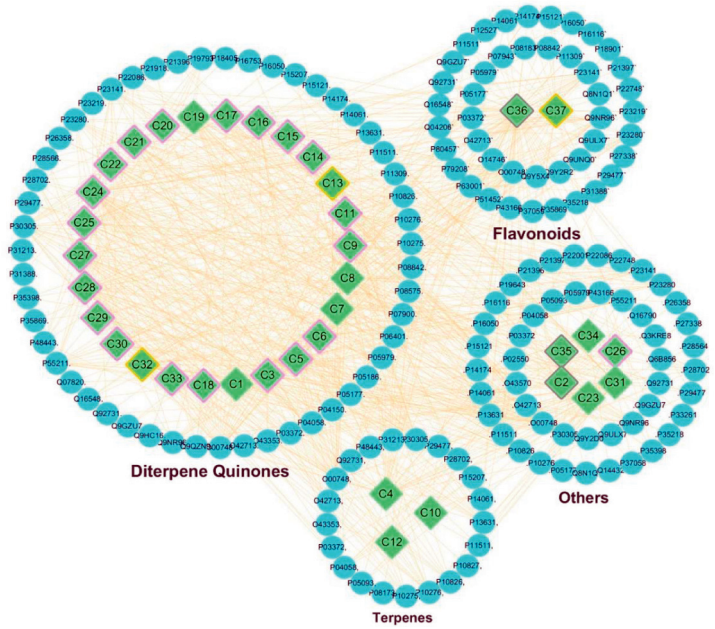
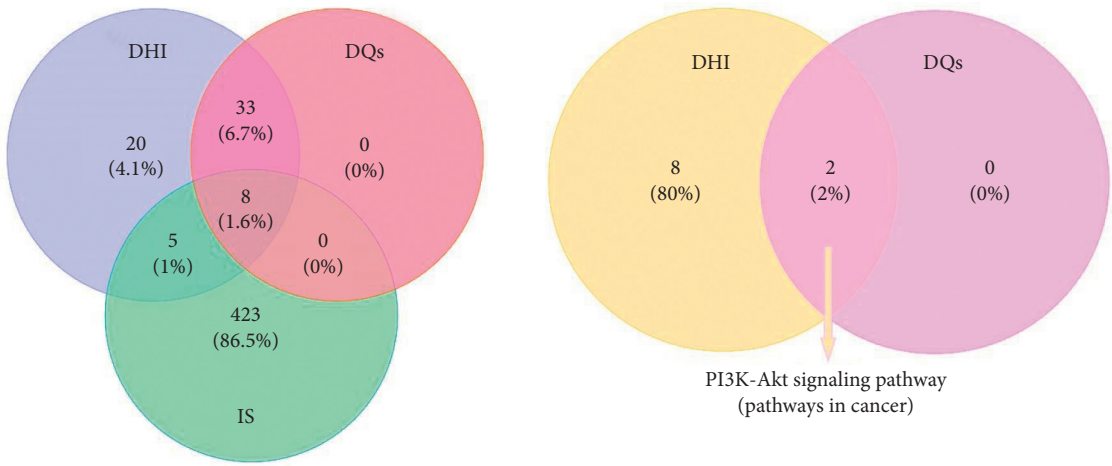
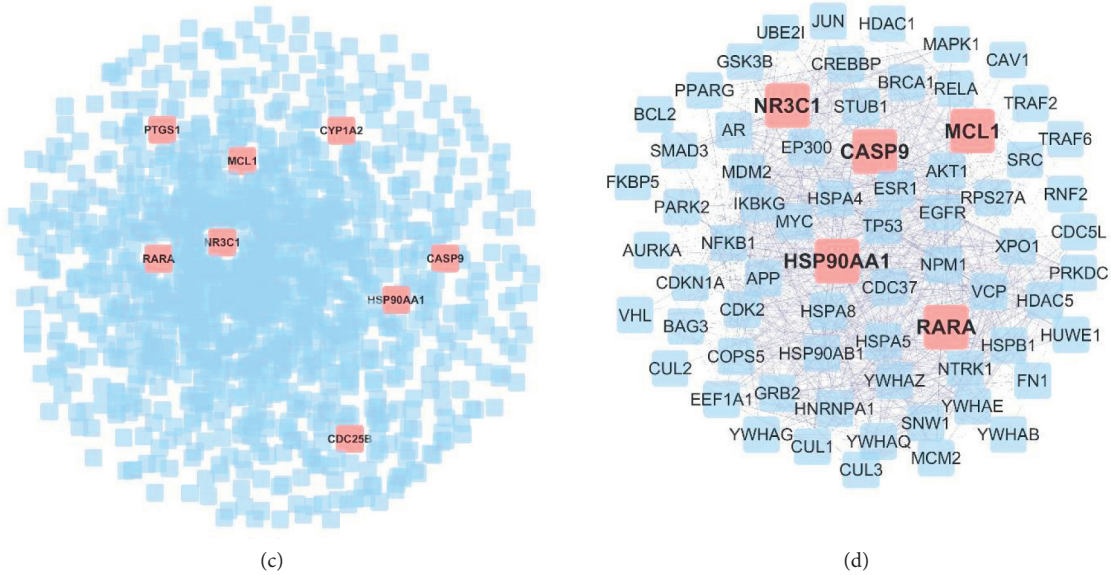


Figure 3: Construction of a compound-target regulatory network and the classification of putative target proteins of DHI compounds. The candidate compounds (diamond mesh node with green) are divided into groups by structural category. The candidate compounds are divided into good (purple border), moderate (yellow border), week (gray border), and N/A (no border). Similarly, the putative target proteins (the ellipse mesh node with blue) are grouped and surrounded with corresponding compounds.



(a) (b)

Figure 4: Continued.



(c) (d)

Figure 4: Topological analysis of the protein-protein interaction network. (a) Overlapping target proteins Venn diagram between DHI targets, DQs targets, and IS targets. (b) Overlapping KEGG signaling Venn diagram between DHI and DQs. (c) PPI network constructs based on 8 overlapping targets of DQs associated with IS. Herein, 1162 protein nodes were obtained. After extracted by top 20^{th} percentile of DC and top 6^{th} percentile BC, a total of 70 nodes were obtained. (d) Core module identified by PPI network analysis. 5 overlapping protein associated with PI3L/Akt signaling are marketed as red squares.

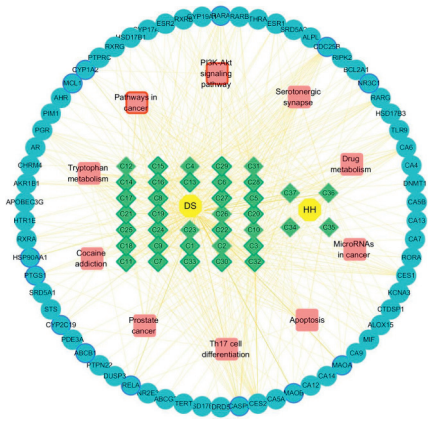


Figure 5: Construction of compound-target-pathway network. Yellow diamond represents herbs in DHI; green diamond represents active compounds, with green border representing good pharmacokinetic properties; red rectangle represents KEGG signaling enriched form 13 overlapping target proteins of DHI associated with IS, and the KEGG signaling enriched from 8 overlapping targets of the DQs associated with IS was annotated with red border; blue ellipse nodes indicate putative targets of DHI, and targets of DQs annotated with blue border.

and myeloid cell leukemia-1 (MCL-1) [30–33]. MCL-1 involved in proapoptotic function and led to the activation of the downstream caspase cascade [34–36]. Finally, our label-free mass spectrometry results show that Hsp90aa1 may be modulating apoptosis via modulation of PI3K/Akt signaling.

Therefore, our results indicated that DHI and its major category DQs effectively exert antiapoptosis functions by regulating HSP90AA1-induced PI3K/Akt signaling and other downstream molecules like MCL-1 and caspase-9 (Figure 8). In summary, our findings offer new insights on future DHI research and its applications in IS treatment. However, some shortcomings of our study should be considered. Apoptosis is a complicated process regulated by multitargets. Although our results were verified using label-free mass spectrometry and transcriptome RNA-sequencing, the experimental work with large sample size and multiple-time-point will be done in future study to find more evidence.

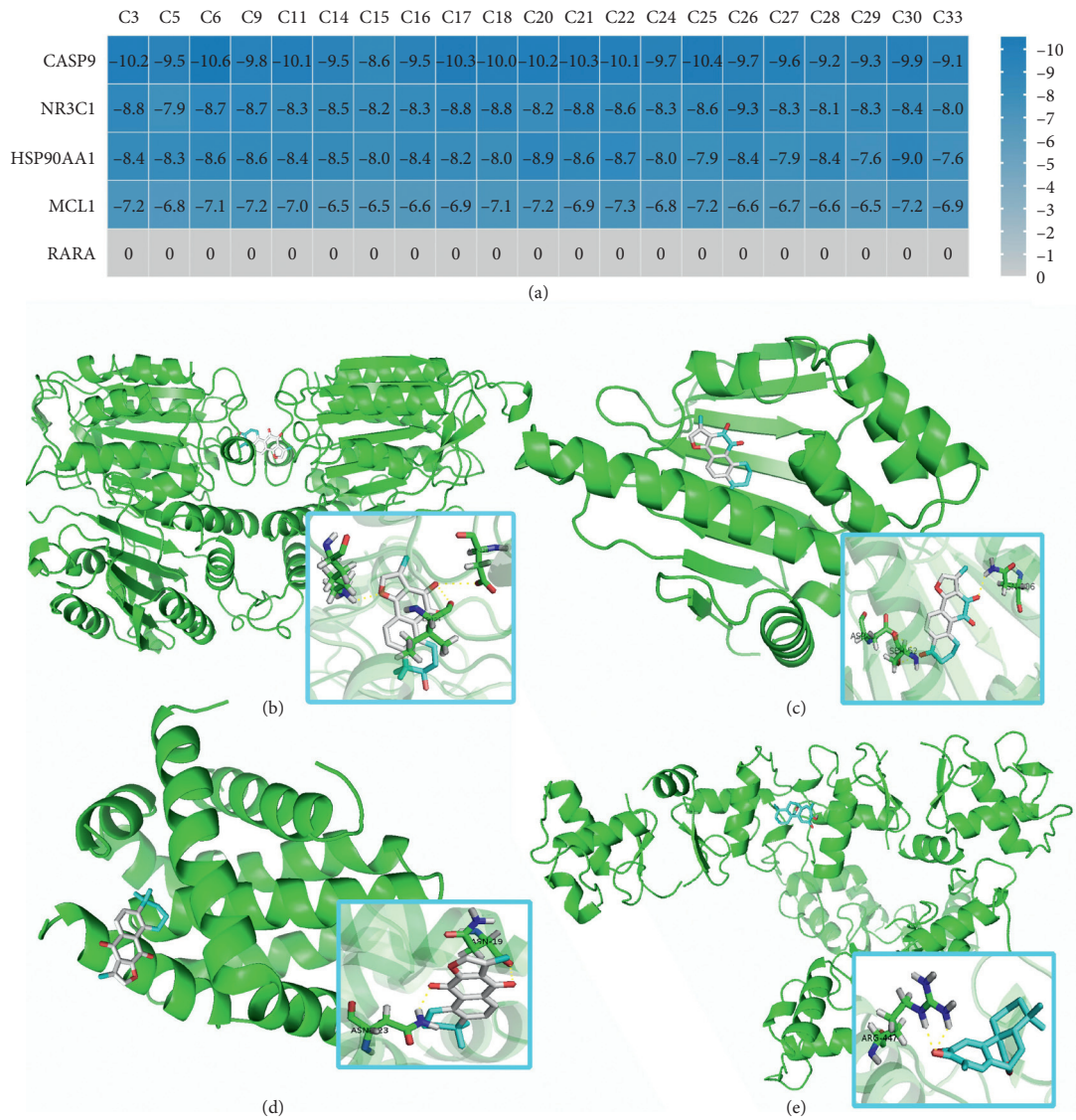


Figure 6: Results of molecular docking and docking simulation. (a) Heat map of the binding energies. (b) 3alpha-hydroxytanshinone IIa (C6) in the protein caspase-9 (PDB ID:2AR9). (c) Nortanshinone (C30) with HSP90AA1 (2BTH). (d) Isotanshinone IIa (C22) with MCL-1 (3MK8). (e) Miltipolone (C26) with NR3C1 (6CFN).

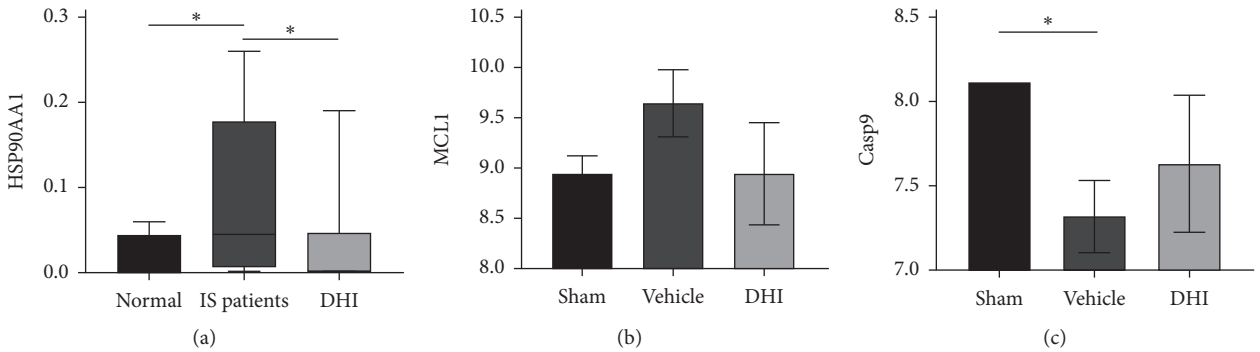


Figure 7: The expression results of 3 nodes in core module associated with PI3K/Akt signaling. (a) The mass spectrometric quantification result of HSP90AA1. (b, c) The MCL-1 and Casp9 mRNA expression levels from mRNA-sequencing results, respectively ($^*P < 0.05$).

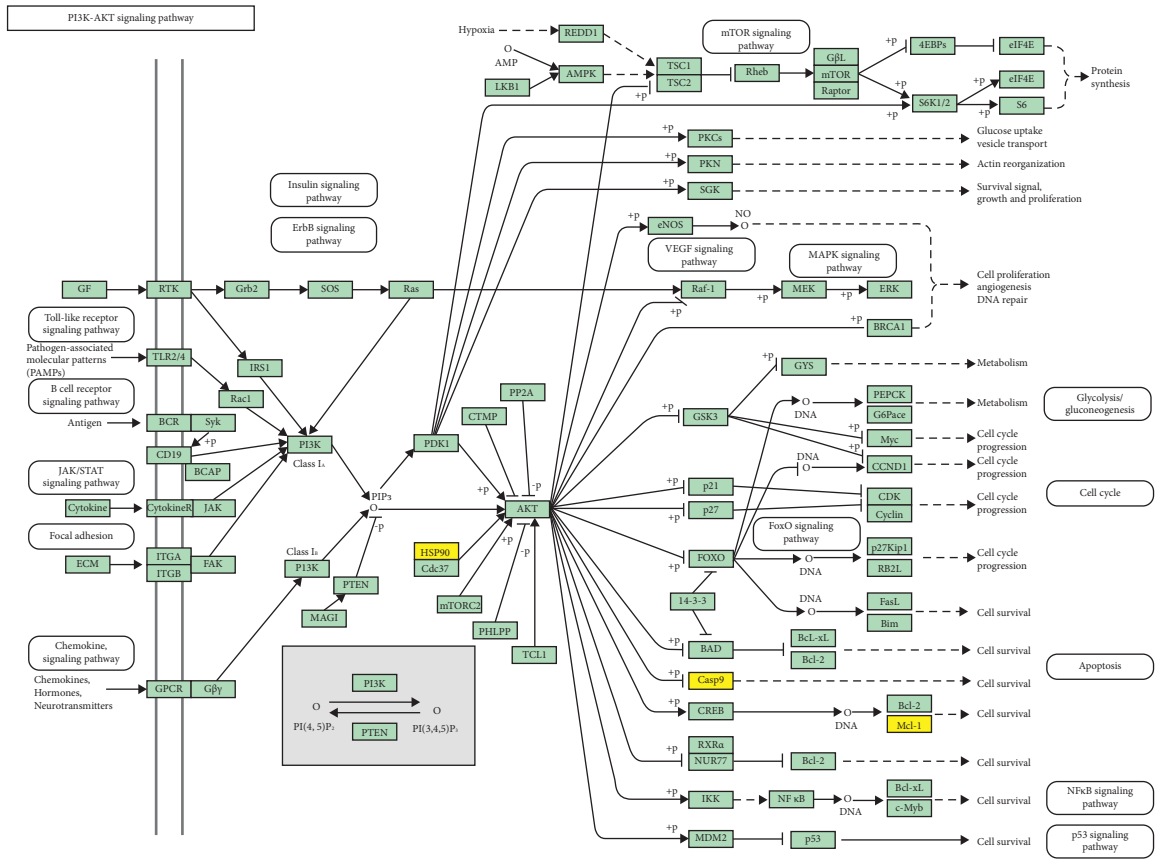


Figure 8: Modulating PI3K-Akt signaling pathway. Key receptors are shown in yellow, and other protein targets are in green (picture derived from KEGG database).

5. Conclusion

Here, we used network pharmacology to investigate the potential mechanisms underlying DHI effects on IS. Our data show that DHI is antiapoptotic via multifaceted activity. DHI, especially the main category (diterpenoid quinones), appears to promote cell survival effects via PI3K-Akt signaling. It targets on both upstream and downstream of the PI3K-Akt signaling, which may be its main mechanism against IS. These results offer rationale for future DHI research and applications in treating IS.

Abbreviations

IS:	Ischemic stroke
r-TPA:	Recombinant tissue plasminogen activator
DHI:	Danhong injection
DS:	Danshen
HH:	Honghua
BBB:	Blood-brain barrier
TCMSP:	Traditional Chinese Medicine Systems Pharmacology database
ETCM:	Encyclopedia of Traditional Chinese Medicine
OB:	Oral bioavailability
DL:	Drug-likeness
2D:	Two-dimensional
CTD:	Comparative Toxicogenomics database
QSAR-TargetNet:	Quantitative structure activity relationships-TargetNet
WebGestalk:	WEB-based gene set analysis toolkit
DQs:	Diterpenoid quinones
GO:	Gene Ontology
KEGG:	Kyoto Encyclopedia of Genes and Genomes
3D:	Three-dimensional
PDB:	Protein data bank
CT:	Compound-targets
BP:	Biological process
CC:	Cellular component
MF:	Molecular function
CTP:	Compound-targets-pathway
DC:	Degree centrality
BC:	Betweenness centrality
PI3K/Akt:	Phosphatidylinositol-3 kinase/protein kinase B
HSP90:	Heatshock protein 90
MCL-1:	Promotes myeloid cell leukemia-1
NF-κB:	Nuclear factor kappa-B.

Data Availability

All data used to support the findings of this study are included within the figure and supplementary tables.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Table S1: the 37 candidate compounds of Danhong injection. Table S2: the 371 putative target proteins for the compounds. Table S3: the 413 IS-associated *Homo sapiens* target proteins from CTD with an inference score of ≥50. Table S4: the 61 IS-associated target proteins of *Homo sapiens* from Genecards with an inference score of ≥30. Table S5: degree centrality of nodes in PPI network. Table S6: betweenness centrality of nodes in the PPI network. Table S7: the GO functional enrichment analysis of diterpenoid quinones. Table S8: the KEGG pathway enrichment of diterpenoid quinones. Table S9: the KEGG pathway enrichment of DHI compounds. (*Supplementary Materials*)

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Review Article

The Protective Effect of Traditional Chinese Medicine on Liver Ischemia-Reperfusion Injury

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Liver ischemia-reperfusion (I/R) injury occurs during transplantation and major hepatic surgery, which may lead to postoperative liver dysfunction. More and more traditional Chinese medicines (TCMs) have been used to treat liver ischemia-reperfusion injury. The purpose of this review is to evaluate the different protective effects of TCMs in the treatment of liver ischemia-reperfusion injury and to summarize its possible mechanisms. The results indicate that TCMs attenuate liver I/R injury via multiple mechanisms, including antioxidation stress, anti-inflammatory response, antiapoptosis, and inhibiting endoplasmic reticulum stress. However, the in-depth mechanism of the protective effects of these traditional Chinese medicines still remains unknown.

1. Introduction

Ischemia-reperfusion (I/R) injury is a two-stage phenomenon in which blood flow into the organ is reduced, leading to tissue hypoxia and cell damage, followed by aggravated injury when oxygen delivery is restored [1]. Liver ischemia-reperfusion injury (I/RI) occurs during transplantation and major hepatic surgery, which may result in postoperative liver dysfunction [2, 3]. The cessation of blood flow to an organ can lead to oxygen and nutrient deficiencies which can induce inflammatory cell infiltration, production of oxygen-derived reactive oxygen species (ROS) and nitrogen-derived reactive nitrogen species, and process during the reperfusion period [4]. Reperfusion injury mainly comes from toxic ROS produced by ischemic tissue when oxygen is reintroduced. ROS can be derived intracellularly and extracellularly, and mitochondria in liver cells are the main source of ROS [5].

Herbal medicine has drawn more and more attention in recent years. According to the World Health Organization, approximately 80% of the global population relies on traditional herbal medicines as part of standard health care [6]. A series of traditional Chinese medicine ingredients have been used to treat liver ischemia-reperfusion injury and have achieved good results (see Table 1). However, traditional Chinese medicine is a multicomponent medicine and plays an effective role through multiple targets and pathways, including antioxidation stress, anti-inflammatory response, antiapoptosis, and inhibiting endoplasmic reticulum stress. In this review, we summarize the effects of TCMs on I/R-induced liver injury, with focus on the possible underlying mechanisms.

2. The Protective Effect of TCMs on Liver Ischemia-Reperfusion Injury and Potential Mechanisms

During I/R, some functional changes that occur at the cellular level may cause cell damage via production of ROS,

Table 1: The protective effect of TCMs on liver ischemia-reperfusion injury and potential mechanisms.

Traditional Chinese medicine	Major active ingredients	Models	Animals	Protective effects	Potential mechanisms	Ref.
Atractylodes macrocephala	*Atractylodes macrocephala* polysaccharide (AMP)	Hepatic I/RI model	SD rats	Antioxidation stress	NF-κB signaling pathway	Jin et al. [7]
Saffron	Saffron ethanol extract (SEE)	Hepatic I/RI model	Wistar rats	Antioxidation stress; inhibition of endoplasmic reticulum stress	—	Pan et al. [8]
Breviscapus	Breviscapine	Hepatic I/RI model	SD rats	Antioxidation stress	Mfn2/Ras-PI3K-Akt pathway	Lin et al. [9]
Salvia miltiorrhiza	Caffeic acid (CA)/3, 4-dihydroxycinnamic acid	Hepatic I/RI model	SD rats	Antioxidation stress; Anti-inflammatory response	Sirt3 signaling pathway	Mu et al. [10]
		Liver transplantation model	SD rats, hepatocyte	Antioxidation stress; anti-inflammatory response	PDIA3-NADPH signaling pathway	Mu et al. [11]
	Magnesium lithospermate B (MLB)	Hepatic I/RI model	C57BL/6 mice	Anti-inflammatory response	NF-κB signaling pathway	Song et al. [12]
	Tanshinone IIA (Tan IIA)	Hepatic I/RI model	C57BL/6 mice	Anti-inflammatory response	TLR4 signaling pathway	Qi et al. [13]
Huperzia serrata	Huperzine A (HupA)	Hepatic I/RI model	Wistar rats	Antioxidation stress; antiapoptosis	—	Xu and Wang [14]
Gynostemma pentaphyllum	Gypenoside (GP)	Hepatic I/RI model	C57BL/6 mice	Antioxidation stress; antiapoptosis	—	Zhao et al. [15]
Glycyrrhiza uralensis	Glycyrrhizin (GL)/ Glycyrrhizic acid	Hepatic I/RI model	SD rats	Antioxidation stress	Nrf2/HO-1 signaling pathway	Kou et al. [16]
Tripterygium wilfondii Hook F	Triptolide (diterpenoid triepoxide)	Hepatic I/RI model	C57BL/6 mice, splenocytes	Anti-inflammatory response	STAT3 signaling pathway	Wu et al. [17]
Kudzu	Puerarin/7, 4-dihydroxyisoflavone-8β-glucopyranoside	Hepatic I/RI model	SD rats	Anti-inflammatory response	TLR4/NF-κB pathway	Xiao et al. [18]
Corydalis yanhusuo	Levo-tetrahydropalmatine (L-THP)	Hepatic I/RI model	BALB/*c* mice	Anti-inflammatory response; antiapoptosis	ERK/NF-κB pathway	Yu et al. [19]
Astragalus membranaceus	Astragaloside IV (AST-IV)	Liver transplantation model	SD rats	Anti-inflammatory response	NF-κB signaling pathway	Chen et al. [20]
Chinese medicine mixture	Xuebijing (XBJ)	Hepatic I/RI model	C57BL/6 mice	Anti-inflammatory response	NF-κB signaling pathway	Liu et al. [21]
Ginkgo biloba leaf	*Ginkgo biloba* Dropping Pill (GBDP)	Hepatic I/RI model	C57BL/6 mice, hepatocytes	Antiapoptosis	—	Wang et al. [22]
Chinese medicine mixture	Berberine	Liver transplantation model	Wistar rats	Inhibiting endoplasmic reticulum stress	—	Zhang et al. [23]

inflammatory cytokines, and chemokines. These events trigger the apoptotic pathway and ultimately lead to organ failure [24]. According to current researches, the protective effect of TCMs on liver I/R is mainly involved in several mechanisms: antioxidation stress, anti-inflammatory response, antiapoptosis, and inhibiting endoplasmic reticulum stress. Also, there are two main models used in animal experiments: the hepatic I/RI model and liver transplantation model.

2.1. Antioxidative Stress. It is well known that oxidative stress and reactive oxygen intermediates play important roles in liver ischemia-reperfusion injury. Free radicals formed by oxidative stress damage the cell membrane of hepatocytes through lipid peroxidation or/and other means. Furthermore, these free radicals can cause extensive damage to DNA and proteins, which can eventually lead to acute and chronic liver damage [25–27]. Lots of research studies are focused on antioxidant compounds extracted from herbal

medicines to address the mechanism of its clinical protective effect to liver I/R injury.

In I/RI model rats, *Atractylodes macrocephala* polysaccharide (AMP), the principal bioactive component of *Atractylodes macrocephala*, significantly inhibited lipid peroxidation and altered the activities of the antioxidant enzyme, superoxide dismutase, and malondialdehyde level, which is associated with its antioxidant properties and inhibition of NF-κB activation [7]. Saffron ethanol extract (SEE) contains abundant flavonoid compounds with antioxidant effect [28]. A study found that SEE could reduce liver IR damage by scavenging free radicals, maintaining physiological ROS level, and attenuating oxidation-mediated chaperone carbonylation [8]. Mitochondria are the main source of ROS in cells. Mitochondrial damage leads to an increase in ROS production, which results in oxidative stress [29]. Mitofusin 2 (Mfn2), located in the outer mitochondrial membrane, has the function of controlling mitochondrial metabolism [30]. Lou et al. found that breviscapine, a flavonoid compound extracted from the natural plant *Erigeron breviscapus* [31], could attenuate liver I/R injury by reducing lipid peroxidation and downregulating the expression of Mfn2 via inhibiting the Ras-PI3K-Akt pathway [9]. Caffeic acid (CA), a single phenolic acid derived from *Salvia miltiorrhiza* [32], is associated with chondriosome. It was found to have a protective effect on I/RI by reducing liver microcirculation disturbance and oxidative damage through regulating Sirt3 and the mitochondrial respiratory chain [10]. Further research shows that PDIA3 (protein disulfide isomerase A3) activates NADPH oxidase and causes the burst of ROS. CA may protect the transplanted liver by inhibiting PDIA3-NADPH oxidase [11]. TCMs exert hepatic ischemia-reperfusion injury protection through antioxidative stress which is also observed in huperzine A (HupA), gypenoside (GP), and glycyrrhizin (GL) [14–16].

2.2. Anti-Inflammatory Response. The liver undergoes a strong inflammatory process during ischemia and reperfusion injury. This liver inflammation is initially triggered by ischemia. However, the inflammation mainly occurs during the reperfusion phase and is characterized by the recruitment of large numbers of neutrophils in the liver. The production of cytokines, chemokines, and danger signals activates resident liver cells, white blood cells, and Kupffer cells [33]. The following research studies have authenticated that TCMs attenuate liver ischemia and reperfusion injury through an anti-inflammatory response pathway.

Ischemia reperfusion is considered to be a complex cascade of inflammatory mediators involved in the pathogenesis of liver injury. Different from caffeic acid, although magnesium lithospermate B (MLB) and Tanshinone IIA (Tan IIA) are also the main components of *Salvia miltiorrhiza*, they mainly exert anti-inflammatory effects. MLB can prevent the activation of inflammatory signaling pathways, reduce the expression of inflammatory mediators, and decrease the infiltration of macrophages and neutrophils, thereby reducing the damage of liver cells induced by IR [12]. It was reported that IL-17 contributes to the accumulation of neutrophils in the inflammatory liver. Triptolide, a purified ingredient of shrub-like vine *Tripterygium wilfondii* Hook F, can reduce the expression of IL-17 by inhibiting transcription 3 (STAT3) phosphorylation, thereby inhibiting the recruitment of neutrophils in the process of liver I/R [17]. In addition, there is evidence that Tan IIA pretreatment can reduce inflammation infiltration and liver damage. The underlying mechanism may be that Tan IIA inhibits the Toll-like receptors 4 (TLR4) signaling pathway, thereby enhancing the expression of HO-1 and reducing the expression of liver proinflammatory cytokines [13]. In Xiao's experiment, puerarin nanoparticle synthesis significantly decreased the TLR4 and NF-κB expressions, which showed that puerarin can display its protective role by restraining the activation of proinflammatory factors through the TLR4/NF-κB fashion [18].

Proinflammatory cytokines such as TNF-α and IL-6 play a key role in liver I/R injury. It has been found that levo-tetrahydropalmatine (L-THP), an active component of *Corydalis yanhusuo*, can inhibit the release of TNF-α and IL-6 induced by liver I/R, and this protective effect is partly dependent on the inhibition of the TNF-α-mediated ERK/NF-κB pathway [19]. Similarly, astragaloside IV (AS-IV), a small molecular saponin, protects liver against ischemia-reperfusion injury by inhibiting the activation of NF-κB in the reperfusion phase and reducing TNF-α [20]. Liu et al. found that Xuebijing (XBJ) with protective function of liver I/RI is largely due to its direct effect on the activation of hepatocyte inflammasomes and caspase-1-dependent IL-1β production, in addition to affecting the production of inflammatory factors/chemokines by Kupffer cells through NF-κb-dependent mechanisms [21].

2.3. Antiapoptosis. Oxidative stress and/or mitochondrial dysfunction induced by hepatic ischemia reperfusion can eventually activate apoptotic cascade. Caspase-3 and -8 are key members of the cysteine-aspartate-specific protease family and have been shown to be essential for apoptosis [34]. Hepatocyte apoptosis is one of the most important cell death types in the process of liver I/R injury [35]. The activation of caspase-3 and caspase-8 was found in various apoptotic cells [36], and the upregulation of caspase-3 and caspase-8 was also found in I/R-induced liver injury, indicating that caspase-mediated apoptosis is essensial in organ I/R injury [37]. In addition to the caspase pathway, Bcl-2 family proteins also play a key role in the regulation of neuronal apoptosis. The following studies have demonstrated that TCMs have an effective performance in hepatic ischemic reperfusion injury through the antiapoptosis pathway.

Moreover, HupA, an alkaloid extracted from *Huperzia serrata*, can reduce liver I/R damage by reducing the expression of apoptosis-related proteins caspase-3, Bcl-2, and Bax [14]. The antiapoptotic effect of GP, the main ingredient of *Gynostemma pentaphyllum*, is related to the inhibition of I/R-induced increase in the activities of proapoptotic proteins Bax, cytochrome c, and caspase-3/8, as well as the

decrease in the level of antiapoptotic protein Bcl-2 [15]. In Wang's study, it was shown that the *Ginkgo biloba* Dropping Pill (GBDP) can inhibit the expression of apoptosis-related protein markers in vitro. Consistent with the results of in vitro experiments, animal experiments confirmed that GBDP can downregulate the expression of proapoptotic proteins and reduce hepatocyte apoptosis caused by liver I/R injury [22].

2.4. Inhibition of Endoplasmic Reticulum Stress. Endoplasmic reticulum (ER) stress refers to the continuous accumulation of misfolded or unfolded proteins in the endoplasmic reticulum lumen, which activate the unfolded protein response (UPR) under pathological conditions. In a steatotic liver, endoplasmic reticulum stress is considered to be the main cause of posttransplant injury [38].

Zhang et al. found that berberine (BBR), a compound derived from the traditional Chinese medicine plants, inhibits endoplasmic reticulum stress-mediated phagocytosis in steatotic liver transplantation. It is the first report that addresses the protective function of BBR on steatosis liver transplantation, but the specific mechanism involved still remains unclear [23]. Besides, saffron ethanol extract can also relieve the endoplasmic reticulum stress and protein ubiquitination induced by liver I/R [8].

3. Conclusions and Prospects

Through this review, we get some similarities from articles published in recent years. Firstly, traditional Chinese medicines with protective effect of liver I/R injury are mostly the main active substances of Chinese medicines. Secondly, TCMs protect liver function via multiple mechanisms, including antioxidation, anti-inflammatory, inhibition of cell apoptosis, and inhibition of endoplasmic reticulum stress, of which antioxidant and anti-inflammatory effects are most commonly reported. Also, the NF-κB signaling pathway is the most frequently involved signaling pathway. Thirdly, different components of traditional Chinese medicines may exert protective effects through different mechanisms. Lastly, the specific signaling pathways involved in these mechanisms remain unkown.

In conclusion, traditional Chinese medicine has protective effect on liver I/R injury. However, future research should pay more attention to in-depth mechanism exploration rather than just descriptive observations. Moreover, research should also clarify which components of Chinese medicine mainly play a protective role in liver ischemia reperfusion. Furthermore, it is necessary to conduct experiments both in vivo and in vitro to increase the convincing power of the experimental results. Finally, large sample, randomized, double-blind, placebo-controlled, and multicenter clinical trials are still in need.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Wen Ma and Songling Tang contributed equally to this study.

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Research Article

Resveratrol Alleviates Vascular Endothelial Damage Caused by Lower-Extremity Ischemia Reperfusion (I/R) through Regulating Keap1/Nrf2 Signaling-Mediated Oxidative Stress

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The present study aims to investigate the protective effects of Resveratrol (RSV) against vascular endothelial damage caused by lower-extremity I/R and the underlying preliminary mechanism. The in vitro hypoxia reoxygenation (HR) model was established on HUVECs. Lower-extremity I/R model was established on rats followed by being treated with RSV and the pathological state of artery was evaluated by HE and EVG staining, while the apoptotic state of artery was detected by TUNEL assay. The cell viability was detected by MTT assay and the apoptotic state of cells was determined by Hoechst test and flow cytometry assay. DCFH-DA staining was used to measure the level of ROS and the production of MDA and SOD was measured by commercial kits. The expression level of Nrf2, Keap1, HO-1, Bcl-2, Bax, and Caspase-3 in cells was determined by Western blot. Nrf2 was knocked down by siRNA technology. Overall, our data indicated that increased cell viability, declined apoptotic rate, and alleviated oxidative stress were observed in RSV treated HR HUVECs, which were significantly reversed by knocking down Nrf2. Animal experiment revealed that the pathological and apoptotic state of femoral artery were dramatically ameliorated by the treatment of RSV, accompanied by the alleviated oxidative stress, which were abolished by the co-administration of ML385, an inhibitor of Nrf2. Taken together, our data revealed that RSV might alleviate vascular endothelial injury induced by lower-extremity I/R injury through regulating Keap1/Nrf2 signaling-mediated oxidative stress.

1. Introduction

Acute lower extremities ischemia mainly results from interrupted flow of the distal artery in the limb induced by arterial thrombosis, arterial injury, and arterial emboli of lower limbs, which is a common arterial disease in vascular surgery department [1]. Myocyte necrosis, as well as the loss of sensation and movement, will be observed approximately 3 hours post-ischemia, with irreversible injury induced about 6 hours later [2]. Due to high disability rate and mortality, public health and safety are being severely threatened by acute lower extremities ischemia [3]. The normal metabolism and function of tissues are significantly impacted by the prolonged tissue or organ ischemia and restoring blood supply to ischemic tissues and organs is an indispensable condition to recover normal functions. However, further damage will be induced to tissues by restoring blood supply following prolonged ischemia, which is defined as lower-extremity ischemia reperfusion (I/R) injury [4]. Lower-extremity I/R damage is mainly pathologically characterized as cell swelling, inflammatory cell infiltration, and cell apoptosis, which further contribute to systemic inflammation, damaged respiratory, renal, and hepatic function [5]. It is reported that series of inflammatory processes are involved in the development of lower-extremity I/R injury, including infiltration of neutrophile granulocytes, injured endothelial cells, excessive released cytokines, and superabundant production of reactive oxygen species (ROS) [6], among which ROS, as well as the induced oxidative stress, plays an important role as the initiating step of oxidative stress [7]. ROS is mainly generated from neutrophile granulocytes, myocytes, vascular endothelial cells, and perivascular tissues. Under normal circumstances, the production and elimination of ROS are maintained as an

equilibrium state. However, once the excessive produced ROS is not cleared, oxidative stress state will be induced, which contributes to the peroxidation of intracellular components in multiple tissues, oxidative damage, and finally tissue dysfunctions [8]. When ischemia occurs, aerobic respiration will be suppressed and anaerobic respiration will be facilitated due to hypoxia, which result in weakened oxidative reaction and increased release of lactic acid. However, as the reperfusion of blood flow, a great deal of oxygens will be instantly released into the body, which contributes to the excessive production of ROS. As a consequence, the cellular components will be oxidized and tissue injury will be induced by series of inflammatory reactions [9]. Therefore, oxidative stress might be an important target for the treatment of lower-extremity I/R injury.

Resveratrol (RSV) is a natural polyacid rich in red wine and has been proved to be healthful [10]. More and more bodies of evidence have claimed that the biofunction of RSV is closely related to its anti-oxidative stress property [11]. Rio reported that oxidative stress was significantly ameliorated in dystrophin-deficient *mdx* mice by RSV through alleviating mitophagy [12]. Huang reported that oxidative stress and mitochondrial dysfunction were dramatically prevented by RSV through regulating the PKA/LKB1/AMPK signaling [13]. This study aimed to investigate the protective function of RSV against in vitro hypoxia reoxygenation model and in vivo ischemia reperfusion model to explore the potential therapeutic property of RSV against lower-extremity I/R injury.

2. Materials and Methods

2.1. Cells and Hypoxia Reoxygenation (HR) Treatment. In the present study, for in vitro experiments, human umbilical vein endothelial cells (HUVECs) were obtained from Bio-Vector NTCC (Beijing, China) and cultured in DMEM medium (Gibco, California, USA) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin. The cultural condition for HUVECs was 5% CO_2 and 37°C. To establish the in vitro HR model, the cells were incubated under the condition of 1% O_2, 5% CO_2, and 94% N_2 for 2 hours, followed by incubation under normal condition (5% CO_2 and 94% N_2). Resveratrol and other chemicals were obtained from Sigma-Aldrich (California, USA).

2.2. Animals and the Establishment of Lower-Extremity I/R Injury Model and Design of Animal Experiments. The establishment of lower-extremity I/R injury model on rats was conducted according to the instruction described previously [14]. Forty-eight Sprague Dawley (SD) rats were obtained from Kay Biological Technology Co., Ltd. (Shanghai, China) and adapted for 7 days after arrival. The animals were anesthetized by intraperitoneal injection of 1% pentobarbital sodium and were fixed on the operation table. A 2 cm incision was opened on the root position of the right hind limb of rats, followed by ligating the superficial arteries and veins of the abdominal wall. Subsequently, the femoral sheath was opened and the femoral artery was separated, followed by carefully closing the proximal end of the femoral artery with a vascular clamp near the inguinal ligament. Then, the blood circulation of the collateral was blocked by a limb ring under the femoral sheath with a rubber band, followed by removing the clamp and rubber band 6 hours after the femoral artery was blocked. The state of blood flow was observed until the pulse and blood flow of rats returned to normal, followed by sewing up the incisions immediately. After the different treatment strategies, the animals were executed with euthanasia and the femoral artery tissues were collected. The incision was also opened on the animals in Sham group followed by sewing up the incisions immediately. The animals in I/R group were administered with normal saline orally for an 8-week consecutive dosing. The rats in RSV + I/R were dosed orally with 1 mg/kg/day RSV for an 8-week consecutive dosing [15, 16]. And the animals in RSV + I/R + ML385 group were administered orally with 1 mg/kg/day RSV combined with 30 mg/kg/week ML385 [17] for 8 weeks.

2.3. MTT Assay. The treated HUVECs were planted on 96-well plates as 2×10^4 cells/well, followed by being added with 5 mg/mL MTT solution (MedChemExpress, New Jersey, USA) for 3-4 hours. Subsequently, approximately 150 μL DMSO solution was added to each well to terminate the reaction. Finally, the optical density (OD) values were detected using the microplate reader (Thermo Fisher, MA, USA).

2.4. Hoechst Assay. Clean coverslips were put in 6-well plates and the cells were planted. Following different treating strategies, the medium was removed and approximately 500 μL polyformaldehyde fixing solution was added to fix the cells for over 10 min. After blotting up the fixing solution, the cells were washed by PBS on decolorization shaker twice and about 500 μL Hoechst solution was added for staining 5 min, followed by being washed twice. Finally, DAPI dyes were added to sealing and the pictures were taken under the fluorescence microscope (Thermo Fisher, MA, USA).

2.5. Flow Cytometry Assay. The treated HUVECs were collected and adjusted in the centrifuge tube at a density of 2×10^6/mL, followed by centrifugation at 4°C and 1000 r/min for 10 min. Subsequently, approximately 200 μL binding buffer was instilled and 5 μL Annexin V-FITC and PI solution were instilled, respectively. After being incubated for 15 min, the samples were loaded in the flow cytometry (BD, New York, USA) for detection.

2.6. Detection of ROS Level. Levels of ROS in treated HUVECs were evaluated by DCFH-DA fluorescence probe assay. In brief, the cells were planted on 6-well plates, followed by different treating strategies. Subsequently, 10 μmol/L DCFA-DA fluorescence probe was added to be further incubated for 30 min. After being washed using serum-free DMEM medium, the images were taken under the inverted microscope (Olympus, Tokyo, Japan).

2.7. MDA and SOD Detection. After the cells were planted on 24-well plates, treated HUVECs following different treating strategies were crushed by ultrasound, followed by collecting the supernatant. The concentration of MDA and SOD in the samples was detected utilizing the commercial kit according to the instruction of the manufacturers.

2.8. Western Blot Assay. The treated HUVECs and the isolated vascular were collected and added with lysis buffer (PMDF : RIPA = 1 : 99) to isolate the total proteins, which were further quantified utilizing the BCA kit (Thermo fisher, MA, USA). Subsequently, the samples were loaded and separated by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which were further transferred to PVEF membrane (Thermo Fisher, MA, USA) and incubated with 5% skim milk to block the non-specific binding proteins. Then, the membrane was incubated with primary antibodies against Nrf2 (1 : 1000, Santa Cruz Biotechnology, California, USA), Keap1 (1 : 1000, Santa Cruz Biotechnology, California, USA), HO-1 (1 : 1000, Santa Cruz Biotechnology, California, USA), Bcl-2 (1 : 1000, Santa Cruz Biotechnology, California, USA), Bax (1 : 1000, Santa Cruz Biotechnology, California, USA), Caspase-3 (1 : 1000, Santa Cruz Biotechnology, California, USA), or GAPDH (1 : 1000, Santa Cruz Biotechnology, California, USA) at 4°C overnight, followed by being incubated with secondary antibody (1 : 2000, CST, Boston, USA) at room temperature for 2 hours. Lastly, the membrane was added with ECL solution and exposed to Tanon 4600 (Tanon, Shanghai, China). Images were analyzed by Image *J* software.

2.9. Transfection. The Nrf2 knockout (Nrf2 KO) HUVECs were established by transfecting the siRNA targeting Nrf2. Briefly, the cells were planted on 6-well plates and were transfected with siRNAs along with the transfection reagents (Lipofectamine 3000, Thermo Fisher, MA, USA), which were further incubated for 24 hours. The efficacy of transfection was confirmed by Western blot assay. The sequence for siRNA targeting Nrf2 was listed as follows: forward: 5′-GGCGCCTAATTGTCAACTTCTG-3′; reverse: 5′-GTGCA GGGTCCGAGGT-3′.

2.10. TUNEL Assay. The isolated femoral artery tissues were fixed in 4% paraformaldehyde solution for 6 hours and embedded by paraffin, followed by being stored at 4°C in 30% sucrose solution for 3 days. Subsequently, the tissues were sectioned (18 μm) and washed by PBS buffer, followed by being added with TUNEL reaction solution at 37°C for 1 hour in the dark. After washing the slides with PBS 3 times, DAB reagent was added for chromogenic experiment, followed by taking images with optical microscope (Olympus, Tokyo, Japan) to evaluate the apoptotic state of femoral artery tissues.

2.11. HE and EVG Staining. The slides were soaked in xylene solution twice, followed by soaking in 100%, 95%, 85%, and 70% ethanol solution successively.

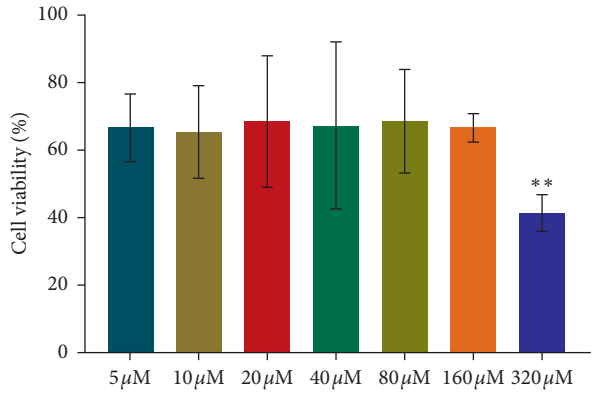
For HE staining, after washing with PBS buffer several times, the slides were stained with Hematoxylin dye for 3–5 min, followed by incubating in acid for 40 s and in ammonia solution for 40 s. Subsequently, the slides were stained in eosin dye for 2 min, followed by soaking in xylene solution for 5 min. Finally, the sections were sealed with neutral gum and observed under the inverted microscope (Olympus, Tokyo, Japan).

For EVG staining, the slides were stained with EVG solution (Hematoxylin, iodine solution, and ferric chloride mixed at a ratio of 5 : 2:2), followed by incubation for 30 min and washed with water. The background was differentiated by ferric chloride differentiation solution. The above steps were repeated until the background was grey white. Subsequently, the slides were re-dyed with VG solution (saturated picric acid and fuchsin solution mixed at a ratio of 9 : 1), followed by being washed several times and quick dehydration using 100% ethanol. Finally, the sections were sealed with neutral gum and observed under the inverted microscope (Olympus, Tokyo, Japan).

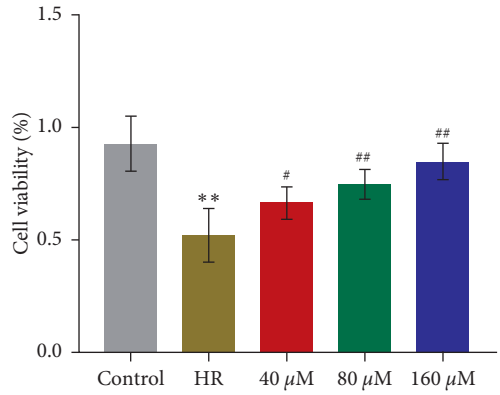
2.12. Statistical Analysis. The data obtained in the present study was analyzed using the SPSS 22.0 software and plotted utilizing GraphPad Prism 8. The data was presented as mean ± SD. The data between two groups was compared with *t*-test and the data among multiple groups was compared with one-way ANOVA analysis. $p < 0.05$ was regarded as significant in the present study.

3. Results

3.1. RSV Protected HUVECs from Injury Induced by HR. Firstly, we explored the highest tolerant concentration for RSV to be incubated in HUVECs. As shown in Figure 1(a), no significant difference was observed on cell viability as the concentration of RSV was increased from 5 μM to 160 μM. However, as the concentration of RSV increased from 160 μM to 320 μM, the cell viability was dramatically suppressed ($^{**}p < 0.01$ vs. 5 μM). To further investigate the protective effect of RSV against HR treated HUVECs, cells were cultured under HR condition in the absence or presence of RSV (40, 80, and 160 μM) for 24 hours. As shown in Figure 1(b), the cell viability was significantly inhibited by HR culture, which was greatly elevated by the introduction of RSV ($^{**}p < 0.01$ vs. control, $\#p < 0.05$ vs. HR, $\#\#p < 0.01$ vs. HR). We further investigated the state of apoptosis of treated HUVECs utilizing Hoechst assay and flow cytometry. As shown in Figure 1(c), compared to control, round nucleus and accumulated chromatin were observed in HR treated HUVECs, which were significantly alleviated by the treatment of RSV, indicating an obviously inhibitory effect of RSV against apoptosis induced by HR. In addition, compared to control, the apoptotic rate (Figure 1(d)) was increased from 6.01% to 36.65% in HR treated HUVECs, which was suppressed to 28.64% and 14.01% by the introduction of 80 and 160 μM RSV, respectively. Lastly, we found that the expression of Bcl-2 was significantly inhibited and the expression of Bax and Caspase-3 was dramatically



(a)



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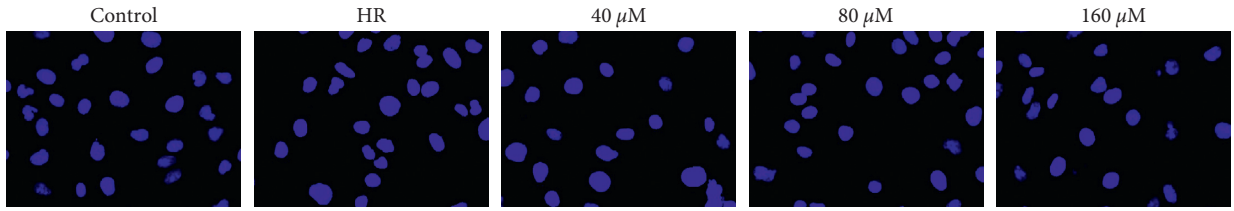
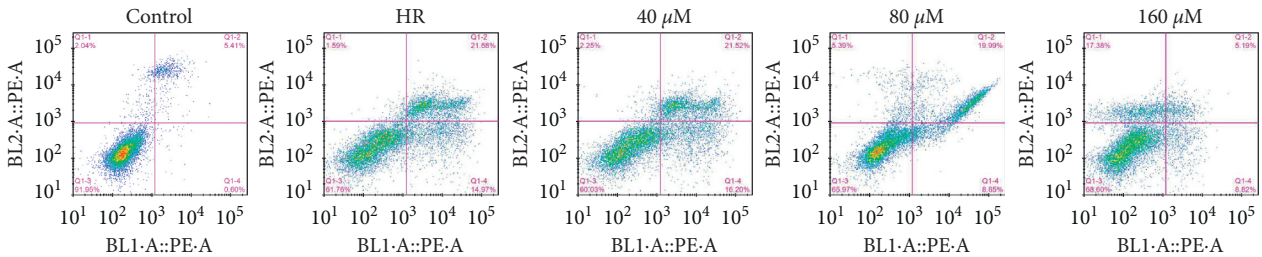


(c)



(d)

Figure 1: Continued.

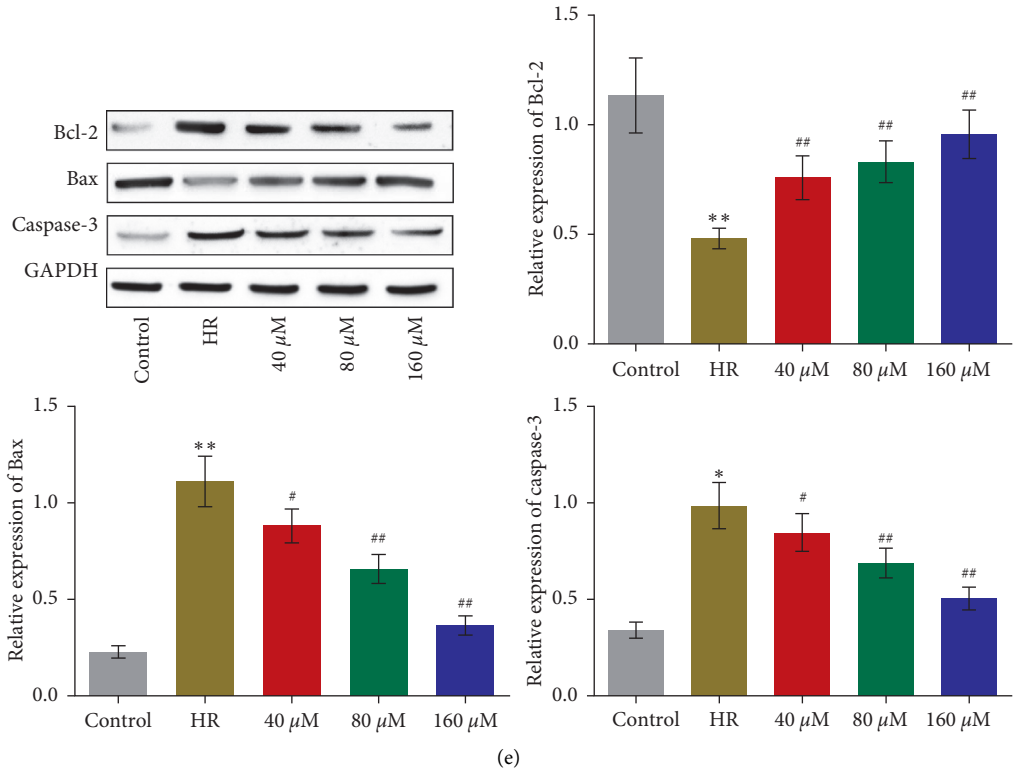


Figure 1: The HR-induced injury on HUVECs was alleviated by RSV. (a) The cell viability of HUVECs treated with different concentration of RSV was evaluated by MTT assay (** $p < 0.01$ vs. 5 μM). (b) The cell viability of HUVECs treated with different strategies was detected by MTT assay (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, and ## $p < 0.01$ vs. HR). (c) The apoptotic state of HUVECs treated with different strategies was measured by the Hoechst test. (d) The apoptotic rate of HUVECs treated with different strategies was determined by flow cytometry. (e) The expression level of Bcl-2, Bax, and Caspase-3 was detected by Western blot (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, and ## $p < 0.01$ vs. HR).

elevated induced by HR treatment, which were greatly reversed by the incubation of RSV (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, ## $p < 0.01$ vs. HR).

3.2. RSV Alleviated Oxidative Stress in HUVECs Induced by HR. To evaluate the state of oxidative stress in HUVECs, the level of ROS, MDA, and SOD was detected. As shown in Figure 2(a), the fluorescence intensity of DCFA-DA was significantly increased in HR treated HUVECs, which was greatly declined by the introduction of different dosage of RSV. Compared to control, the level of MDA (Figure 2(b)) was increased from 8.7 ng/mL to 40.9 ng/mL by the treatment of HR, which was suppressed to 26.3, 17.0, and 13.5 ng/mL by the introduction of 40, 80, and 160 μM RSV, respectively (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, ## $p < 0.01$ vs. HR). In addition, compared to control, the concentration of SOD was significantly inhibited from 4.5 ng/mL to 2.1 ng/mL in HR treated HUVECs, which was elevated to 2.8 and 3.7 ng/mL by the administration of 80 and 160 μM RSV, respectively (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, ## $p < 0.01$ vs. HR). To further investigate the underlying mechanism, the impact of RSV on Nrf2 signaling was evaluated. As shown in Figure 2(c), the expression of Nrf2, Keap1, and HO-1 was significantly suppressed by the treatment of HR, which was greatly elevated by the introduction of RSV (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, ## $p < 0.01$ vs. HR). These data indicated that oxidative stress induced by HR was dramatically ameliorated by RSV.

3.3. The Protective Effect of RSV against HR-Treated HUVECs Was Abolished by the Knockout of Nrf2. To further verify the involvement of Nrf2 signaling in the protective effect of RSV against HR-treated HUVECs, the HR incubated Nrf2 KO HUVECs were established and treated with RSV (RSV + Nrf2 KO HR). As shown in Figure 3(a), the declined cell viability in HR-treated HUVECs was significantly elevated by the introduction of RSV, which was further suppressed by the knocking down of Nrf2 (** $p < 0.01$ vs. control, ## $p < 0.01$ vs. HR, & $p < 0.05$ vs. RSV + HR). We further detected the apoptotic state of HUVECs from different groups. As shown in Figure 3(b), compared to RSV + HR group, round nucleus and accumulated chromatin were observed in RSV + Nrf2 KO HR group. In addition, compared to RSV + HR group, the apoptotic rate of HUVECs was increased from 18.72% to 34.49% in the RSV + Nrf2 KO HR group (Figure 3(c)). Lastly, as shown in Figure 3(d), compared to RSV + HR group, Bcl-2 was significantly downregulated and Bax and Caspase-3 were dramatically upregulated in RSV + Nrf2 KO HR group

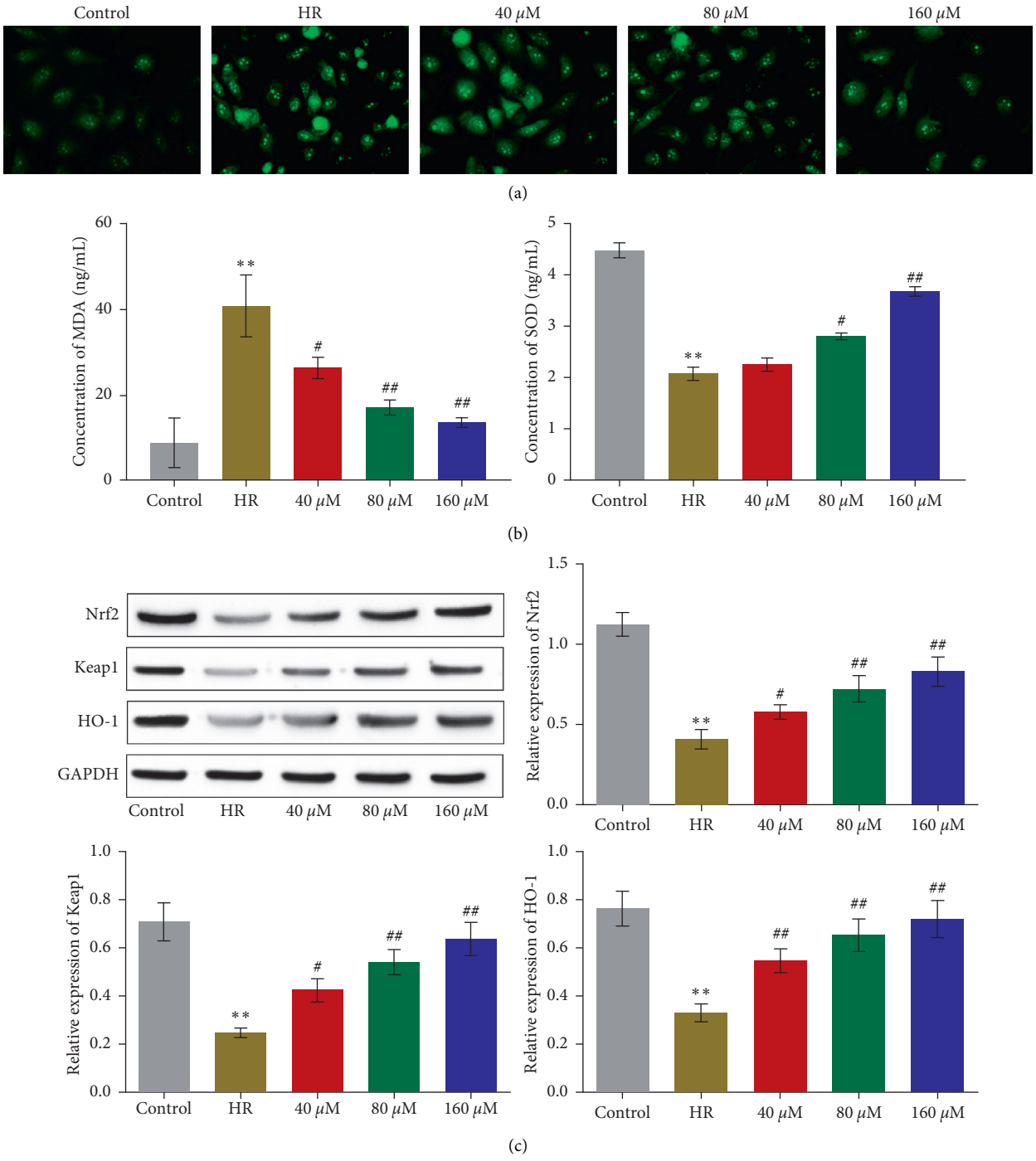
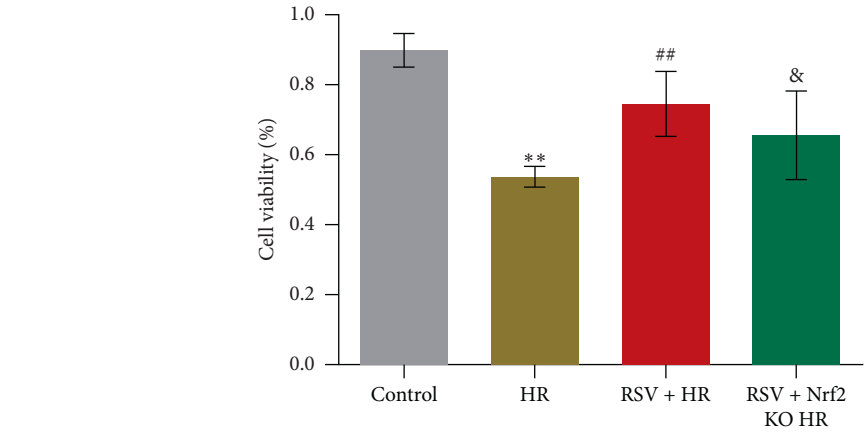


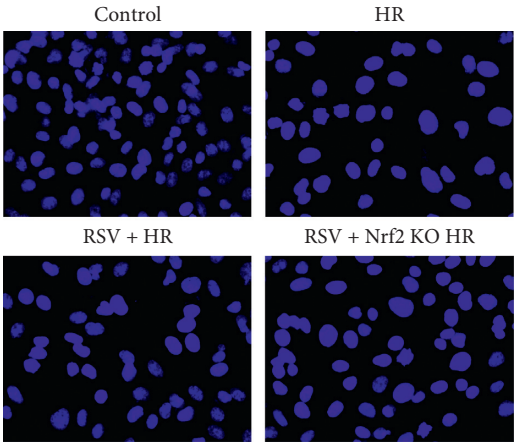
Figure 2: HR-induced oxidative stress on HUVECs was ameliorated by RSV. (a) The ROS level in HUVECs treated with different strategies was measured by DCFH-DA assay. (b) The concentration of MDA and SOD was determined by commercial kits. (c) The expression of Nrf2, Keap1, and HO-1 was evaluated by Western blot (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, and ## $p < 0.01$ vs. HR).

(& $p < 0.05$ vs. RSV + HR). These data indicate that the protective effect of RSV against HR-treated HUVECs was abolished in Nrf2 KO HUVECs.

3.4. The Protective Effect of RSV against HR-Induced Oxidative Stress Was Abolished by the Knockout of Nrf2. We further detected the state of oxidative stress in HUVECs treated with different strategies. As shown in Figure 4(a), decreased fluorescence intensity in RSV + HR group was significantly elevated in RSV + Nrf2 KO HR group (& $p < 0.05$ vs. RSV + HR), indicating that the suppressed production of ROS induced by RSV was greatly reversed by knocking down the expression of Nrf2. In addition, compared to RSV + HR group, the concentration of MDA (Figure 4(b)) was increased from 71.48 ng/mL to



(a)



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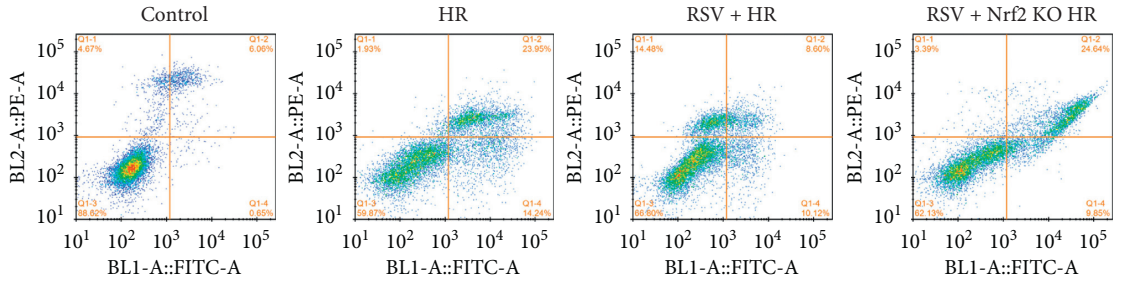

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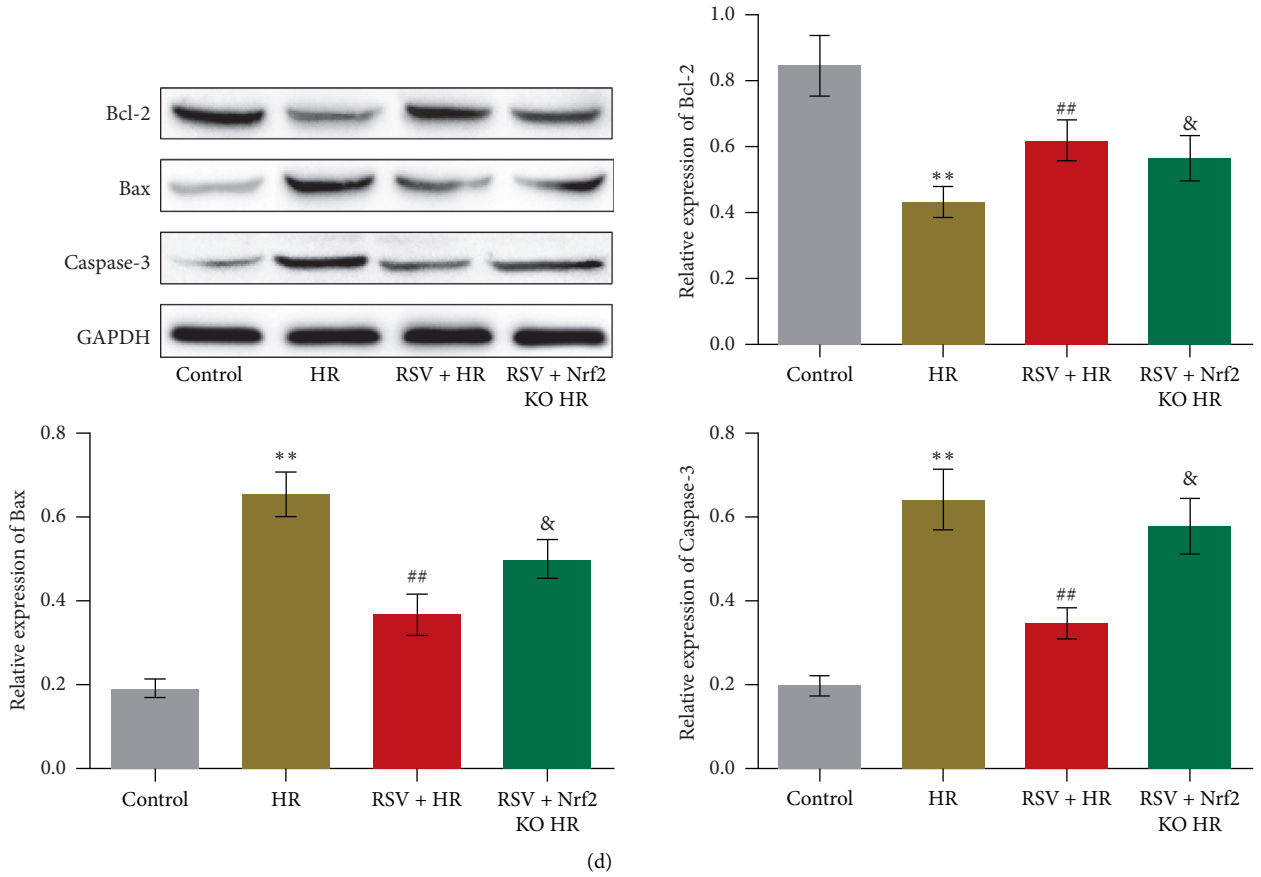

Figure 3: The protective effect of RSV against HR-treated HUVECs was abolished by knocking down Nrf2. (a) The cell viability of HUVECs treated with different strategies was detected by MTT assay. (b) The apoptotic state of HUVECs treated with different strategies was measured by Hoechst test. (c) The apoptotic rate of HUVECs treated with different strategies was determined by flow cytometry. (d) The expression level of Bcl-2, Bax, and Caspase-3 was detected by Western blot (** $p < 0.01$ vs. control, ## $p < 0.01$ vs. HR, and & $p < 0.05$ vs. RSV + HR).

79.57 ng/mL and the concentration of SOD was decreased from 2.56 ng/mL to 1.74 ng/mL in RSV + Nrf2 KO HR group (& $p < 0.05$ vs. RSV + HR). As for the state of Nrf2 signaling (Figure 4(c)), compared to RSV + HR group, the expression of Nrf2, Keap1, and HO-1 was dramatically inhibited in RSV + Nrf2 KO HR group (& $p < 0.05$ vs. RSV + HR). These data indicated that knocking down Nrf2 abolished the protective effect of RSV against HR-induced oxidative stress.

3.5. RSV Ameliorated the Pathological Symptom in Lower-Extremity I/R Rats. To explore the potential therapeutic effect of RSV against lower-extremity I/R, the lower-extremity I/R model was established followed by RSV treatments. As shown in Figures 5(a) and 5(b), in Sham group, the boundary among intima, media, and adventitia was clear. The structure of these membranes was integrated. Monolayer of endothelial cells in alignment was observed in the intima, connected by tight junctions. Collagen fiber and elastic fiber were abundant, and the structure was clear without fracture in media membrane. The adventitia was composed of connective tissues and integrated with no significant thickening, fracture, or defect. In I/R group, indistinct boundary and incrassation were observed in intima, media, and adventitia. Increased volume of endothelial cells, rough lumen surface, and small amount of platelet adhesion were observed in intima. Disordered thickening, degeneration and fracture of fiberboard, and infiltration of inflammatory cells were observed in media membrane. Fiber connective tissues characterized with edema, thickening, local fracture, and defect were observed in adventitia. In RSV + I/R group, the degree of endothelium edema, thickening vascular wall, infiltration of inflammatory cells, and damaged elastic fiber layer were obviously alleviated, which, however, were not observed in RSV + I/R + ML385 group. ML385 is a promising Nrf2 inhibitor widely used in experimental studies. We further investigated the apoptotic state in femoral artery tissues. As shown in Figure 5(c), compared to Sham group, the increased number of apoptosis bodies was observed in I/R group and was decreased in RSV + I/R group, which was greatly suppressed in RSV + I/R + ML385 group. In addition, the downregulated Bcl-2 and upregulated Bax and Caspase-3 in I/R group were significantly reversed by the treatment

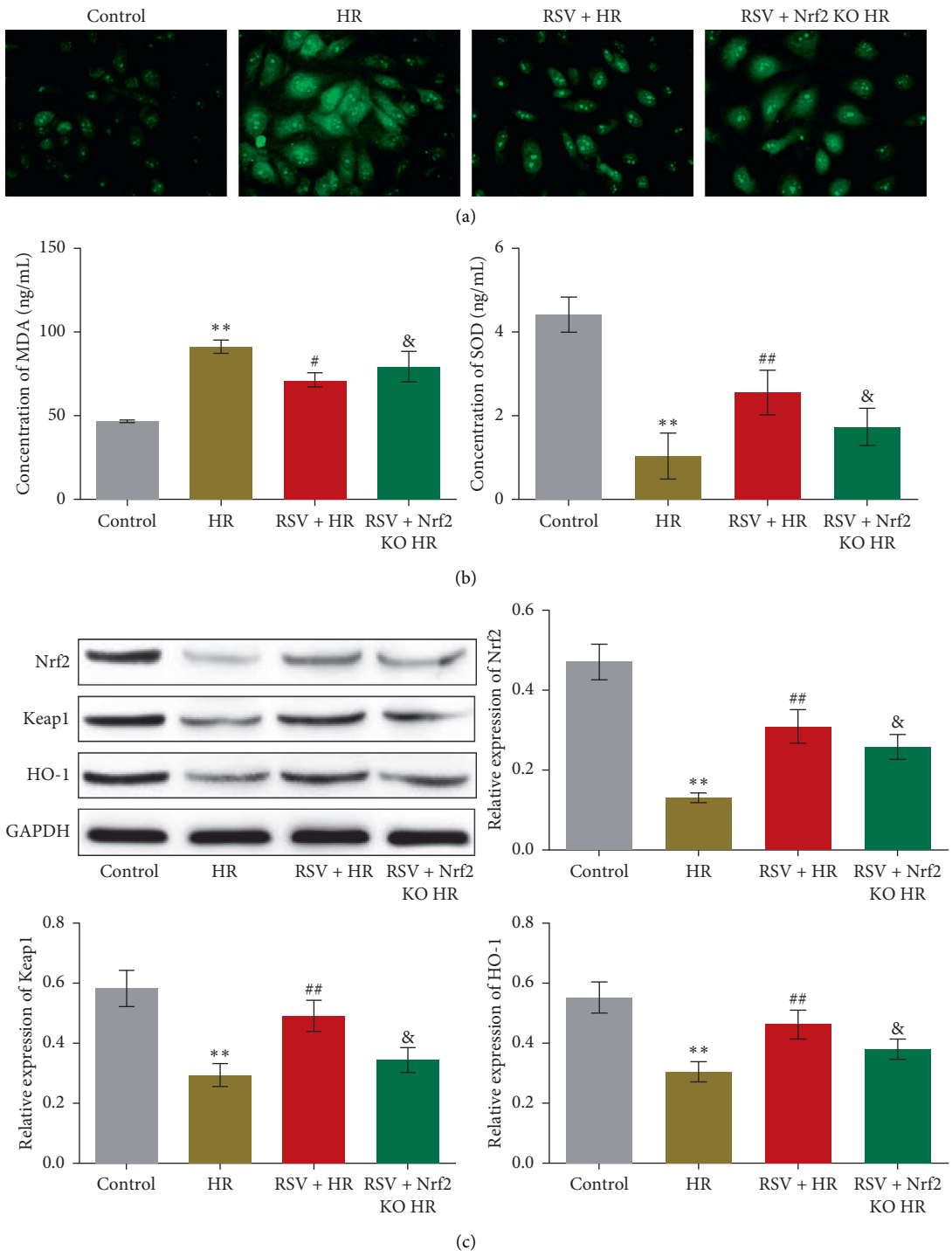

Figure 4: The protective effect of RSV against oxidative stress in HUVECs induced by HR was abolished by knocking down Nrf2. (a) The ROS level in HUVECs treated with different strategies was measured by DCFH-DA assay. (b) The concentration of MDA and SOD was determined by commercial kits. (c) The expression of Nrf2, Keap1, and HO-1 was evaluated by Western blot (** $p < 0.01$ vs. control, # $p < 0.05$ vs. HR, ## $p < 0.01$ vs. HR, and & $p < 0.05$ vs. RSV + HR).

of RSV, which were further abolished by the coadministration of ML385 (** $p < 0.01$ vs. Sham, ## $p < 0.01$ vs. I/R, & $p < 0.05$ vs. RSV + I/R). These data indicated that the pathological symptom in lower-extremity I/R rats was significantly ameliorated by RSV, which was further abolished by blocking Nrf2 signaling.

3.6. RSV Alleviated Oxidative Stress in the Femoral Artery Tissues of Lower-Extremity I/R Rats. As shown in Figure 6(a), compared to Sham group, the concentration of MDA in femoral artery tissues was increased from 43.36 ng/mL to 87.27 ng/mL in I/R group, which was further decreased to 58.83 ng/mL in RSV + I/R group. In

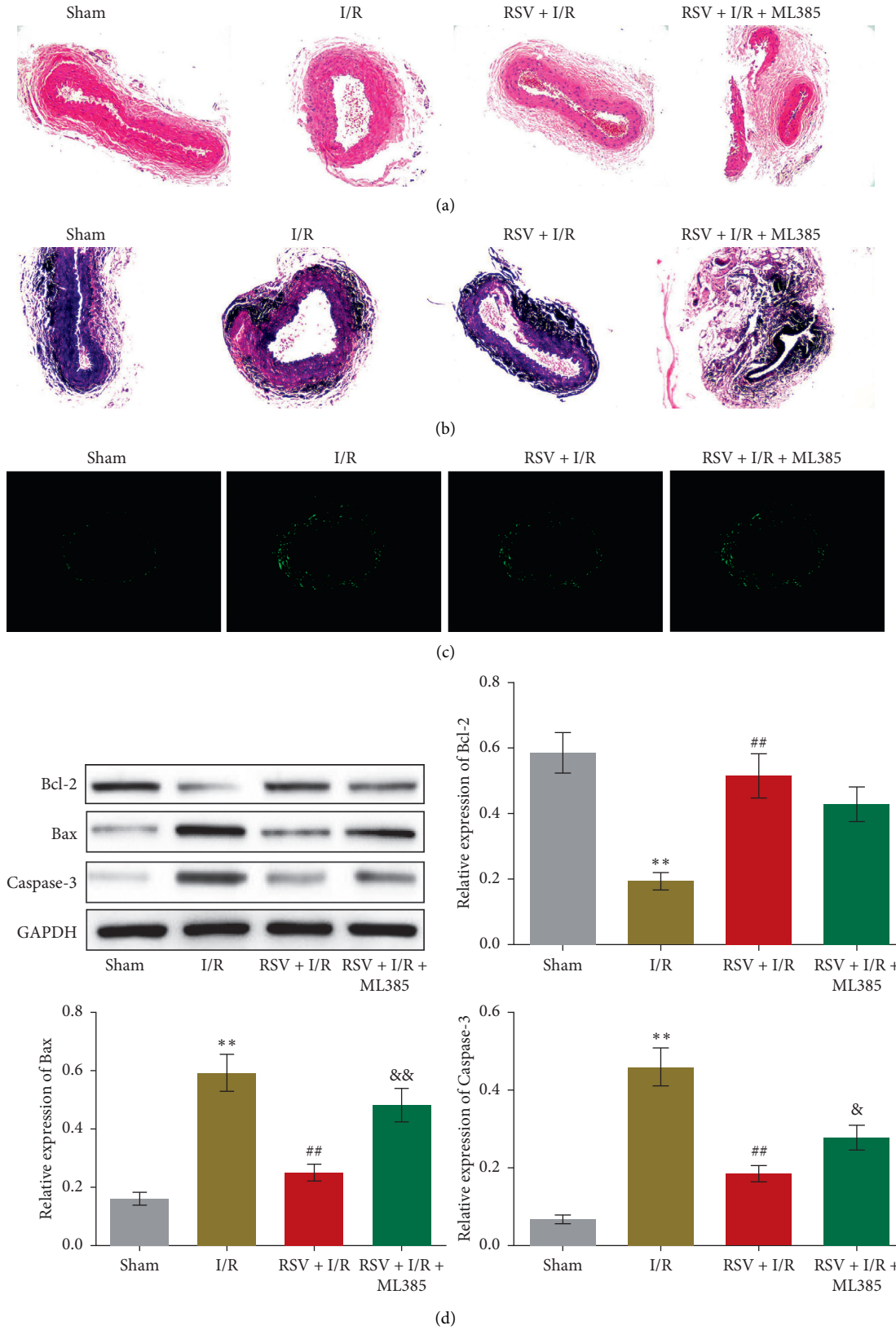

Figure 5: RSV alleviated the pathological changes in femoral artery tissues from low-extremity I/R rats. (a, b) The pathological state of femoral artery tissues was evaluated by HE and EVG staining. (c) The apoptotic state of femoral artery tissues was measured by TUNEL staining assay. (d) The expression level of Bcl-2, Bax, and Caspase-3 was detected by Western blot (** $p < 0.01$ vs. Sham, ## $p < 0.01$ vs. I/R, & $p < 0.05$ vs. RSV + I/R, and && $p < 0.01$ vs. RSV + I/R).

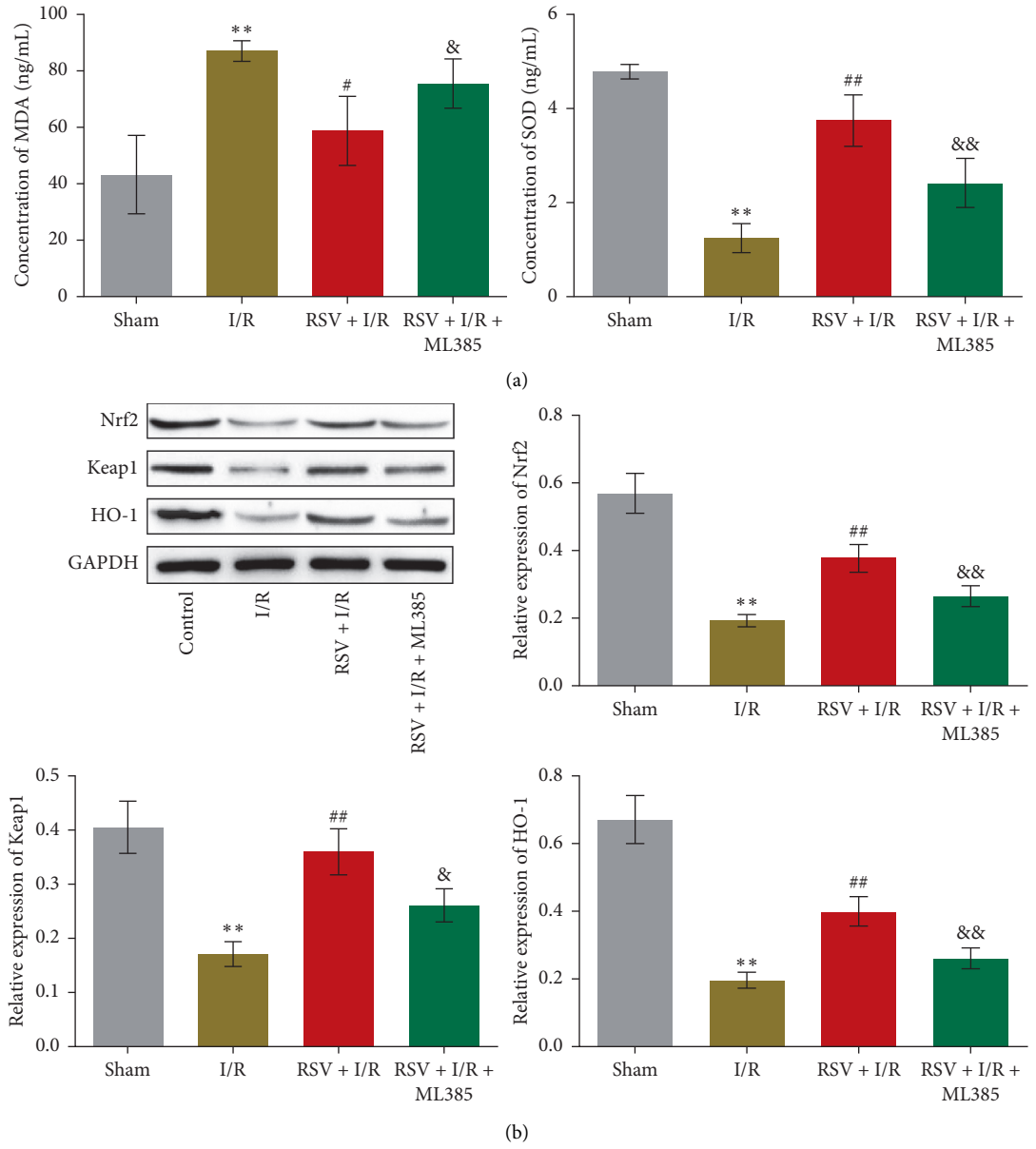

Figure 6: RSV alleviated oxidative stress in femoral artery tissues from low-extremity I/R rats. (a) The production of MDA and SOD in femoral artery tissues was detected by commercial kits. (b) The expression level of Nrf2, Keap1, and HO-1 was evaluated by Western blot (** $p < 0.01$ vs. Sham, # $p < 0.05$ vs. I/R, ## $p < 0.01$ vs. I/R, & $p < 0.05$ vs. RSV + I/R, and && $p < 0.01$ vs. RSV + I/R).

addition, the concentration of MDA in femoral artery tissues was elevated to 75.74 in RSV + I/R + ML385 group. Compared to Sham group, the concentration of SOD in femoral artery tissues was declined from 4.79 ng/mL to 1.26 ng/mL in I/R group and was promoted to 3.75 ng/mL in RSV + I/R group, which was further decreased to 2.43 ng/mL in RSV + I/R + ML385 group. As shown in Figure 6(b), the decreased expression of Nrf2, Keap1, and HO-1 in femoral artery tissues in I/R group was significantly elevated in RSV + I/R group, which was further suppressed in RSV + I/R + ML385 group (** $p < 0.01$ vs. Sham, ## $p < 0.01$ vs. I/R, & $p < 0.05$ vs. RSV + I/R, && $p < 0.01$ vs. RSV + I/R). These data indicated that oxidative stress in femoral artery tissues of lower-extremity I/R rats was significantly alleviated by the treatment of RSV, which was abolished by blocking Nrf2 signaling.

4. Discussion

Vascular endothelial cells are considered the first barrier for the protection of vessels, which play an important role in wound healing, thrombosis, and neovascularization [18]. It is widely reported that injures on vascular endothelial cells are involved in the pathological progress of lower-extremity I/R injury [19]. In the process of I/R injury, apoptosis of vascular endothelial cells is commonly observed. Wu et al. reported that apoptosis induced vascular endothelial cell injury was involved in the pathological process of lower-

extremity I/R injury, which was closely related to AMPK signaling [20]. In addition, the upregulation of Bax and Caspase-3, proapoptotic factors [21], and the downregulation of Bcl-2 [22], an anti-apoptotic factor, in vascular endothelial cells are reported to be observed in I/R injury animal models [23]. In the present study, we used HR cultural condition to simulate the pathological state of HUVECs in lower-extremity I/R, which was verified by the decreased cell viability and elevated apoptotic rates. By the treatment of RSV, the cell viability and apoptotic rate were significantly alleviated, indicating a promising protective effect of RSV against endothelial injury induced by HR condition. Further verifications were conducted in lower-extremity I/R rats. Significant pathological changes, including endothelium edema, thickening vascular wall, infiltration of inflammatory cells, damaged elastic fiber layer, and increased number of apoptotic body, were observed in lower-extremity I/R rats, which were consistent with the description reported previously [24]. Following 8 weeks' consecutive treatment of RSV, the pathological changes were significantly reversed, indicating a pronounced protective property of RSV against lower-extremity I/R endothelial injury. In our further work, more indexes will be included to fully evaluate the therapeutic effect of RSV on lower-extremity I/R injury, including measuring the thickness of intima, media, and adventitia of arterial wall, as well as the measurement of arterial lumen area.

In ischemia diseases, the rapid onset, the duration, and severity of ischemia are regarded as the main elements responsible for the tissue's damages. As the blood flow is restored, abundant oxygens and nutrient substances get into the cells, which contribute to the rebooting of aerobic oxidation and the abnormal activation of tissue metabolism. Subsequently, the production of ROS was accelerated, which further results in the activation of nicotinamide adenine dinucleotide phosphate (NADPH), the excessive production of nitric oxide (NO), and the release of cytochrome C. As a consequence, the irreversible damage on tissues and cells will be aggravated [25]. Several mechanisms are reported on the injury induced by excessive released ROS and oxidative stress. Firstly, the permeability of cell membrane and lysosomal membrane can be changed by ROS, which induces transmembrane transport disorder. As a result, the proteases are activated, DNA dissolution is trigged, and the protein denaturation is induced, which finally activate the apoptotic signaling and the onset of apoptosis. Secondly, excessive release of inflammatory factors and chemotaxis of leukocytes will be mediated by ROS, which contribute to the development and processing the severe inflammatory reactions. Thirdly, the permeability of mitochondrial membrane will be changed by ROS, which subsequently blocks the respiratory chain, induces the metabolic disorder, and disrupts the cellular structure [26]. In the present study, oxidative stress was observed both in HR treated HUVECs and in femoral artery tissues isolated from lower-extremity I/R rats, which were consistent with the description reported previously [27, 28]. After 8 weeks' consecutive treatment of RSV, the state of oxidative stress both in HR treated HUVECs and in femoral artery tissues isolated from lower-extremity I/R rats was dramatically alleviated, indicating a promising protective effect of RSV against oxidative stress, which was consistent with the reports proposed by Zhuang et al. [29] and Sebori [12]. To further confirm that the protective effect of RSV against I/R injury was related to the inhibition of oxidative stress, we verified the effects of RSV by blocking the Nrf2 signaling, which is a well-known regulatory signal pathway against oxidative stress [30]. We found that the protective effect of RSV against both I/R injury and oxidative stress was significantly abolished, indicating that RSV might alleviate the I/R injury by ameliorating oxidative stress in endothelial cells. In our future work, the downstream impact of oxidative stress, such as excessive release of inflammatory factors and infiltration of inflammatory cells, will be investigated to further confirm the mechanism concluded in the present study.

5. Conclusion

Our data indicated that RSV might alleviate vascular endothelial injury induced by lower-extremity I/R injury through regulating Keap1/Nrf2 signaling-mediated oxidative stress.

Data Availability

All the data in this study are included within the manuscript.

Conflicts of Interest

All the authors declare that there are no conflicts of interest.

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Research Article

MiR-203 Targets to the 3′-UTR of SLUG to Suppress Cerebral Infarction-Induced Endothelial Cell Growth and Motility

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Cerebral infarction is one of the leading causes of death worldwide, in which angiogenesis plays a critical role. On the other hand, accumulating evidence has demonstrated that microRNAs (miRNAs) function as key modulators in the formation and progression of cerebral infarction. However, the molecular mechanisms of miRNAs underlying cerebral infarction-associated angiogenesis remain unclear. In the present study, we indicated that the expression of miR-203 was significantly downregulated in serum samples derived from patients with cerebral infarction and in mice brain samples following middle cerebral artery occlusion (MCAO) compared with healthy controls. *In vitro*, the expression of miR-203 was obviously downregulated in hypoxia-induced human umbilical vein vascular endothelial cells (HUVECs). Functionally, ectopic expression of miR-203 drastically suppressed HUVEC proliferation, invasion, and migration. In addition, SLUG, a zinc finger transcriptional repressor, was identified as a direct target of miR-203 and was negatively correlated with miR-203 expression in MCAO mice and in hypoxia-induced HUVECs. Furthermore, overexpression of SLUG reversed the inhibitory effect of miR-203 on proliferation, invasion, and migration abilities of HUVECs. Taken together, our research provides a novel insight of the miR-203-SLUG axis into cerebral infarction-associated endothelial behaviors and may offer a powerful therapeutic target of cerebral ischemia.

1. Introduction

Cerebral infarction is one of the leading causes of death for people over 60 years old worldwide [1–3]. Cerebral infarction frequently results in irreversible neurological deficits and is difficult to diagnose as powerful diagnostic markers are lacking. Although accumulating studies have demonstrated that a wide range of genes and signaling pathways are involved in cerebral ischemia, the precise molecular mechanisms remain not fully elucidated. Previous studies have proved that ischemic stroke can initiate angiogenesis to recover the oxygen and nutrient supply and facilitate functional recovery in damaged brain tissues [4–7]. However, the molecular mechanisms underlying cerebral infarction-related angiogenesis are not well documented.

miRNAs represent a class of endogenous, single-strand, small noncoding RNAs with approximately 22 nucleotides in length, which posttranscriptionally suppress target gene expression by targeting its 3′-untranslated region (3′-UTR) [8–10]. miRNAs have been widely reported to be implicated in multiple physiological and pathological processes, including cell proliferation, apoptosis, tissue homeostasis, organ development, carcinogenesis, inflammation, and ischemic diseases. Emerging evidence has indicated that miRNAs are of great importance in cerebral infarction. For instance, Cai et al. have reported that miR-146b-3p regulates the development and progression of cerebral infarction with diabetes through RAF1/P38MAPK/COX-2 signaling pathway [11]. On the other hand, miRNAs also play an essential role in angiogenesis and other biological behaviors of endothelial cells. For example, Zhao et al. have demonstrated

that miR-124 aggravates failing hearts by suppressing CD151-facilitated angiogenesis in the heart [12]. However, limited research has paid attention to mechanisms of miRNAs underlying cerebral infarction-associated angiogenesis.

MiR-203 (also named miR-203a-3p) is a tumor suppressor miRNA playing an important role in various types of cancers and serving as an epidermis-specific miRNA essential for skin development. Much evidence has shown that miR-203 modulates cancer initiation and progression by targeting SLUG [13–16]. Moreover, miR-203 has been reported to regulate angiogenesis in cancers and the placenta [14, 17, 18] by targeting VEGFA, VEGFR2, and SLUG. However, little research has paid attention to the role of the miR-203-SLUG axis in ischemia, especially in cerebral infarction and cerebral infarction-related angiogenesis.

In the present study, we found that miR-203 was significantly downregulated in patients with cerebral infarction, MCAO mice, and hypoxia-induced HUVECs. Meanwhile, a reverse correlation between miR-203 and SLUG was observed in these samples. Importantly, overexpression of miR-203 in hypoxia-induced HUVECs suppressed cell proliferation, invasion, and migration, which could be reversed by ectopic expression of SLUG. Our findings not only demonstrate the function of the miR-203-SLUG axis in cerebral ischemia but also provide a putative therapeutic target for related diseases.

2. Materials and Methods

2.1. Patient Sample Collections. Patients with cerebral infarction ($n = 18$) were collected by the Department of Vascular Surgery, Second Hospital of Hebei Medical University from January 2017 to December 2017, which was approved by the Ethics Committee of Hebei Medical University. Sixteen healthy volunteers were enrolled as normal controls. Venous blood samples (5–10 ml) were collected from all the participants, which were centrifuged at 2000 rpm at 4°C for 30 min. Then the serum samples were isolated and stored at −80°C until analysis. Patients with other CNS diseases were excluded.

2.2. MCAO Mouse Model Constructions. Six- to eight-week-old male mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal procedures were approved by the Institutional Animal Care and Use Committee of Hebei Medical University. Mice were anesthetized with an intraperitoneal injection of a mixture of medetomidine hydrochloride (0.315 mg/kg), midazolam (2.0 mg/kg), and butorphanol tartrate (2.5 mg/kg), which was maintained at 37°C. Then, the cerebral ischemia was produced by intraluminal occlusion of the right middle cerebral artery using a nylon filament. The mice were randomly divided into two groups, with one group treated for 1 hour, the other for two hours. Later, the filament was withdrawn to allow reperfusion for 4 h. For the control group, the internal carotid artery was dissected without occlusion with a nylon filament. After that, all the animals were sacrificed with analgesics to obtain the brain tissues for further analyses.

2.3. Hypoxia-Induced HUVECs. The HUVECs were purchased from Life Technologies (Carlsbad, CA, USA) and cultured in Medium 200 with a low serum growth supplement. After being cultured in good condition with the medium containing 10% FBS (Gibco), 20 μg/ml VEGF (Gibco), 100 U/mL penicillin, and 100 μg/ml streptomycin, and the confluency being up to 80%, the cells for hypoxia models were incubated in the hypoxic medium in the absence of FBS and glucose, and the culture dish was put into a sterilized hypoxic box for 2 h, 4 h, and 6 h, with the concentration of oxygen kept less than 1% with 5% CO_2 and 95% N_2.

2.4. RNA Isolation and Quantitative RT-PCR. For mRNA detection, total RNA was isolated from the serum samples, brain tissues, or harvested cells by using TRIzol total RNA isolation kit (Tiangen, Beijing, China). Quantitative RT-PCR was performed using the One-Step SYBR® PrimeScript™ RT-PCR kit (Takara, Dalian, China) on the Lightcycler Real-time PCR detection system (BioRad, Hercules, CA). GAPDH was used as an internal control. Primer sequences were as follows: 5′- ATGAGGAATCTGGCTGCTGT-3′ (SLUG, forward), 5′- CAGGAGAAAATGCCTTTGGA-3′ (SLUG, reverse), 5′- TCGGAGTCAACGGATTTGGT-3′ (GAPDH, forward), and 5′- TTGGAGGGATCTCGCTCCT-3′ (GAPDH, reverse). The TaqMan Human miRNA Assays were applied for miR-203 detection. Briefly, 5 ng of total RNA was reversely transcribed to cDNA with stem-loop primers by using the TaqMan miRNA Reverse Transcription Kit (Ambion, Carlsbad, California, USA). Quantitative real-time PCR (qRT-PCR) was carried out by TaqMan Universal PCR Master Mix. All PCR primers were from the TaqMan miRNA Assays. U6 RNA was used as an internal control.

2.5. Luciferase Reporter Assay. Reporter plasmid containing wild-type SLUG or mutant plasmid was transfected into HUVECs along with miR-203 or control mimic, using the FUGENE HD reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. Twenty-four hours later, cells were lysed with 100 μl of Passive Lysis Buffer (Promega, Fitchburg, WI), and their Renilla luciferase levels were analyzed using the Dual Glo Luciferase Assay System (Promega). The firefly luciferase activity was used as an internal control.

2.6. Lentiviral Production. The cDNA of SLUG was cloned into the pSin4-EF2-IRES-Pur vector at the *SpeI* and *EcoRI* sites. The pSIN lentiviral system was used for ectopic expression of SLUG in HUVECs. The pSIN-SNAI, psPAX2, and pMD2G plasmids were cotransfected into HEK-293T cells using the Fugene HD reagent. The supernatant containing lentivirus was harvested and then filtered at 48 h after transfection.

2.7. Cell Proliferation Assay. Cell proliferation assay was performed using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's manual. Briefly, HUVEC suspensions (2×10^4 cells) were seeded into a 96-well cell culture plate. 20 μl of MTT (5 mg/ml) was added to each well. Then, the plates were incubated at 37°C for 4 h to allow the MTT to react with viable cells to form formazan crystals. After that, the medium was removed, the formazan crystals were dissolved in 100 μl of DMSO at 37°C for 15 min. The absorbance was measured by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm.

2.8. Western Blot. Western blot was performed as previously described [19]. Rabbit polyclonal SLUG antibody was purchased from Abcam (Cat# 27568, Cambridge, MA, USA). Mouse monoclonal β-actin antibody was from Santa Cruz Biotechnology (Cat# 47778, Santa Cruz, CA, USA).

2.9. Invasion and Migration Assay. For invasion assay, 1×10^5 cells were suspended into 100 μl of fresh culture medium and seeded into the top chamber of a 24-well transwell insert (pore size: 8 μm, BD Biosciences, Franklin Lakes, NJ, USA) precoated with 25 μl of growth-factor reduced Matrigel (diluted into three volumes of serum-free culture medium). For migration assay, cells (1×10^5) were suspended into 100 μl of fresh medium and seeded in the top chamber of an insert, which was inserted into a 24-well plate. Five hundred μl of culture medium with 10% FBS was added to the bottom chambers. Twenty-four hours later, the cell on the upper layer of the insert was removed by a cotton swab. The invading or migrated cells were fixed in 4% paraformaldehyde (Beyotime), stained with 0.1% crystal violet (Beyotime), and photographed under the Nikon inverted microscope. Staining was then dissolved with 10% acetic acid and quantified at a microplate reader at 570 nm.

2.10. Statistical Analysis. The statistical significance among the groups was determined by an unpaired Student's t-test. The correlation between miR-203 and SLUG was analyzed by the Pearson method. A P-value smaller than 0.05 was considered statistically significant.

3. Results

3.1. MiR-203 Expression Is Reversely Correlated with SLUG in Serum Samples Derived from Patients with Ischemic Infarction, Brain Tissues Derived from MCAO Mice, and Hypoxia-Induced HUVECs. Firstly, to evaluate the function of the miR-203-SLUG axis in cerebral infarction, we determined the expression level of circulating miR-203 in patients with ischemic infarction ($n = 18$) and healthy controls ($n = 16$) by using quantitative RT-PCR. We found that miR-203 was significantly downregulated in patients with cerebral infarction compared with healthy controls ($P = 0.0035$, Figure 1(a)). We then established an MCAO model in mice and determined the expression levels of miR-203 and SLUG in brain tissues. Compared with the sham group, the expression of miR-203 was significantly reduced in both 1h- and 2h-occlusion groups (Figure 1(b)), while the expression of SLUG was notably elevated (Figures 1(c), 1(e) and 1(f)) and a reverse correlation between miR-203 and SLUG was observed (Figure 1(d)). Next, we examined miR-203 and SLUG expression in hypoxia-induced HUVECs. The results demonstrated that miR-203 was obviously downregulated in HUVECs with hypoxic treatment compared with normal cells (Figure 1(g)), while SLUG was upregulated (Figure 1(h) and 1(i)). These findings indicate that downregulation of miR-203 and upregulation of SLUG may be attributed to ischemia.

3.2. SLUG Is Direct Target of MiR-203 in HUVECs. To elucidate whether miR-203 directly targets SLUG in HUVECs, we constructed a luciferase reporter containing the wild-type SLUG 3′-UTR or mutant plasmid for miR-203 binding sites (Figure 2(a)) and performed a luciferase report assay in HUVECs. The results indicated that cotransfection of miR-203 mimic suppressed the luciferase activity of the reporter with wild-type SLUG 3′UTR but failed to suppress that with mutant reporter plasmid (Figure 2(b)). In addition, transfection of miR-203 mimic in HUVECs (Figure 2(c)) significantly inhibited the protein level of SLUG, which was determined by using Western blot analysis (Figure 2(d)). These results demonstrate that endogenous SLUG in HUVECs is directly targeted by miR-203.

3.3. The MiR-203-SLUG Axis Functions as a Key Regulator in Hypoxia-Induced Cell Proliferation and Migration in HUVECs. It is well known that cell proliferation and migration play an important role in hypoxia-induced angiogenesis. We next determined the effect of miR-203-SLUG in hypoxia-induced cell proliferation and migration in HUVECs. The MTT assay demonstrated that hypoxic treatment induced HUVEC proliferation (Figure 3(b)), which was suppressed by transfection of miR-204 mimic (Figures 3(a) and 3(b)). However, when we overexpressed SLUG in miR-203-transfected cells, hypoxic treatment reelevated HUVEC proliferation again (Figure 3(b)). A similar phenomenon was seen in the invasion and migration assay whereby the HUVECs transfected with miR-203 suppressed hypoxia-induced cellular invasion and migration, which was erased by ectopic expression of SLUG (Figures 3(c)–3(e)). Taken together, these findings suggest that the miR-203-SLUG axis functions as a key modulator in the biological behavior of HUVECs, and thus contributes to angiogenesis in cerebral infarction.

Moreover, to determine the effect of SLUG on the biological behaviors of HUVEC induced by hypoxia, we silenced the expression of SLUG in hypoxia-treated HUVECs (Figure S1A). The results demonstrated that the silence of SLUG drastically attenuated the proliferation and migration rates of HUVECs induced by hypoxia (Figures S1B and S1C).

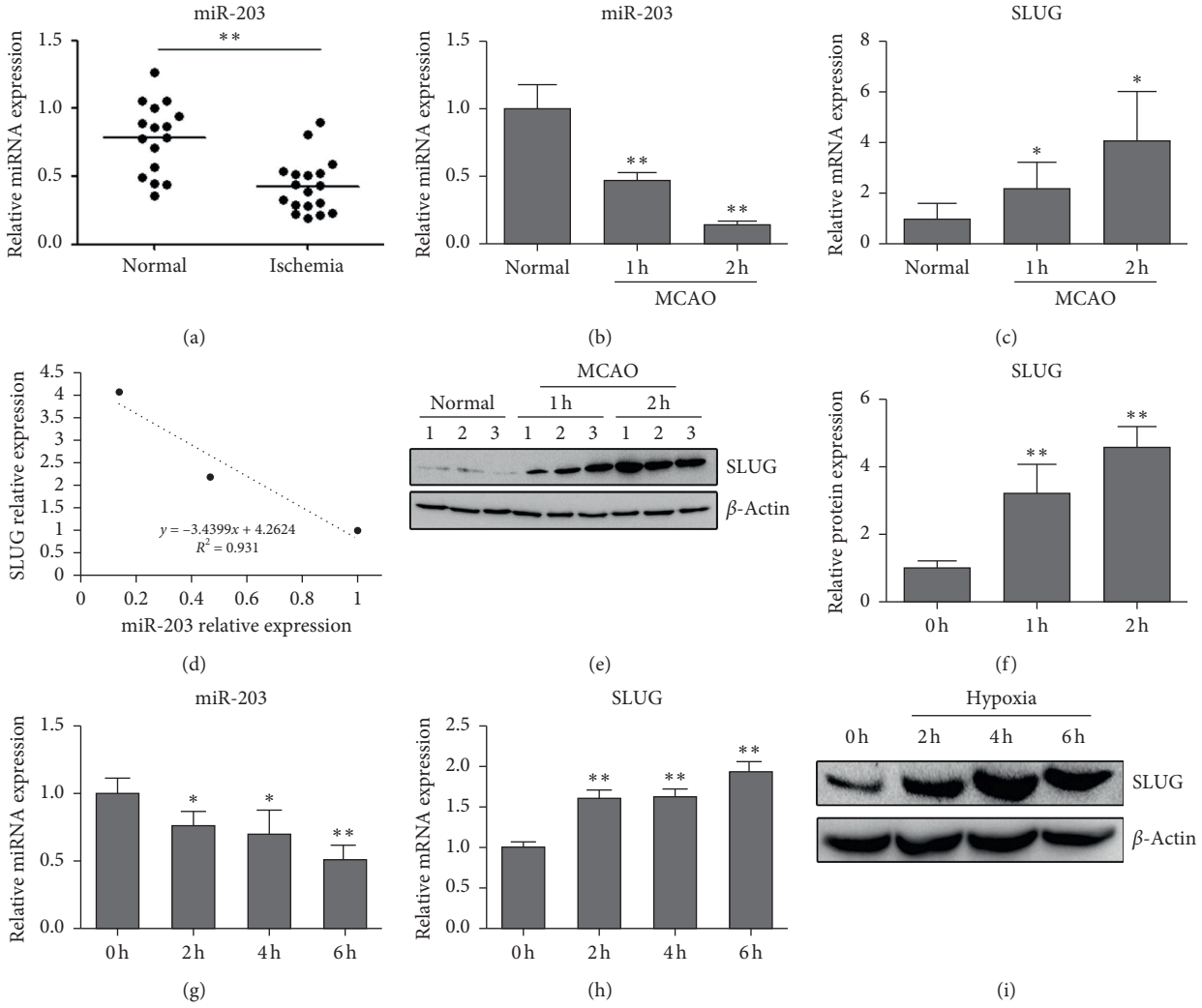

Figure 1: MiR-203 expression is downregulated in serum samples derived from patients with ischemic infarction, brain tissues from MCAO mice, and hypoxia-induced HUVECs, and its expression is negatively correlated with SLUG. (a) The expression of circulating miR-203 in patients with ischemic infarction compared with healthy control. (b) The expression of miR-203 in brain tissues of MCAO mice in 1 h and 2 h experimental groups compared with normal control. (c) SLUG expression in brain tissues of MCAO mice treated with 1h- and 2h-occlusion compared with normal control. (d) Correlation analysis of miR-203 and SLUG expression in brain tissues of MCAO mice. (e, f) Western blot to determine the protein level of SLUG in brain tissues of two groups of MCAO mice compared with normal control ($n = 3$). The relative protein level of SLUG was shown in (f). (g) miR-203 expression in hypoxia-induced HUVECs, compared with normal cells. (h) SLUG expression in hypoxia-induced HUVECs compared with normal cells. (i) Western blot analysis to evaluate the protein level of SLUG in hypoxia-induced HUVECs compared with normal cells. $^{*}P < 0.01$, $^{**}P < 0.01$ versus control.

4. Discussion

Deregulation of miRNAs and transcription factors contributes to a wide range of pathologic diseases, including ischemia. In the present study, we found that the expression of miR-203 was drastically downregulated in patients with ischemic infarction, MCAO mice and hypoxia-treated HUVECs compared with normal controls, while SLUG was upregulated. Moreover, we demonstrated that miR-203 suppressed HUVEC proliferation, invasion, and migration via targeting SLUG. These results suggest that the miR-203-SLUG axis is of great importance in the pathogenesis of cerebral infarction.

In this study, we focused on miR-203, as this miRNA has been previously reported as a tumor suppressor miRNA in carcinogenesis associated with tumor angiogenesis. Several studies have also demonstrated that miRNAs play a critical role in the nervous system. Tripathi et al. revealed that riboflavin treatment increased miR-203 expression, which in turn inhibited the c-Jun expression and increased neuronal cell survival [20]. Yang et al. reported that miR-203 negatively regulated ischemia-induced microglia activation by targeting MyD88, an important adapter protein involved in most Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) pathways [21]. For the relationship between miR-203 and ischemia, only one literature reported that miR-203

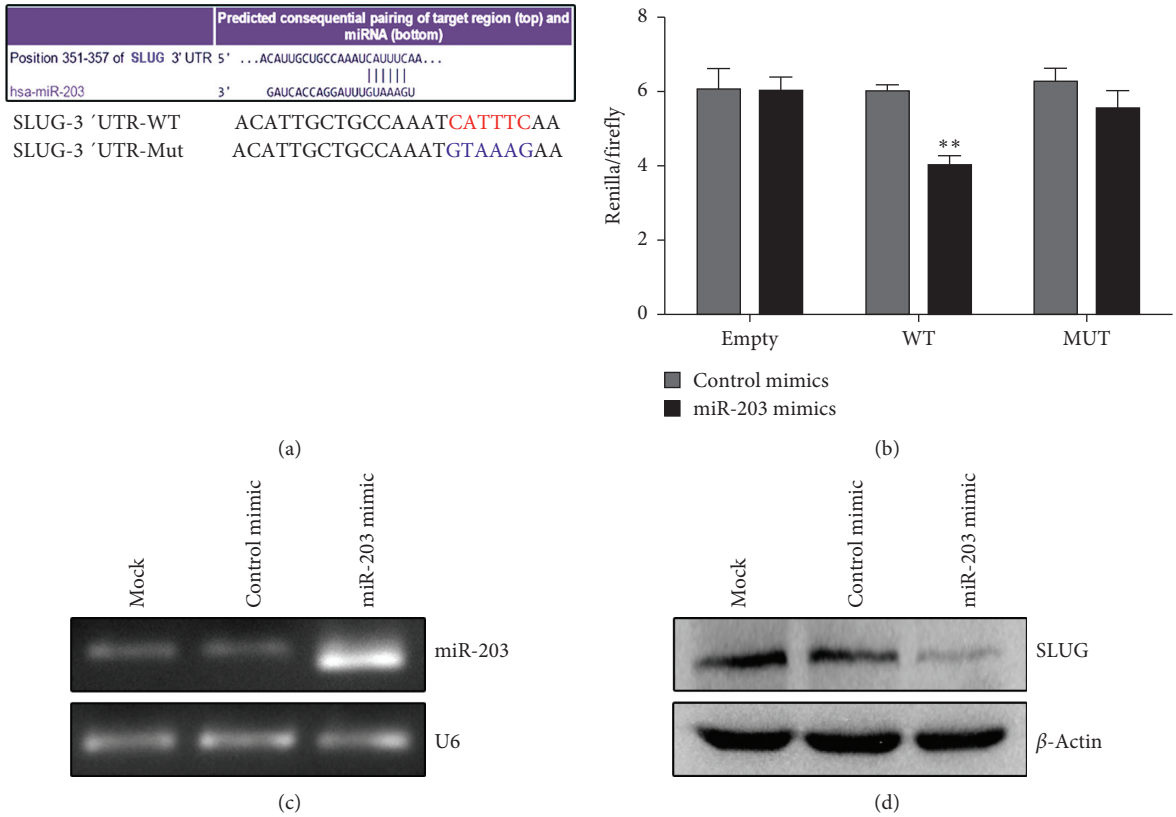

Figure 2: SLUG is a direct target of miR-203 in HUVECs. (a) Schema of miR-203 binding sites within the SLUG 3′UTR. Mutated sites were shown in blue. (b) The psiCHECK-2 sensor plasmids containing the SLUG 3′UTR or mutated 3′UTR were cotransfected in HUVECs with miR-203 mimic or control mimic. Overexpression of miR-203 selectively decreased the luciferase expression compared to controls. No downregulation of luciferase was observed for the negative control or mutated plasmids with miR-203 overexpression. (c) Semiquantitative RT-PCR to determine the expression of miR-203 in HUVECs transfected with miR-203 mimic compared with control. (d) Western blot to determine the protein level of SLUG in HUVECs transfected with miR-203 mimic compared with control. $^{**}P < 0.001$ versus control.

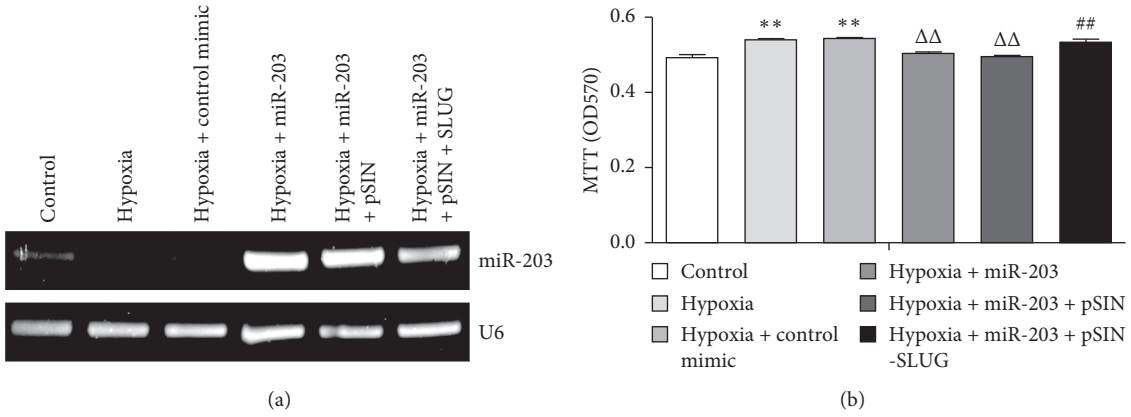

Figure 3: Continued.

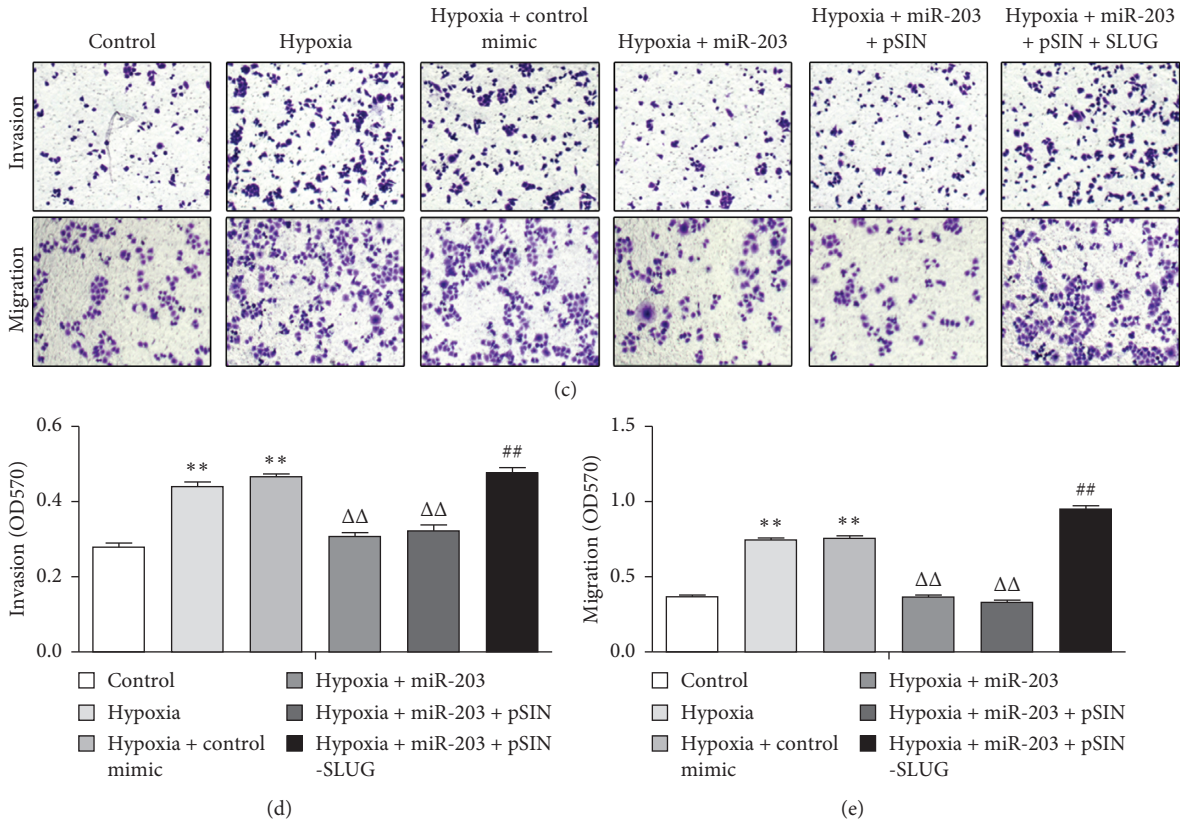

Figure 3: The miR-203-SLUG axis functions as a key regulator in hypoxia-induced cell proliferation, invasion, and migration. (a) Semiquantitative RT-PCR to determine the expression of miR-203 in HUVECs with indicated treatment. (b) MTT assay to determine the cell proliferation rate of HUVECs with indicated treatment. (c–e) Transwell invasion and migration assays to determine the invasion and migration abilities of HUVECs with indicated treatment. Cells invading or migrating to the bottom side of the upper chamber were stained with crystal violet, dissolved with acetic acid, and quantified at OD570 (D and E). $^{**}P < 0.001$ versus control; $^{\Delta\Delta}P < 0.001$ versus hypoxia + control mimic; $^{\#\#}P < 0.001$ versus hypoxia + miR-203 mimic.

functioned as a regulator in acute kidney injury, in which Aldosterone induced NRK-52E cell apoptosis in acute kidney injury via rno-miR-203 hypermethylation and Kim-1 upregulation [22]. In the present study, we systematically identified the expression, function and underlying mechanisms of miR-203 in cerebral infarction.

On the other hand, no literature has demonstrated the role of SLUG in the nervous system and ischemia. Only one study implicated snail as a potential target molecule in cardiac fibrosis after I/R injury [23]. In that paper, Lee et al. demonstrated that I/R injury to mouse hearts significantly increased the expression of snail. Importantly, the cell source of snail induction is endothelial cells. When snail was overexpressed in endothelial cells, they underwent endothelial-to-mesenchymal transition. Snail overexpression-mediated EMT-like cells noticeably stimulated trans-differentiation of fibroblasts to myofibroblasts. The injection of a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, a selective snail inhibitor, remarkably suppressed collagen deposition and cardiac fibrosis in mouse I/R injury and significantly improved cardiac function and reduced snail expression *in vivo*. In our present study, we also demonstrated that ischemia and hypoxia-induced SLUG expression in brain tissues and HUVECs. Overexpression of SLUG induced cell invasion and migration in HUVECs.

In order to further verify that the cytological mechanism of the reverse correlation between miR-203 and SLUG is related to cell proliferation, invasion, and migration, the study established an in vitro hypoxia-induced HUVECs model and confirmed cell proliferation, invasion, and migration, which were reversed by ectopic expression of SLUG, in such a hypoxic condition. The above research findings further support the role of the miR-203-SLUG axis in the angiogenesis of cerebral infarction. Our study first elucidates the role of SLUG in cerebral infarction.

Data Availability

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

Conflicts of Interest

All authors declare that they have no conflicts of interest to this work.

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Supplementary Materials

Figure S1: Silencing of SLUG attenuates the proliferation and migration rates of HUVECs induced by hypoxia. (a) Western blot to determine the protein level of SLUG in indicated groups. (b) MTT assay to determine cell proliferation rate of HUVECs with indicated treatment. $^{**}P < 0.001$ versus control; $^{\#\#}P < 0.001$ versus hypoxia + Ctrl siRNA. (c) Transwell migration assays to determine the migration abilities of HUVECs with indicated treatment. (*Supplementary Materials*)

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Review Article

Renal-Protective Effects and Potential Mechanisms of Traditional Chinese Medicine after Ischemia-Reperfusion Injury

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Renal ischemia-reperfusion (I/R) injury mainly causes acute kidney injury (AKI) after renal transplantation, trauma, sepsis, and hypovolemic shock. Patients with renal I/R injury are frequently associated with a poor prognosis. Traditional Chinese medicine (TCM) has been used for the prevention and treatment of various diseases in China and other Asian countries for centuries. Many studies have shown the protective effect of TCM on renal I/R injury, due to its diverse bioactive components. The potential mechanisms of TCMs on renal I/R injury include anti-inflammation, antioxidative effect, anti-cell death, downregulation of adhesion molecule expression, regulation of energy metabolism by restoring Na^+-K^+-ATPase activity, and mitochondrial fission. This review summarizes the major developments in the effects and underlying mechanisms of TCMs on the renal I/R injury.

1. Introduction

Renal ischemia-reperfusion (I/R) injury is the main cause of acute kidney injury (AKI) and is reported to be associated with high morbidity and mortality in both adults and children. It often occurs after renal transplantation, as well as in sepsis, trauma, hypovolemic shock, etc. [1]. During the renal I/R process, excess reactive oxygen species (ROS) are likely to cause oxidative stress, which subsequently triggers lipid peroxidation [2] and cell death due to apoptosis, autophagy, pyroptosis, and ferroptosis caused by DNA and protein damage in ischemic tissue [3]. In addition, inflammation plays an important role in renal I/R injury, along with leukocyte infiltration and tissue damage in the kidney, followed by excessive production of proinflammatory cytokines [4]. Energy metabolism dysfunction, such as decreased Na^+-K^+-ATPase activity, has been considered as one of the critical mechanisms of renal I/R injury [5].

Increase in blood urea nitrogen (BUN) and creatinine (Cr) [3] and a higher urinary total protein level are reported to occur after renal I/R injury [6]. Histopathology of renal I/R injury shows luminal narrowing, intraluminal necrotic cellular debris, tubular basal membrane rupture, tubular vacuolization, interstitial congestion, apparent cell swelling and nuclear infiltration in the H&E staining of renal tissues [7], and higher Kim-1 expression, thus causing a higher renal tubular injury score [2].

Traditional Chinese medicine (TCM) has been used in China, Korea, Japan, and other Asian countries for the treatment of various diseases, such as cerebrovascular diseases, cardiac diseases, liver diseases, renal disorders, etc. In the theory of TCM, renal I/R injury is attributed to Qi and blood deficiency and blood stasis syndrome [8]. From the perspective of TCM, renal I/R injury should be treated through the replenishment of Qi and activation of blood circulation [7]. Several TCMs have been applied for the prevention and treatment of renal I/R, and they show different renal-protective effects in Table 1, including anti-inflammation (*Cordyceps sinensis*, cordycepin, Yisheng injection, cryptotanshinone, *Panax notoginseng*, total glucosides of paeony, hydroxysafflor yellow A, farnesiferol B, anemoside B4, etc.), antioxidative stress (ligustrazine, berberine, glycyrrhizin, Sheng Mai San, hyperoside, salvianolic acid A, salvianolic acid B, arctigenin, farnesiferol B,

Table 1: The protective effects and potential mechanisms of TCM and the major ingredients on renal I/R injury.

TCM or its ingredients	Experiment object	Protective effects	Potential mechanisms	Ref.
Cordyceps sinensis	SD rats	Decreased inflammation	—	[9]
Cordycepin	SD rats	Decreased inflammation, apoptosis, oxidative stress	Nrf2-HO-1	[10]
Ligustrazine	C57BL/6 mice	Decreased apoptosis, oxidative stress	—	[11]
Yisheng injection	C57BL/6 mice	Reduced neutrophil infiltration, decreased inflammation	—	[12]
Fufang Shenhua Tablet	Wistar rats	Decreased inflammation	Downregulation of TLRs	[8]
	Wistar rats	Decreased inflammation	MyD88 signaling pathway	[13]
	Wistar rats	Decreased Na + -K + -ATPase level	—	[5]
Berberine	NRK-52E cells	Decreased mitochondrial oxidative stress and apoptosis	Sirt1/p53 signaling pathway	[14]
Glycyrrhizin	C57BL/6 mice	Inhibition of inflammation and renal cell apoptosis	p38 MAPK signaling pathway	[15]
Honokiol	Wistar albino rats	Inhibition of oxidative stress and inflammation	—	[16]
Cryptotanshinone	C57BL/6 mice	Inhibition of cell apoptosis and inflammatory response	NF-κB-p38 MAPK signaling pathway	[17]
Hyperoside	C57BL/6 mice HK-2 cells	Attenuated tubular cell apoptosis, oxidative stress, inhibited mitochondrial fission	OMA1-OPA1 axis	[18]
Notoginsenoside R1	SD rats	Reduced apoptosis and inflammatory response	NF-κB-p38 MAPK signaling pathway	[19]
Brazilin	SD rats	Decreased inflammation attenuated apoptosis and oxidative stress	NF-κB signaling pathway	[20]
Salvianolic acid A	SD rats HK-2 cells		Akt/mTOR/4EBP1 pathway	[21]
Salvianolic acid B	BALB/c mice	Reduced oxidative stress and inflammation, inhibition of pyroptosis	Nrf2/NLRP3 signaling pathway	[22]
Total glucosides of paeony	SD rats NRK-52E cells	Attenuated apoptosis and inflammation	XIST/miR-124-3p/ITGB1 axis	[23]
Hydroxysafflor yellow A	SD rats	Decreased inflammation	TLR4/NF-κB pathway	[4]
Sanqi oral solution	SD rats	Enhanced autophagy, attenuated apoptosis	ERK/mTOR pathway	[7]
Arctigenin	C57BL/6 mice	Alleviated inflammatory response and oxidative stress	NF-κB signaling pathway	[3]
Farnesiferol B	Female C57/BJ mice	Attenuated inflammation	TGR5/NF-κB signaling pathway	[2]
Polydatin	BALB/c mice Primary renal tubular epithelial cells	Antiapoptosis and antioxidative effects	Sonic hedgehog (shh) signaling pathway	[24]

polydatin, Shenfu injection, etc.), anti-cell death (honokiol, cryptotanshinone, brazilin, salvianolic acid B, Sanqi oral solution, etc.), downregulation of adhesion molecule expression (ligustrazine and Yisheng injection), regulation of energy metabolism by restoring Na^+-K^+-ATPase activity (Fufang Shenhua Tablet), and mitochondrial fission (hyperoside).

In this review, we have discussed the studies in the past decades on the protective effects and potential mechanisms of TCMs and the major ingredients on renal I/R injury.

2. Protective Effects of TCMs and Major Ingredients on Renal I/R Injury

Generally speaking, almost all the following TCMs and the major ingredients play a protective role in renal I/R injury, including the improvement of renal function and histological changes in renal tissues, inhibiting inflammatory cytokine release and macrophage/neutrophil infiltration, decreasing production of oxidative stress and lipid oxidation, regulating programmed cell death (apoptosis, autophagy, pyroptosis, or ferroptosis), decreasing release of adhesion molecules, regulating energy metabolism, endothelial injury, and mitochondrial dysfunction [25].

2.1. Renal Function and Renal Histological Examination. In previous studies, renal function was often measured by the levels of Scr and BUN. In some cases, urinary total protein level was also used for renal function measurement. It was reported that Scr and BUN levels were significantly increased in the I/R model group, compared with the sham group. TCMs or CCMs, such as Fufang Shenhua Tablet, cryptotanshinone, and notoginsenoside R1, could inhibit the increased levels of Scr and BUN, and thus improve renal function [13, 17, 19]. In addition, anemoside B4 could decrease the urinary total protein level [6], indicating improvement of renal function.

Several histological abnormalities were observed after renal I/R injury, including tubular brush border loss, epithelial cell dilatation and necrosis, cytoplasmic

vacuolization, and cast formation [3]. Most TCMs were reported to alleviate renal histological changes and decrease renal tubular injury score, caused by I/R injury. Furthermore, TCMs, such as Sanqi oral solution, hydroxysafflor yellow A, and total glucosides of paeony, showed dose-dependent effects, such that higher concentrations showed greater alleviating effects [4, 7, 23]. In contrast, ATG played a detrimental role in renal I/R injury since renal function and histology worsened with increasing concentration [3].

2.2. Anti-Inflammatory Activity. Most TCMs play an anti-inflammatory role in renal protection after I/R injury. Increase in expressions of inflammatory genes, such as MCP-1 and TNF-a mRNA, and their proteins were observed in the renal I/R group. *Cordyceps sinensis* treatment was reported to reverse these effects [9, 26]. Additionally, cordycepin, the extract from *Cordyceps sinensis*, could decrease secretion of proinflammatory factors and alleviate inflammatory reaction [10], including IL-1β, IL-6, and TNF-α, at the protein and mRNA levels. The same effect was observed after treatment with Yisheng injection, Fufang Shenhua Tablet, glycyrrhizin, honokiol, cryptotanshinone, notoginsenoside R1, brazilin, total glucosides of paeony, hydroxysafflor yellow A, arctigenin, farnesiferol B, and anemoside B4 [2–4, 6, 8, 12, 13, 15–17, 19, 20, 23]. Moreover, the infiltration of neutrophils and macrophages was observed in some studies. ATG decreased the infiltration of CD11b + Gr1+ neutrophils and CD68+ macrophages in renal tissue after I/R injury [3], and the neutrophil infiltration was decreased after pretreatment with Yisheng injection [12].

2.3. Antioxidative Stress. Oxidative stress and lipid oxidation increase after renal I/R injury, while treatment with some TCMs inhibits the production of oxidative stress and promotes the production of antioxidant factors. In Feng Han and AR Shahed's studies [10, 26], *Cordyceps sinensis* and its extract cordycepin increased the levels of antioxidative factors, including GSH, GSHPx, and SOD, and decreased the levels of oxidative stress products, including MDA, NO, and iNOS. Other TCMs also showed similar effects, such as MDA decrease and SOD increase in the ligustrazine treatment group [11], berberine treatment group, and Sal A and Sal B treatment groups [21, 22], SOA decrease and SOD increase after glycyrrhizin treatment [15], MDA/MPO decrease and SOD/CAT increase after honokiol treatment [16], decrease in iNOS and $ONOO^-$levels after SMS treatment [27], and decrease in ROS levels after hyperoside treatment. CS treatment decreased NGAL levels both in kidney tissues and in urine, which was increased in the I/R group [28]. In addition, farnesiferol B reduced oxidative stress (diminished levels of NAGL and H2O2) and lipid oxidative signaling pathways (diminished levels of lipid peroxidation markers, including 4-HNE and MDA) in renal I/R [2].

2.4. Anti-Cell Death. There are several types of programmed cell death, including apoptosis, autophagy, pyroptosis, and ferroptosis. Previous studies have reported that the protective effect of TCMs on renal I/R injury was associated with programmed cell death, especially apoptosis.

Antiapoptosis was observed after treatment with some TCMs before or after renal I/R injury, with downregulation of the levels of apoptosis proteins and upregulation of the levels of antiapoptosis proteins. In the *Cordyceps sinensis* treatment group [26], Fas and FasL mRNA expression levels were decreased, while caspase-3 activity was decreased after glycyrrhizin and honokiol treatment [15, 16]. TUNEL-positive cells were increased after Sal A treatment, indicating a reduction of apoptotic activity [21]. An *in vitro* study found that berberine significantly inhibited the expression of apoptotic proteins, such as Bax [14].

Treatment with Sanqi (SQ) oral solution showed decrease in apoptosis and increase in autophagy [7]. Lower Bax and caspase-3 levels and higher Bcl-2 level were observed in the SQ treatment group in a dose-dependent manner, demonstrating the antiapoptotic activity of SQ. Meanwhile, compared to the I/R model group, higher dose of SQ significantly increased the levels of LC3II/LC3I and Beclin1, indicating an enhanced autophagy activity after renal I/R injury. In addition, treatment with SQ and 3-MA, an autophagy inhibitor, showed worse histological damages and renal function, compared with SQ alone treatment, indicating the essential role of autophagy in SQ treatment during renal I/R injury.

In Yu Pang's study [22], the levels of GSDMD, caspase-1, and IL-1β, the pyroptosis-related proteins, were strongly upregulated in the model group. The pyroptosis-related proteins were significantly decreased after treatment with Sal B, demonstrating the antipyroptosis activity of Sal B.

Lastly, ferroptosis plays an important role in the protective effects of farnesiferol B treatment on renal I/R injury. The mRNA expression of Gpx4, the key ferroptosis regulator, was significantly reduced after renal I/R injury, while farnesiferol B treatment increased its expression. However, the intergroup difference was not significant [2]. So, further studies are needed.

2.5. Downregulation of Adhesion Molecules. In the I/R model group, expression of ICAM-1 was upregulated but was greatly diminished after ligustrazine treatment [11]. Moreover, ICAM-1 was downregulated in a dose-dependent manner after pretreatment with Yisheng injection in the renal I/R group [12].

2.6. Recovery of Na^+-K^+-ATPase Activity. Yang Yang et al. [5] found a significant decrease in Na^+-K^+-ATPase activity in the I/R model group when compared with the sham group. However, Shenhua Tablet (SHT) was observed to increase the decreased Na^+-K^+-ATPase activity after renal I/R, both in the low-dose group and the high-dose group. Combined with other results in this study, the authors concluded that SHT could recover the number of Na^+-K^+-ATPase and improve its activity in the tubular epithelial cells, promote epithelial cell polarity in renal tubular cells, and thereby improve renal function.

2.7. Endothelial Protective Effects. Salvianolic acid A (SAA) can protect peritubular capillary endothelium from renal I/R injury [29]. VEGFA expression was increased in tubular epithelial cells, which was associated with peritubular capillary density in the SAA treatment group, as well as the reduced levels of plasma vWF and lower platelet activation after I/R injury. Moreover, significant increase in Klotho protein expression was observed, indicating less endothelial injury after renal I/R with SAA treatment.

3. Potential Mechanism of TCMs and Major Ingredients in Renal I/R Injury

Several signaling pathways were considered to be involved in the renoprotective effects of TCMs, especially the anti-inflammation effect, antioxidative effect, antiapoptotic activity, and some others. These pathways included the NF-κB/TLR4, NF-κB-p65, NF-κB-p MAPK, Nrf2-NLRP3, Nrf2-HO-1, Shh signaling pathway, PI3K-Akt [30], ERK/mTOR [7], Akt/mTOR/4EBP1 [21], OMA1-OPA1 [18], XIST/MicroRNA-124-3p/ITGB1 [23], etc. The most important pathways involved in renal I/R injury are discussed below.

3.1. NF-κB Signaling Pathway. NF-κB is believed to play an important role in the anti-inflammatory effects and anti-apoptotic and antioxidative activity of TCMs in renal I/R process.

Activation of the NF-κB signaling pathway was inhibited in the cryptotanshinone (CTS) treatment group after renal I/R [17]. I/R significantly increased the phosphorylated p65 and IκBα levels, indicating the activation of NF-κB signaling pathway. After CTS treatment, the phosphorylation of p65 and IκBα was inhibited. Brazilin was also proven to downregulate the inflammatory activity by inhibiting NF-κB signaling pathway activation *in vitro* [20].

Hydroxysafflor yellow A (HSYA) was reported to decrease the renal inflammation after I/R injury by suppressing the TLR4/NF-κB signaling pathway activation [4], as phospho-IKKβ, phospho-IκBα, and NF-κB-p65 expression levels were upregulated in the I/R group but were downregulated after treatment with HSYA *in vitro*. Similar effect of arctigenin (ATG) and polydatin on TLR4 and NF-κB-p65 expression was observed [3, 31].

Notoginsenoside R1 (NR1) decreased inflammatory factors and apoptosis by inhibiting the phosphorylation of p38 MAPK and NF-κB activation in renal tissue after I/R injury [12]. The anti-inflammatory effects and antioxidative activities of farnesiferol B on renal I/R injury were based on the activation of TGR5 and inhibition of the NF-κB pathway [2].

3.2. Nrf2 Signaling Pathway. In renal I/R process, Nrf2 plays a central role in renal-protective effects of TCMs, including the anti-inflammatory effects and antioxidative and anti-cell death activity.

Cordycepin is known to alleviate inflammation, oxidative stress, and apoptosis in the I/R process. The antioxidative mechanism of cordycepin was analyzed by measuring the levels of Nrf2 and HO-1, the key proteins in antioxidative stress process. Western blotting results showed an increase in the expression of Nrf2 and HO-1 after cordycepin treatment, indicating that cordycepin may suppress the oxidative stress during renal I/R injury through the Nrf2/HO-1 signaling pathway [10].

Another study found that Sal B could inhibit caspase-1/GSDMD-mediated pyroptosis and thereby alleviate I/R injury in mice, by activating Nrf2/NLRP3 signaling pathway [22]. Sal B was reported to inhibit pyroptosis by downregulating pyroptosis-related proteins GSDMD, caspase-1, and IL-1β. Moreover, Sal B showed its antioxidative property by inhibiting the expression of Keap1 protein, thus increasing Nrf2 nuclear expression and HO-1 protein levels *in vivo*. *In vitro*, Sal B reduced NLRP3 protein expression and increased Nrf2 nuclear import and activated expression of downstream antioxidant components. Moreover, the Nrf2/NLRP3 signaling pathway was demonstrated to be involved through Nrf2 knockdown *in vivo* and siNrf2 transfection *in vitro*.

3.3. Sonic Hedgehog (Shh) Signaling Pathway. Shh signaling pathway is important in the antioxidative and antiapoptotic effects of TCM treatment after renal I/R injury. Polydatin alleviated apoptosis and oxidative stress after renal I/R injury through activating the sonic hedgehog signaling pathway [24]. Polydatin was found to dose-dependently induce increase in the Shh gene and protein expression compared with the I/R group, both *in vitro* and *in vivo*. Moreover, the mRNA and protein expression of Ptch 1 and Smo, two key elements in the Shh signaling pathway, were reduced by cyclopamine (an inhibitor of Smo) combined with polydatin. Meanwhile, higher apoptotic activity and oxidative effects were observed. Therefore, inhibition of Shh signaling impaired the antioxidative and antiapoptotic effects of polydatin.

4. Summary

This review discusses the protective effects of TCMs and their active components on renal I/R injury. The mechanisms of the protective effects of TCMs include inhibition of inflammation, decrease of oxidative stress and lipid oxidation products, regulation of programmed cell death, reduction of adhesion molecule release, and regulation of energy metabolism and endothelial injury by activating NF-κB and Nrf2 signaling pathways, which are considered to be multiple targets for successful treatment. However, the existing studies are either animal experiments *in vivo* or *in vitro* studies, with lower level of evidence and strength of recommendation. Therefore, further clinical studies are needed to explore the effects of TCMs and the underlying mechanisms.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Demin Liu and Songling Tang contributed equally to this study.

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Research Article

Protective Role of Sulodexide on Renal Injury Induced by Limb Ischemia-Reperfusion

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Background. Though widely known as a potent antithrombin agent with protective effects on the kidney and other remote organs, it is currently ambiguous when it comes to sulodexide's function on ischemia-reperfusion (I/R) injury. With this research, we pursued to further explore how sulodexide exerts its influence on limb I/R injury, in which deleterious effects on the kidney were what we primarily focused on. *Methods*. We randomized twenty-four C57BL/6 male rats into three groups, namely, sham operation group (control group), I/R group, and sulodexide pretreatment group. Hematoxylin and eosin staining was applied for discovery of renal histological changes. Serum creatinine (Cr) and serum urea nitrogen (BUN) were measured. Apoptotic parameters were detected by the TdT-mediated dUTP Nick-End Labeling method. To what extent and levels that antiapoptotic and proapoptotic proteins were expressed could be sensitively revealed by immunohistochemistry assay. Lipid peroxidation product propylene glycol and inflammatory factors were examined by enzyme-linked immunosorbent assay. Additionally, an extracorporeal hypoxia-reoxygenation (H/R) model of human renal proximal tubule epithelial HK2 cells was established. Our targets lay in cell proliferation and apoptosis, and we used western blotting to reflect apoptosis-related gene expression. *Results*. The levels of serum BUN, Cr, and inflammatory factors in sulodexide-intervened rats manifested significant reduction when compared with the I/R group. Also, sulodexide could protect the kidney from histological changes and could effectively inhibit intraparenchymal apoptosis. Furthermore, adding 2 μl/mL or 5 μl/mL of sulodexide to H/R model cells in vitro gave rise to significant restoration of the degenerative proliferation capacity of the HK2 cells following H/R injury and late cellular apoptosis experienced dramatic reduction versus the H/R group. When treated with 5 μl/mL of sulodexide at a dose of 10 mg/kg, the levels of the antiapoptotic proteins were increased, while the proapoptotic proteins showed opposite trends. Notable escalation on antiapoptotic protein expression level, in contrast with the opposite trends exhibited in proapoptotic proteins, was observed with 5 μl/mL sulodexide pretreatment with the dosage being 10 mg/kg. *Conclusion*. Sulodexide can protect against kidney damage caused by I/R injury of the lower limbs by enhancing cell proliferation, inhibiting apoptosis, reducing inflammatory reactions, and scavenging oxygen free radicals.

1. Introduction

Ischemia-reperfusion (I/R) is a common phenomenon in clinical practice associated with high morbidity and mortality [1]. Due to its high incidence and devastating systemic effects [1], ischemia-reperfusion (I/R) injury has grabbed extensive attention in a host of clinical situations, as ischemia induces serious damage to not only the local organ but other involved organs as well, which is subsequently exacerbated by reintroduction of oxygen upon reperfusion [2]. In this process, a series of pathophysiological steps are strongly interacted with final postoperative death, among which distant multiple organ dysfunction is considered to be a fatal initiator [3–6].

In addition to remote injury in lung and intestinal tissues, acute kidney injury (AKI) also usually occurred following limb I/R injury [7–9]. Although efforts had been made to avoid or at least partly attenuate AKI, associated morbidity and mortality rates remain high over the decades [10]. Therefore, identifying novel preventive measures to lower the incidence of AKI and improve clinical outcomes is of urgent demand.

To date, even if awaiting to be fully clarified, the precise mechanisms underlying I/R injury have increasingly been disclosed, with inflammatory reaction, bursting release of reactive oxygen species and large scales of apoptotic cells generation predominantly composing this pathophysiological condition [11–13]. Moreover, the confirmed evidence that medications such as heparin [14, 15] may attenuate I/R injury exactly implies the crucial role played by microvascular thrombosis formation in this I/R injury-induced nonequilibrium status [12]. Therefore, clinically, it is imperative to identify pharmacological agents with multifunctional properties such as anticoagulation, anti-inflammatory, and antioxidant activities that may be effective treatments for AKI attributed to lower extremities ischemia-reperfusion injury.

Sulodexide, as a new antithrombotic agent, to be more precise, a glycosaminoglycan under very purification, has reportedly shown positive effects in many conditions that result from vascular surgery, with heparin sulfate and dermatan being its two major molecular components [16]. The pharmacological effects of sulodexide are made apparent by its capacity to repair the inner wall of injured blood vessels including by rebuilding the barrier structure of the vascular endothelial lumen surface and the glycocalyx and by restoring basic functions and selective permeability of the vascular endothelium and the ability to significantly reduce inflammatory factors, such as matrix metalloproteinase (MMP)-9, thereby lessening damage to the endothelium caused by inflammatory factors [17–21]. Also, with heparan sulfate and dermatan sulfate, respectively, possesses great affinity for antithrombin III and heparin cofactor II, the mutually strengthened anticoagulant effect makes sulodexide a dual antithrombotic agent [16, 22]. Moreover, blood rheology is improved by the drug's lipid-lowering effect triggered by activating lipoprotein esterase activity [16, 23]. Sulodexide may also exert positive effects in renal disease [24–27] by significantly reducing the level of proteinuria, preventing the onset of a nephropathy hypercoagulable state, inhibiting renal fibrosis, and protecting residual renal morphology and function. Our research aims to explore whether sulodexide could avoid or attenuate lower-limb I/R injury and kidney malfunction caused by this event and further investigate the associated pathophysiological mechanism.

2. Materials and Methods

2.1. Experimental Animals. Eighteen male C57BL/6 mice (8–10 weeks old, with weight ranging from 20 g–25 g) were obtained from the Experimental Animal Center of the Chinese Academy of Sciences (Beijing, China). All of the work was carried out in accordance with the regulations required by ethics committee of Hebei Medical University. The study animals were accommodated in a relatively homothermal and humidity-controlled room, where food and water was provided ad libitum.

2.2. Experimental Design. We randomly arranged the eighteen rats into three groups of six animals each ($n = 6$). All rats were intraperitoneally treated with 40 mg/kg of sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA) and kept at 37°C. In the sham-operated control (sham) group, the femoral artery in the right leg was exposed and the incision was then closed without inducing I/R injury. In the group of I/R, the right femoral artery was also exposed and a rubber band was used to occlude the right hind femoral artery, thus inducing limb perfusion dificits for three hours, followed by band release to allow reperfusion for four hours. Finally, in the I/R + sulodexide group, exposure to rubber band application to limb ischemia for three hours was in combination with intravenous injection of 10 mg/kg of sulodexide; the block was then released for four hours to allow reperfusion. After seven hours, intraperitoneal administration of sodium pentobarbital injection at 100 mg/kg was implemented on the rat for euthanization.

2.3. Blood Collection and Detection. Prior to euthanasia, the right eyeball of each animal was obtained for 0.5 mL of the blood sample, allowed to stand for 15 minutes in a thermostatic water bath at 35°C, with rotating speed at 3,000 r/min for five minutes for centrifugation. The supernatant was then collected for following analysis: blood urea nitrogen (BUN) (Jiangcheng Biotechnology, Nanjing, China) and creatinine (Cr) (Jiangcheng Biotechnology) following the manuals.

The levels of MMP-9, IL-1, TNF-α, and IFN-γ in the serum were measured with enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Enzyme-linked immunosorbent assay was secondarily conducted following the manufacturer's guidance, to evaluate serum inflammatory parameters including levels of MMP-9, interleukin (IL)-1, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ.

2.4. Determination of Renal Oxidative Stress Biomarkers. Renal homogenates were prepared according to the method of Wang et al. [28].

2.5. Renal Histology. Immediately after organ procurement, the color, elasticity, degree of swelling, exudation, and the degrees of necrosis and adhesion were assessed and photographed. 4% paraformaldehyde was used to immerse and fix the renal tissue, which was then embedded in paraffin. Having been exposed to the above process, tissue sections measuring 5 μm were stained with hematoxylin and eosin and examined for the renal morphology changes with a light microscope. Findings of interest included the degree of tissue edema, the destruction of nephron structural integrity, the accumulation of inflammatory cells, and the shedding of red blood cells.

2.6. Culture and Maintenance of HK2 Cells. HK2 cells, deriving from human renal proximal tubule epithelial cell line, were cultured in an extracellular matrix (ECM) medium (Gibco Laboratories, Gaithersburg, MD, USA) with fetal

bovine serum at concentration of 10% contained (Gibco Laboratories).

2.7. Establishment of the In Vitro Hypoxia/Reoxygenation (H/R) Model of HK2 Cells. We model the cells to be victim of H/R injury when the growth was close to the confluent state; the number of cells was about 1×10^6. The culture medium was replaced by a sugar-deficient solution (containing 1.8 mmol/L of $CaCl_2$, 0.9 mmol/L of NaH_2PO_4, 6.0 mmol/L of $NaHCO_3$, 20 mmol/L of HEPES, 1.2 mmol/L of $MgSO_4$, 98.5 mmol/L of NaCl, and 10 mmol/L of KCl; pH: 6.8). Smooth gaseous influx of 5% CO_2 combined with 95% N_2 conteneously flowed into the sterilized oxygen-demanding box, where the culture dish was located, until there was less than 0.1% of oxygen inside the box and such environment should be maintained for one hour. Then, the culture medium was replaced by a simulated reperfusion solution (containing 5.0 mmol/L of KCl, 20 mmol/L of $NaHCO_3$, 129.5 mmol/L of NaCl, 55 mmol/L of glucose, 0.9 mmol/L of NaH_2PO_4, 1.2 mmol/L of $MgSO_4$, 1.8 mmol/L of $CaCl_2$, and 20 mmol/L of HEPES; pH: 7.4), followed by incubation with 95% O_2 and 5% CO_2 for reoxygenation for 24 hours.

2.8. Cell Treatment. The experimental cells were divided into the following four groups: control, H/R, 2 μl/mL of sulodexide + H/R and 5 μl/mL of sulodexide + H/R. Being treated with 2 μl/mL or 5 μl/mL of sulodexide before reoxygenation was done where cells in sulodexide treatment groups distinguish from others. Repeatability was ensured with as much as three times of implementation on all experiments.

2.9. Sulforhodamine Experiments. SRB experiments were performed as previously described [29].

2.10. MTT Experiments to Detect Cell Viability. Twenty thousand treated HK2 cells were plated into a 96-well plate ($n = 6$) each of which was added with 20 mL of MTT (5 mg·mL). Later another four hours was spent for incubation of the plates at 37°C. The next step was to remove the MTT and to dissolve formazan crystals for 10 minutes in 150 mL of DMSO at 37°C. Measurement of absorbance was realized by a microplate reader at 570 nm.

2.11. Apoptosis Detection. Apoptosis (annexin V and PI double staining) was detected by flow cytometry according to the manufacturer's protocols (BD Pharmingen™, BD Biosciences, San Diego, CA, USA). Following the guidance of rules drawn by the manufacturer, staining cells with annexin V-FITC and PI was used to quantitatively evaluate apoptotic cells, while flow cytometry for detection (BD Pharmingen™; BD Biosciences, San Diego, CA, USA).

2.12. Reverse Transcription and Quantitative Polymerase Chain Reaction (PCR) to Detect Messenger RNA (mRNA) Expression. The whole spectrum of RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and reversely transcribed as previously referred. The SYBR green (Takara, Dalian, China) method and IQ5 real-time PCR detection system (BioRad Laboratories, Hercules, CA, USA) were used for real-time PCR. The primer sequences were as follows: BAX, forward: CCTTTTCTACTTTGCCAGCAAAC; BAX, reverse: GAGGCCGTCCCAACCAC; BCL-2, forward: TCCGCATCAGGAAGGCTAGA; BCL-2, reverse: AGGACCAGGCCTCCAAGCT; caspase-3, forward: AGAACTGGACTGTGGCATTGAG; caspase-3, reverse: GCTTGTCGGCATACTGTTTCAG; BCL-xL, forward: CATGGCAGCAGTAAAGCAAG; BCL-xL, reverse: TAGAGTTCCACAAAAGTATC; BAD, forward: AAGGCTTGGTCCCATCGGAAGTTT; BAD, reverse: TTAACATTTGGTAGTGAGCACGGCCC; GAPDH, forward: TGCACCACCAACTGCTTAGC; and GAPDH, reverse: GGCATGGACTGTGGTCATGAG.

2.13. Western Blot. As has been introduced, western blotting was orderly performed [28]. The antibodies, sourced from either Abcam (Cambridge, England) or Cell Signaling Technology (CST) (Danvers, MA, USA), were as follows: BAX, Abcam cat no. ab182734; BCL-2, Abcam cat no. ab692; caspase-3, CST cat no. 9662; BCL-xL, CST cat no. 2764; BAD, Abcam cat no. ab32445; β-actin, Abcam cat no. ab8227; goat antimouse horseradish peroxidase secondary antibody, Abcam cat no. ab47827; goat antirabbit horseradish peroxidase secondary antibody, Abcam cat no. ab7090; and enhanced chemiluminescence (ECL) substrate luminescent liquid, Abcam cat no. 133406.

2.14. Statistical Analyses. All of the data are presented as mean ± standard error of the mean. The statistical significance of parameters was assessed by one-way analysis of variance combined with Tukey's post hoc test using the Graph Pad Prism software version 5 (Graph Pad Inc., La Jolla, CA, USA). A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. In an In Vivo Rat Model, Sulodexide Treatment Ameliorated Renal Dysfunction Caused by Hind-Limb I/R Injury. Classic biomarkers were detected to assess the correlation between the renoprotection conferred by sulodexide under the circumstance of I/R injury and inflammation and oxidative stress. Firstly, an in vivo hind-limb I/R injury model was established. As shown in Figure 1(a), the mice were randomly allocated into three groups and each group was treated as described in the Materials and Methods section. As expected, the Cr and BUN levels in serum were significantly increased following hind-limb I/R treatment but attenuated by sulodexide treatment (Figure 1(b)). As Figure 1(a) illustrates, preliminarily, the randomly arranged three groups of animals were treated as afore mentioned in the Materials and Methods chapter, in which way we progressively established an in vivo hind-limb I/R injury model. Aligning with our previous expectation, attenuation of

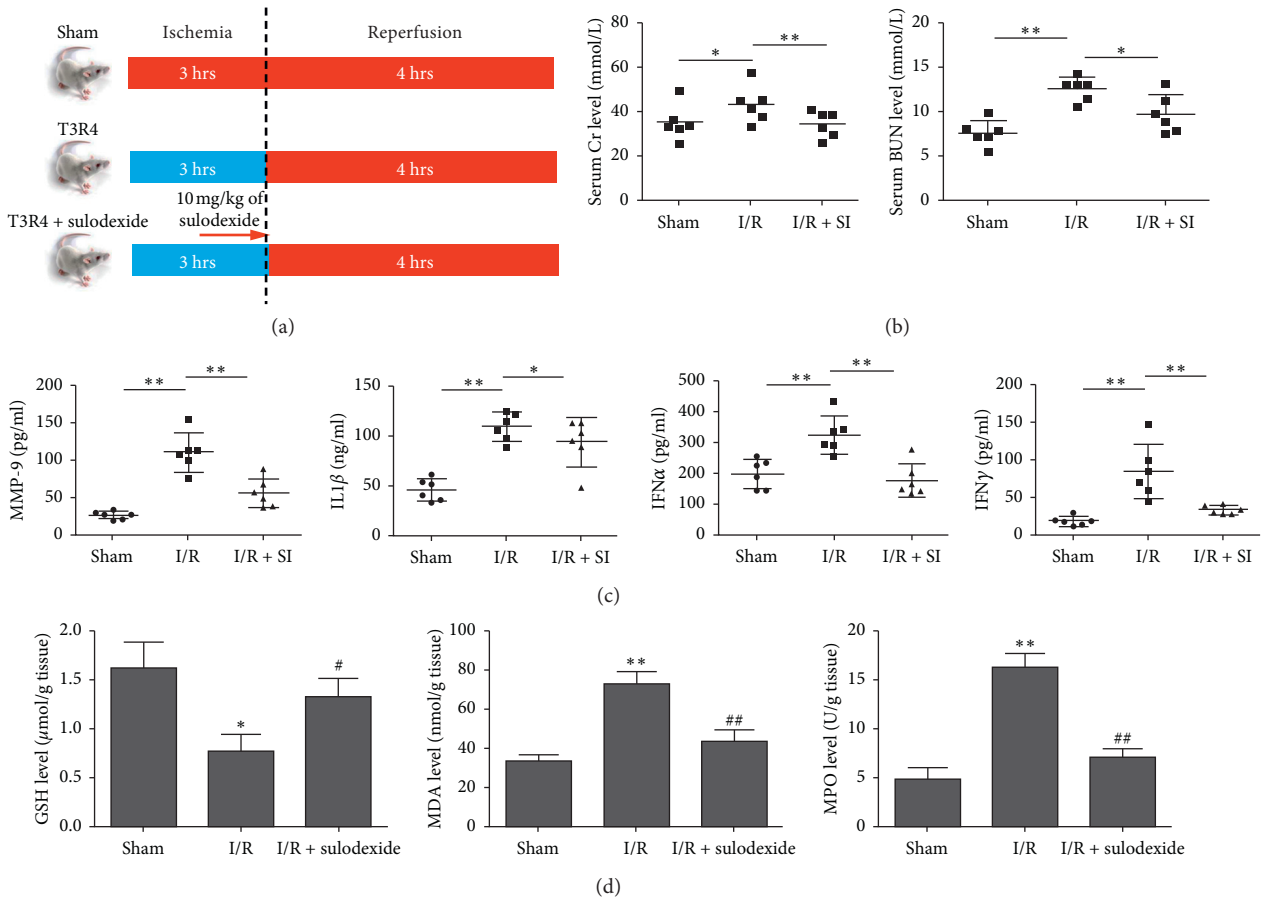

Figure 1: In an in vivo rat model, sulodexide treatment ameliorates renal dysfunction caused by hind-limb I/R injury. (a) A schematic to show hind-limb I/R injury and sulodexide intervention. (b) Evaluation of Cr and BUN levels in serum samples. (c) Evaluation of MMP-9, IL-1β, TNF-α, and IFN-γ levels in the animals' serum samples. (d) Assessment of glutathione, MDA, and MPO expression levels in renal parenchyma. The data were statistically analyzed by Student's t test. *, $P < 0.1$, **, $P < 0.01$, and ***, $P < 0.001$.

marked increase attributed to hind-limb injury was incurred by sulodexide treatment (Figure 1(b)), while similar trends were manifested in Figure 1(c), where dramatic elevation to various degrees on inflammatory factors such as MMP-9, IL-1β, TNF-α, and IFN-γ in serum was closely followed by significant dropping down by sulodexide. The MDA level and myeloperoxidase (MPO) activity in renal tissue also exhibited the same results as the above factors, while the glutathione level in renal tissue was significantly declined following hind-limb I/R treatment but would be improved by sulodexide treatment (Figure 1(d)). In summary, these results suggested that such dysfunction could be ameliorated using sulodexide partially by anti-inflammation and anti-oxidant stress.

3.2. Renal Histological Changes Occurred Following Hind-Limb I/R Injury in In Vivo Rat Models. Hematoxylin and eosin staining of renal tissue from the I/R group in comparison with that from sham rats revealed that the renal tissue of the former group had suffered varying degrees of damage, including tissue edema, destruction of the nephron structural integrity, aggregation of inflammatory cells, and shedding of red blood cells. Yet sulodexide's promising potential to significantly ameliorate this damage was evidently bolstered by the contrary tendency after its application (Figure 2).

3.3. Renal-Cell Apoptosis Was Alleviated in I/R Injury Rats with Sulodexide Pretreatment. TdT-mediated dUTP Nick-End Labeling (TUNEL) assays on the kidney sections was conducted for deeper exploration on whether antiapoptosis process was involved in sulodexide's protective role under I/R injury. Administration of sulodexide remarkably reversed I/R injury induced growth in cell apoptosis compared with rats in the control group (Figure 3). Furthermore, to what extent the proapoptosis proteins BAX and BAD and the antiapoptosis proteins BCL-2 and BCL-xL expressed, together with the activity of caspase-3, were simultaneously tested as well. Here, it was observed that I/R injury led to a substantial decrease in the expression levels of BCL-2 and BCL-xL and an increase in the expression levels of BAX and BAD as indicated in Figure 4, although the total caspase-3 was not changed (Figure 4). Corresponding with precedent findings, BCL-2 and BCL-xL underwent explicit retardation on their expression, while opposite manifestation was seen in BAX and BAD, with the level of caspase-3 remaining

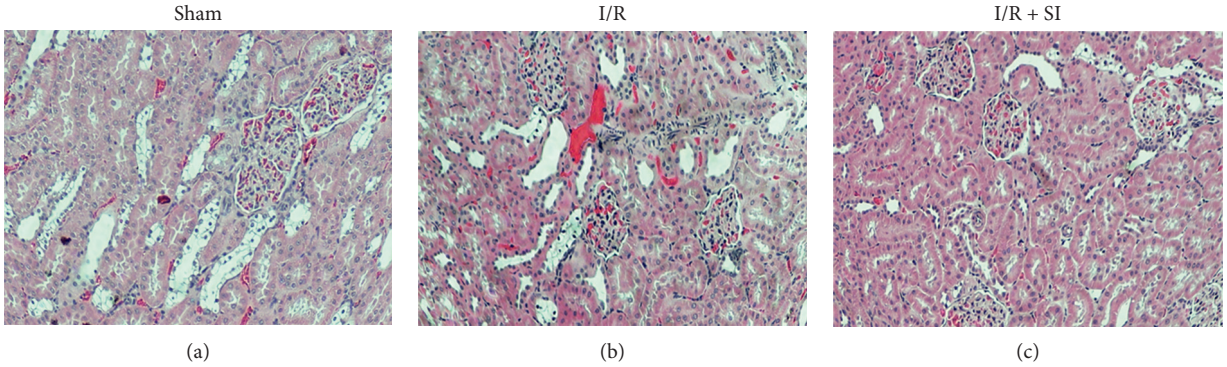

Figure 2: Hematoxylin and eosin staining of renal tissue from the rat with indicated treatment. (a) Sham-group rats. (b) Rats with hind-limb I/R injury. (c) Rats received I/R injury + sulodexide (10 mg/kg). Tissue sections were stained with hematoxylin and eosin. Scale bar, 100 μm.

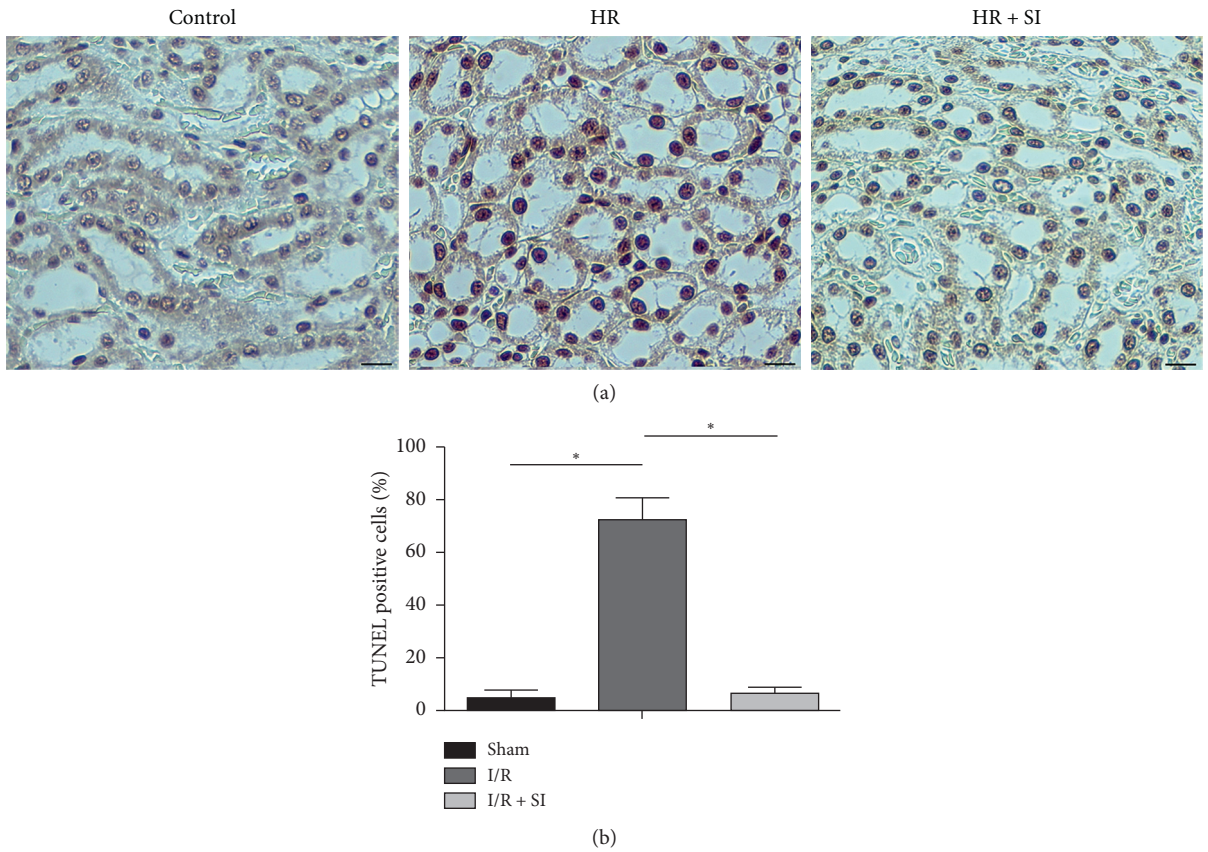

Figure 3: Renal tissues were assessed regarding apoptosis with the TUNEL method. (a) The proportion of cells towards apoptosis was increased significantly following hind-limb I/R injury, yet sulodexide intervention could effectively inhibit the occurrence of apoptotic cells. Scale bar, 50 μm. (b) Statistical result. All of the data were statistically analyzed by Student's t test. *, $P < 0.05$.

stable (Figure 4). Taken together, these results suggest that renal-cell apoptosis in hind-limb I/R injury rats could be effectively ameliorated by sulodexide intervention.

3.4. Sulodexide Attenuated H/R-Induced Cellular Growth Arrest in HK2 Cells. In In Vitro H/R models on HK2 cells, we established that following the previous description, cell survival was subsequently detected to confirm the positive effects that sulodexide exerted on cellular growth arrest. Unsurprisingly, the suppression of cell viability induced by H/R treatment was blocked by sulodexide, whose noteworthy protective capacity against H/R injury was strongly indicated (Figure 5).

3.5. Sulodexide Attenuated H/R-Induced Cellular Apoptosis in HK2 Cells. To further determine the effects of sulodexide on H/R-induced cell apoptosis, we completed annexin V and PI double staining following with flow cytometry to determine

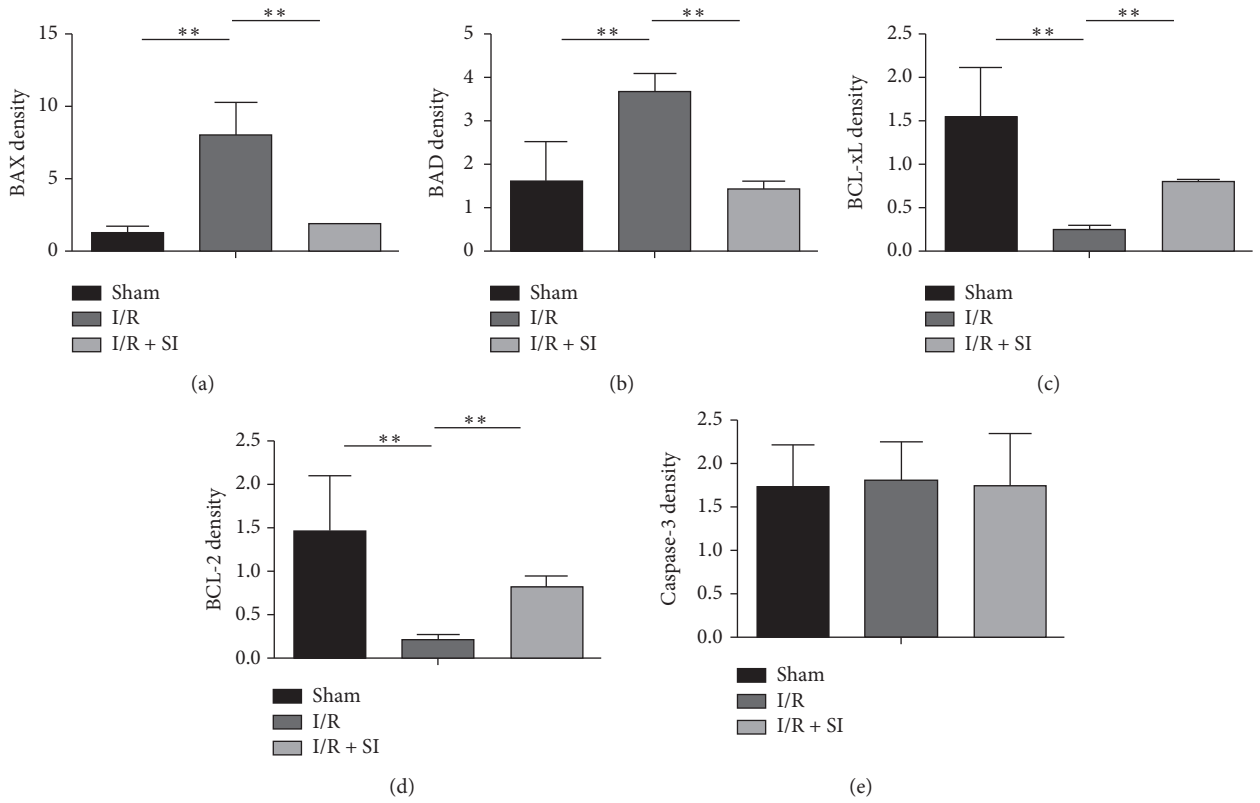

Figure 4: The expression of apoptosis and antiapoptosis proteins of renal tissues in in vivo rat models with western blotting. Hind-limb I/R injury in rats contributed to substantial downregulation in BCL-2 and BCL-xL expression, and correspondingly an uptrend in the expression levels of BAX and BAD. (a) BAX, (b) BAD, (c) BCL-xL, (d) BCL-2, and (e) caspase-3. All of the data were statistically analyzed by Student's t test. **, $P < 0.01$ and ***, $P < 0.001$.

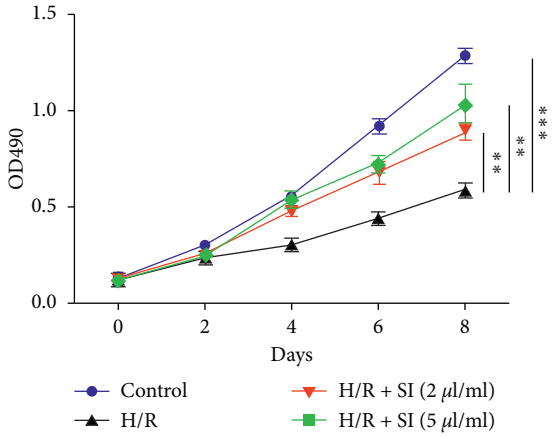

Figure 5: Sulodexide administration blocks H/R-induced HK2 cell growth arrest, which was determined by MTT assay. All data are shown as mean ± standard error of the mean. All of the data were statistically analyzed by Student's t test. **, $P < 0.01$ and ***, $P < 0.001$.

HK2 cell apoptosis rates with indicated treatment. The effects of sulodexide on H/R-triggered HK2 cell apoptosis and necrosis was more accurately illuminated by annexin V/PI double staining, following flow cytometry to examine the apoptosis ratio. As shown in Figures 6(a) and 6(b), the significant late apoptosis (annexin V+/PI+) was pronouncedly inhibited by sulodexide, whereas sulodexide had little effect on early apoptosis (annexin V+/PI−). H/R treatment induced diving on BCL-2 and BCL-xL expression levels emerged with employment of quantitative real-time PCR (qPCR) and western blot analysis, but sulodexide made it possible to be preserved. Meanwhile, at the same time, proapoptotic proteins BAX, BAD, and caspase-3 exhibited the opposite trend (Figures 6(c) and 6(d)).

4. Discussion

In addition to the damage that occurs in local tissues receiving hind-limb I/R injury, distant organs can also be affected. The kidney, as one example, can experience AKI [4, 5]. Thus far, there is no clinically precise and significant treatment. Moreover, the exact underlying molecular mechanism still remains unknown. In combination with previous relevant literature reports, inflammation, oxidative stress, apoptosis, and the generation of microvascular thrombosis play pivotal roles in the pathogenesis of I/R injury [11–13]. Given that sulodexide is a drug with multifunctional properties covering anticoagulation, anti-inflammation, and antioxidant stress, in current study, the therapeutic effects of sulodexide on renal injury following hind-limb I/R injury and the latent mechanisms were determined.

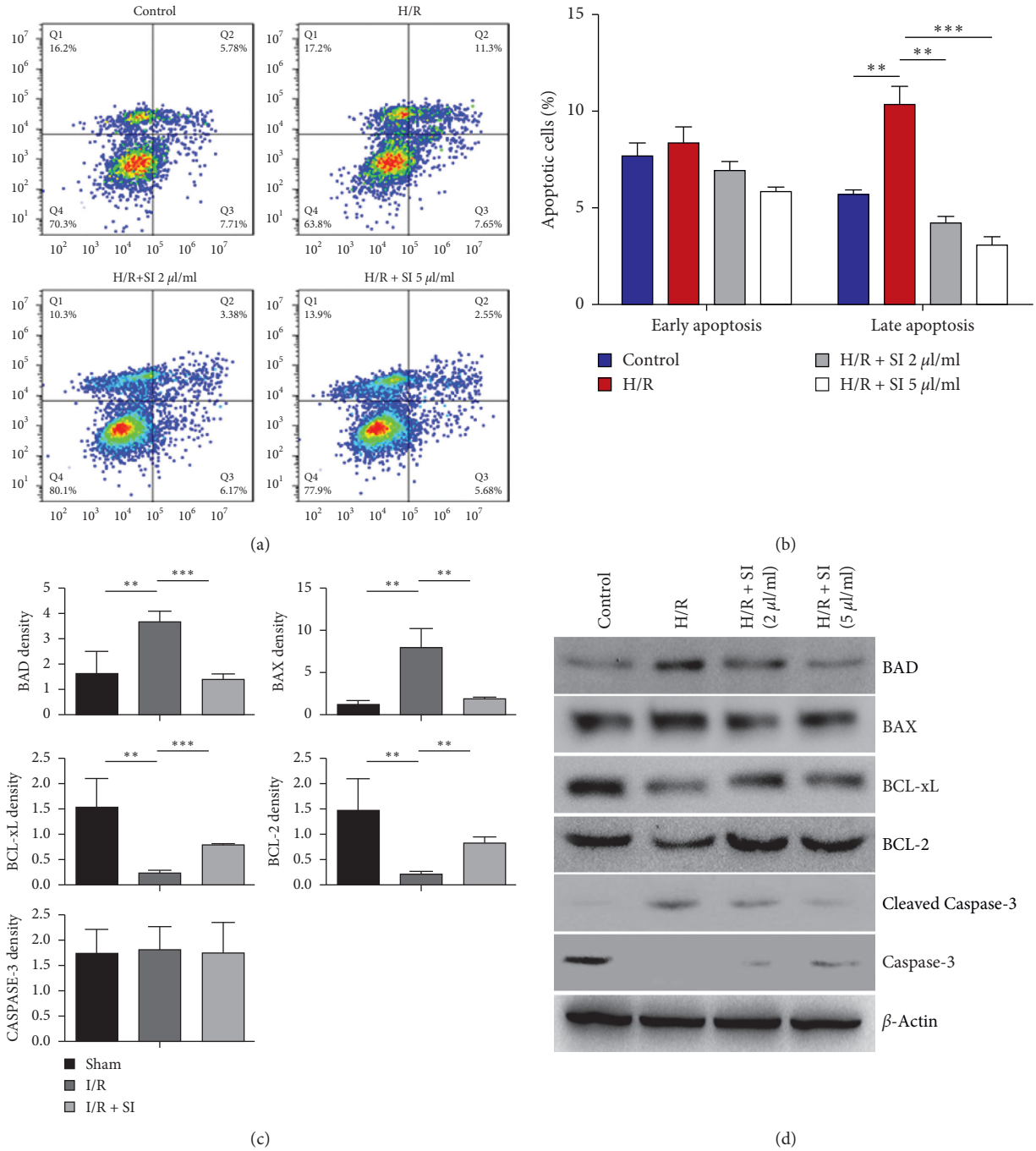

Figure 6: Sulodexide protects HK2 cells against H/R-induced apoptosis. (a) Representative image to show the early and late apoptotic cells in the aforementioned groups. (b) Quantification of apoptotic cells in HK2 cells with H/R and sulodexide treatment in early and late stages. (c) Quantitative PCR to directly reflect the expression levels of BAD, BAX, BCL-xL, BCL-2, and caspase-3. (d) The protein levels of BAD, BAX, BCL-xL, BCL-2, and caspase-3 visualizedly revealed by western blotting. **, $P < 0.01$ and ***, $P < 0.001$.

Characterized with suppressed oxidative reaction, blocked cascade amplifying inflammation, preserved cell viability, and reversed histological and functional renal changes, sulodexide exerted highly protective capacity against hind-limb I/R injury in our research. Moreover, the direct attenuation towards cell growth arrest and apoptosis is H/R-induced HK2 cells also exactly matched with our prediction. Based on this evidence, we believe that the renoprotective effects of sulodexide on I/R injury of the lower limbs may be mediated via the above mechanisms, with sulodexide presented as a promising therapeutic drug.

Apart from its anticoagulation property, sulodexide also possesses anti-inflammatory [17–21] and antioxidative [30] effects. Supplementarily, the present study demonstrated that, in vitro, levels of inflammatory factors such as MMP-9, IL-1β, TNF-α, and IFN-γ levels in serum all increased to

varying degrees following I/R treatment and were attenuated by sulodexide treatment. Meanwhile, the MDA level and MPO activity in renal tissues showed similar trends as those of the above factors, while the glutathione level in renal tissues significantly declined following I/R treatment, yet its trend could be improved by sulodexide treatment. The glutathione level reflects the body's ability to clear out oxygen free radicals in the body, thereby protecting the structure and functional integrity of cell membranes; MDA can increase the level of peroxides in the body, resulting in cell damage, and MPO is unique to neutrophils, indicating that cell-mediated inflammatory responses are suppressed.

In the present study, inhibited BAX and BAD activity and escalated BCL-2 and BCL-xL expression levels in in vivo rat models collectively verified sulodexide being the mitigator of cellular late apoptosis, which was found to be extensively involved in limb I/R injury conditions in our previous studies. Besides, sulodexide also restrained caspase-3 activation and the expression of BAX and BAD in HK2 cells under H/R-induced injury. In conclusion, sulodexide might inhibit kidney cell via directly modulating the expression of apoptosis-related proteins.

There are also some limitations to this study. A multitude of likely signaling transduction pathways supposedly run through the whole damage-repair process, yet most of which have not been clearly clarified so far. For example, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway serves as a crucial pathway in I/R-induced cellular apoptosis [31]. In future research, the research avenue into the activity MAPK/ERK signaling pathway under sulodexide-dominated I/R therapy would be what we are eager to shed light on.

In conclusion, sulodexide can alleviate renal injury caused by hind-limb I/R injury by enhancing cell proliferation and exhibiting anti-inflammatory, antioxidative stress, antiapoptosis, and anticoagulation effects. This indicates that sulodexide may be a potential agent for renal detriment following hind-limb I/R injury prevention and treatment. Even though all above establishes solid foundation for sulodexide to be a promising agent on prophylactic and therapeutic applications towards renal injury and systemic aggravation due to hind-limb I/R injury, whether sulodexide administration can effectively reach ideal clinical outcomes and how to make it optimally realize its unique efficacy remain to be determined and deserve endless attention and endeavor in the future.

Data Availability

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

Disclosure

Tao Yuan and Ni Yang were considered as co-first authors.

Conflicts of Interest

The authors declare no conflicts of interest exist.

Authors' Contributions

Xiang Gao designed the study. Xiang Gao, Tao Yuan, Ni Yang, Wei Bi, and Jinwen Zhang wrote and revised the manuscript. Tao Yuan, Jinwen Zhang, Xueyan Li, Long Shi, and Yang Liu performed the experiments. Tao Yuan and Ni Yang were contributed equally to this work.

Acknowledgments

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Research Article

Protective Effects of Shenfuyixin Granule on H_2O_2-Induced Apoptosis in Neonatal Rat Cardiomyocytes

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Shenfuyixin granule (SFYXG, i.e., Xinshuaikang granule) is a prescription, commonly used in the clinical experience, which plays a significant role in the treatment of heart failure. The purpose of this present research was to investigate the protective effect of SFYXG, and the mechanism about anti-H_2O_2-induced oxidative stress and apoptosis in the neonatal rat cardiomyocytes. Myocardial cells, as is well known, were divided into 4 groups: normal, model, SFYXG, and coenzyme Q10 group, respectively. Cells viability was determined by MTT assay. Flow cytometry and AO/EB staining were implemented to test the apoptosis rate and intracellular reactive oxygen species (ROS) level. Mitochondrion membrane potential (MMP) was evaluated by JC-1 fluorescence probe method. The myocardial ultrastructure of mitochondrion was measured by electron microscope. The related mRNA expression levels of Bax, Bcl-2, Caspase-3, caspase-8, and caspase-9 were detected by real-time polymerase chain reaction (PCR). Also, the expression levels of Bax and Bcl-2 protein were detected by Western blot, and the expression levels of caspase-3, caspase-8, and caspase-9 protein were tested by caspase-Glo®3 Assay, caspase-Glo®8 Assay, and caspase-Glo®9 Assay, respectively. GAPDH was used as the internal reference gene/protein. The results revealed that SFYXG (0.5 mg/ml) raised the viability of myocardial cell, weakened the apoptosis rate and ROS level, corrected the mitochondrion membrane potential stability, and improved cell morphology and ultrastructure of myocardial mitochondrion. Furthermore, SFYXG upregulated the antiapoptosis gene of Bcl-2, but downregulated the proapoptosis genes of Bax, caspase-3, and caspase-9. In conclusion, SFYXG could appear to attenuate myocardial injury by its antioxidative and antiapoptosis effect.

1. Introduction

Reactive oxygen species (ROS) is one of the leading causes of heart failure (HF) and cardiomyocytes' death. The generation and elimination of ROS, under normal circumstances, remain a balance in the process of oxidative metabolism. When under oxidative stress, the overproduction of ROS occurs and could transform the normal physiological signaling process into an abnormal one [1]. Recently, cardiomyocytes apoptosis, induced by ROS, has drawn increasing attention [2, 3]. Studies had shown that excessive ROS causes the disorder of energy metabolism and eventually leads to cellular apoptosis and necrosis, which is one of the pivotal causes of heart failure [4, 5]. Furthermore, H_2O_2, one classic type of ROS, acts as a damaging oxidant. Therefore, the best means to prevent heart failure are to eliminate the excessive production and accumulation of ROS, and to inhibit the apoptosis of myocardial cells. However, traditional Chinese medicine has been widely used in the comprehensive therapy of cardiovascular diseases, such as heart failure. Chinese medicines, with the effect of benefiting Qi and promoting blood circulation, could improve the heart function of rats with HF. SFYXG, a widespread used Chinese medicine prescription, has been demonstrated in the treatment of heart failure by its significant clinical effects. SFYXG is composed of eleven traditional Chinese medicines, including Renshen (Radix Ginseng), Fuzi (Radix Aconiti Carmichaeli), Guizhi

(Ramulus Cinnamomi), Danshen (Radix Ginseng), Chishao (Radix Paeoniae Rubra), Yimucao (Herba Leonuri), Zhuling (Polyporus Umbellatus), Zexie (Rhizoma Alismatis), Tinglizi (Semen Descurainia), Sharen (Fructus Amomi), and Dazao (Fructus Jujubae). Its functions include nourishing Qi and warming Yang, promoting blood circulation, removing stasis, and promoting urination. Researches have shown that SFYXG could block renin-angiotensin system (RAS), improve the myocardial pathomorphology and ultrastructure, and inhibit the expression of c-fos and c-mycso in order to delay or improve cardiac remodeling in chronic HF rats. In addition, SFYXG could enhance the level of adenosine triphosphate (ATP) and improve cardiac in rats with HF by inhibiting the overexpression of uncoupling protein-2 (UCP-2) and weakening mitochondrion membrane potential. With the development of drug extraction technology, recent researches have indicated that GinsenosideRg5 [6], alkaloids [7], and tanshinone IIA [8, 9] have antioxidative properties, which are the main components of Renshen, Fuzi extract, and Danshen, respectively. Though both clinical and experimental studies have demonstrated that SFYXG has various pharmacological effects on heart failure, it remains unknown whether SFYXG could restrain H_2O_2-induced oxidative stress and apoptosis in myocardial cells.

2. Materials and Methods

2.1. Drug. SFYXG was provided by Shandong Buchang Pharmaceutical Co. Ltd. (lot no. 131101). 1g extract is equal to 9.5 g crude drug approximately. The drug was filtered by 0.22 μm cell strainers in order to reach the required concentration by adding culture media.

2.2. Animals. Male/Female Sprague–Dawley rats (1–3 days old) were offered by Henan Laboratory Animal Center (license number: SCXK 2010–0002).

2.3. Isolation and Culture of Neonatal Rat Cardiomyocytes. The hearts of the rats were shredded into 1 mm [3] tissue fragments and digested 7-8 times. The equal amount of DMEM, containing 10% fetal bovine serum, was added to the supernatant solution, obtained through digestion, to make cell suspension. Cells were then collected through centrifugation at 1000 rpm for 10 min at 4°C, 3 times. Nonmyocytes were removed through 200 mesh screen. The majority of myocardial fibroblasts, after 90-minute cell differential adherent culture, were removed, and 0.1 mmol/L Brdu was added in order to inhibit the remnant myocardial fibroblasts growth. Following cells count, the myocardial cell suspension was diluted to required cell density and inoculated into 96-well plates (cell density 5×10^4/mL, 100 μL/hole) or 6-well plates (cell density 1×10^5/mL, 2 mL/hole), and then incubated in CO_2 incubator (37°C, 5% CO_2). Culture media were changed every other day for 3–4 days. Experiments were carried out when cells pulsation rhythm synchronized and overlaid the bottom of the plates.

2.4. Effect of SFYXG, Coenzyme Q10, and H_2O_2 on Cardiomyocytes. Different concentrations of SFYXG (0, 0.1, 0.25, 0.5, 0.75, 1.0, and 1.5 mg/ml) were added to the 96-well plates, which were used to culture the primary cardiomyocytes. After incubation for 24 hours, 10 μL MTT (5 mg/ml) was added to each pole and the incubation continued for 2 hours again. Then, the culture media were discarded and 100 μL DMSO was added to each pole. The plates were oscillated for 10 minutes on the oscillator. Optical density (OD value) was measured with microplate reader at 490 nm.

Coenzyme Q10 at different concentrations (0, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, and 1×10^{-8}M) was added to the 96-well plates. Cell activity was determined by the same assay as shown above.

H_2O_2 at different concentrations (0, 10, 25, 50, 75, 100, and 150 μM) was added to the 96-well plates, respectively, which were used to culture the primary cardiomyocytes to establish cell apoptosis model with the same method as stated above.

2.5. Effect of SFYXG on Oxidative Damage Induced by H_2O_2. The cardiomyocytes, cultured in the 96-well plates, were divided into 4 groups: the normal group, the model group, the SFYXG group, and the coenzyme Q10 group. The model, SFYXG, and coenzyme Q10 groups first dealt with the concentration of 50 μM H_2O_2 for 6 h. Then, all the culture media were changed and the latter 2 groups were added, 0.5 mg/ml SFYXG and 1×10^{-4} M coenzyme Q10, respectively. After 24-hour incubation, OD value was detected by MTT.

In the following experiments, cell grouping and corresponding treatment were in accordance with the 4.5 method.

2.6. Calcein-AM Dyeing Method to Detect Cardiomyocytes Activity. Cells were treated as 4.5 method. 100 μL calcein-AM (50 μM) was added, and then cells were incubated for 25 min at 37°C, after being washed by PBS 3 times. Fluorescence microscope was used to observe cells after being washed by PBS 3 times. The fluorescence excitation wavelength is 490 nm, and the emission wavelength is 515 nm.

2.7. ROS Level in Cardiomyocytes. Cells were collected after corresponding treatment. Then, cells were incubated in DMEM medium with 50 μL DCFH-DA (10uM) at 37°C for 30 min. Cells were washed with PBS 3 times, and the fluorescence intensity was measured.

2.8. AO/EB Staining. Cells were collected after the treatment and were adjusted to 1×10^{10}/L suspension. Then, 5 μL AO/EB equal volume mixture was added to the 95 μL cell suspension. We took a drop of cell suspension and put it on the clean glass slide with a cover slip for observation in 3 min under the fluorescence microscope, whose excitation wavelength was 510 nm blue light.

2.9. JC-1 Assay to Detect the Mitochondrial Membrane Potential. Cells, which had been cultured and treated in 6-well plates afterwards, were harvested and 2 ml DMEM without serum was added and mixed in order to prepare cell suspension. Then, JC-1 detection solution was added to the mitochondrion suspension for chemical reaction in a dark environment for 7 min. According to the instructions, spectrofluorometer was implemented to detect the fluorescence intensity. The results were expressed as reflectivity [ER, ER = fluorescence intensity 590 nm/527 nm].

2.10. Transmission Electron Microscopy to Observe the Ultrastructure of Mitochondrion. After dealing with corresponding treatments, cells, cultured in the 6-well plates, were harvested. The 3% glutaraldehyde was added to centrifuge tube slowly in case of the cell cluster dispersed after being centrifuged at 1000 rpm for 10 min. They were fixed at 4°C for 2 hours.

2.11. The Apoptosis Rate of the Cardiomyocytes. The apoptosis rate of the cardiomyocytes was detected with the flow cytometer. The cardiomyocytes were washed by PBS 3 times and dissolved with trypsin. After that, the $1\times$ binding buffer was added to and diluted the cells to 5×10^6 per milliliter and dyed by Annexin V (10 μg/ml) and PI (10 μg/ml) individually in the dark conditions for 3 min and 15 min, respectively. Lastly, the 1×binding buffer was replenished and it was made sure that the total volume was 500 μl and detected through the flow cytometer.

2.12. The mRNA Expressions of Bcl-2, Bax, Caspase-3, Caspase-8, and Caspase-9. The mRNA expression levels in cardiomyocytes were quantitatively analyzed by rt-PCR. Under the ice bath, the total RNA of cardiac myocytes was extracted with Trizol reagent. The optical density ratio (A260/A280) of 260 nm and 280 nm (1.8–2.0) was measured by ultraviolet spectrophotometer. According to the instructions of reverse transcriptase kit, the reverse transcriptase reaction was carried out. Primer sequences are shown in Table 1

2.13. Protein Expressions of Bcl-2, Bax, Caspase-3, Caspase-8, and Caspase-9. The proteins were extracted by RIPA buffer with cocktail. Protein concentration was measured by the bicinchoninic acid assay (BCA assay). The total protein (5 μg) was separated by SDS-PAGE and was transferred onto PVDF membranes. The membranes were blocked by 5% powdered milk in TBS-Tween 20 for 1 h at 25°C. The expression levels of proteins, related to apoptosis, were detected by using primary antibodies against Bcl-2 and Bax overnight at 4°C, and then combined with secondary antibody for 2 hours at 25°C. Blots, then, were developed by the Pierce ECL Plus Western Blotting substrate. ImageJ software was used to analyze the density. Cells were treated as 4.5 method. According to Caspase-Glo®3/8/9 Assay description, 100 μL Caspase-Glo®3/8/9 reagent was added to the samples and washed with PBS 3 times; then, they would be mixed and incubated for 3 hours at 37°C. The luminescence was measured.

Table 1: The Primer sequences of RT-PCR.

Gene	Sense primer (F)	Reverse primer (R)	Fragment length (bp)
GAPDH	ACA GCA ACA GGG TGG TGG AC	TGAG GGT GCA GCG AACTT	252
Bcl-2	CTT TGA GTT CGG TGG GGT CA	AGT TCC ACA AAG GCA TCC CAG	153
Bax	GCT CAA GGC CCT GTG CAC TAA	GAA GCC TCA GCC CAT CTT CTT	223
Caspase-3	GAG CTG GAC TGC GGT ATT GA	AGG AAT AGT AAC CGG GTG CG	118
Caspase-8	GAC CAC ATC CCG CAG AAG AA	GAT CCC GCC GAC TGA TAT GG	139
Caspase-9	CAT CTT CAA TGG GAC CGG CT	GGT CTT TCT GCT CAC CAC CA	86

2.14. Statistical Analysis. All data are shown as mean ± SD. Statistical analysis was performed by one-way ANOVA when there are three or more groups. $P < 0.05$ is considered to indicate a statistically significant difference.

3. Results

3.1. Effect of SFYXG on the Activity of Neonatal Rat Cardiomyocytes. Based on the OD value, noncytotoxicity was found after SFYXG was applied within the range of concentration 0.1–0.75 mg/ml for 24-hour interaction, and when the concentration increased to 1.0 mg/ml and more, cell activity decreased significantly compared with the normal group ($P^*<0.05$; Figure 1(a)). Therefore, in the follow-up experiments, 0.5 mg/ml SFYXG was chosen as the intervention concentration. Coenzyme Q10 1×10^{-4} M and H_2O_2 50 μM were chosen by using the same method (Figures 1(b) and 1(c)).

3.2. Effect of SFYXG on Oxidative Stress Injury. Compared with normal group, the activity of cardiomyocytes in the model group significantly decreased ($P < 0.05$; Figures 2 and 3). 0.5 mg/ml SFYXG and coenzyme Q10 1×10^{-4} M both promoted activity of cardiomyocytes ($P < 0.05$; Figures 2 and 3).

3.3. Effect of SFYXG on ROS Level. Compared with the normal group (Figure 4), ROS level increased (154.82 ± 10.01 vs. 63.88 ± 3.75 , $P^*<0.05$) after 50 μM H2O2 was applied for 6 hours, whereas ROS levels (89.18 ± 3.45 vs 154.82 ± 10.01, 98.82 ± 1.27; $P^{\#}<0.05$, $P < 0.05^{\blacktriangle}$; Figure 4) decreased after 0.5 Mg/Ml SFYXG and coenzyme Q10

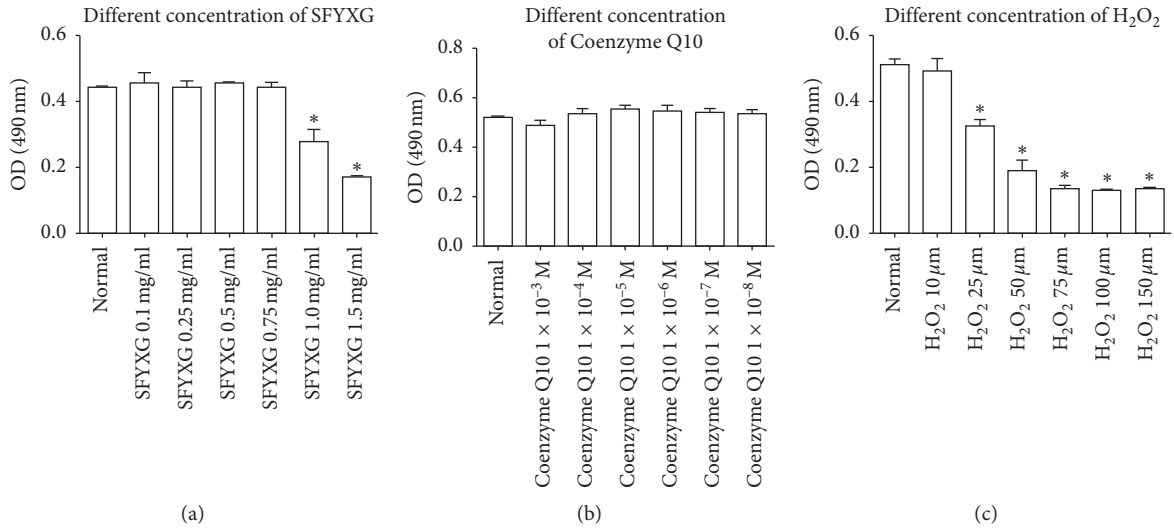

Figure 1: . Effect of SFYXG, coenzyme Q10, and H2O2 on the activity of neonatal rat cardiomyocytes. (a) Different concentration of SFYXG. (b) Different concentration of coenzyme Q10. (c) Different concentration of H_2O_2.

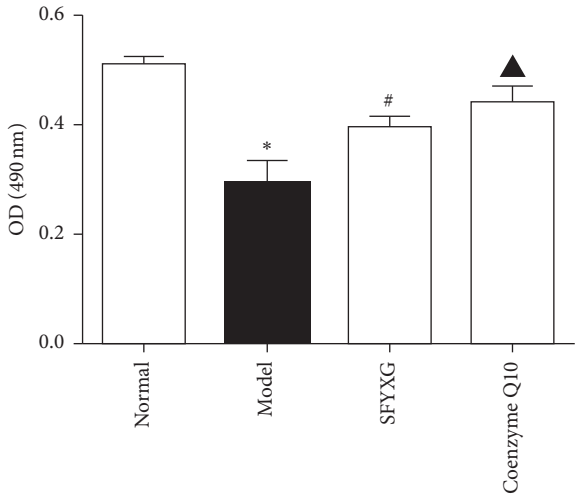

Figure 2: The activity of cardiomyocytes was detected by MTT. Note: P^*<0.05 vs normal group (0.29 ± 0.04 vs 0.51 ± 0.02); $P^{\#}$<0.05 vs model group (0.40 ± 0.02 vs 0.29 ± 0.0 4); $P^{\blacktriangle}$<0.05 vs model group (0.44 ± 0.03 vs 0.29 ± 0.0 4).

1×10^{-4} M were applied, respectively. The results suggested that SFYXG could weaken ROS.

3.4. Effect of SFYXG on Cardiomyocytes Morphology. Under fluorescence microscope, cells in the normal group were full with intact membrane, and the nuclei appeared homogeneous green fluorescence. However, the shape of cells in the model group was irregular, and the apoptotic cells showed inhomogeneous orange red fluorescence. Compared with the model group, the majority of the cells in SFYXG and coenzyme Q10 groups presented green fluorescence and the orange red fluorescence decreased (Figure 5). The results show that SFYXG attenuates cardiomyocytes' morphology injury caused by H2O2.

3.5. SFYXG Influence on Cardiomyocytes MMP. Compared with the normal group, cardiomyocytes MMP caused by H2O2 decreased rapidly in the model group (2.44 ± 0.32 vs 6.45 ± 0.35, P^*<0.05). What is more, in SFYXG and coenzyme Q10 groups, the MMP were enhanced significantly (4.19 ± 0.24, 4.55 ± 0.26; $P^{\#}$ <0.05, $P^{\blacktriangle}$ <0.05; Figure 6). It suggested that SFYXG could reduce the toxicity of H2O2.

3.6. Ultrastructure Changes of Mitochondrion in Each Group. In the normal group, the mitochondria of individuals are homogeneous with integrate structure, and mitochondrion cristae are clear and complete. And the myocardial fibers are aligned. However, in the model group, the mitochondria are in various sizes and shapes, and they swell irregularly with incomplete structure. Above this, mitochondria dissolved into the vacuole, and partial creast and myocardial fibers fracture are broken and fuzzy. Compared with the model group, the SFYXG group mitochondria swell slightly, the cristae and myocardial fibers are in a regular pattern, and there is slight dissolution (Figure 7).

3.7. Apoptosis Rate of Primary Cardiomyocytes In Vitro of Each Group. Compared to the normal group, the apoptosis rate was higher than the model group (10.73 ± 0.46 vs 31.46 ± 0.78, P^*<0.05). What is more, in SFYXG and coenzyme Q10 groups, the apoptosis rate was reduced significantly (17.26 ± 0.31, 15.67 ± 0.43; $P^{\#}$ <0.05, $P^{\blacktriangle}$ <0.05; Figure 8). It suggested that SFYXG could reduce the apoptosis rate of the cardiomyocytes caused by H2O2 (Figure 8).

3.8. Effect of SFYXG on mRNA Expressions of Bax, Caspase-3, Caspase-8, Caspase-9, and Bcl-2. Compared with the normal group, cardiomyocytes showed higher expression levels of Bax, caspase-3, caspase-8, and caspase-9, and lower expression level of Bcl-2 mRNA in the apoptosis model. After

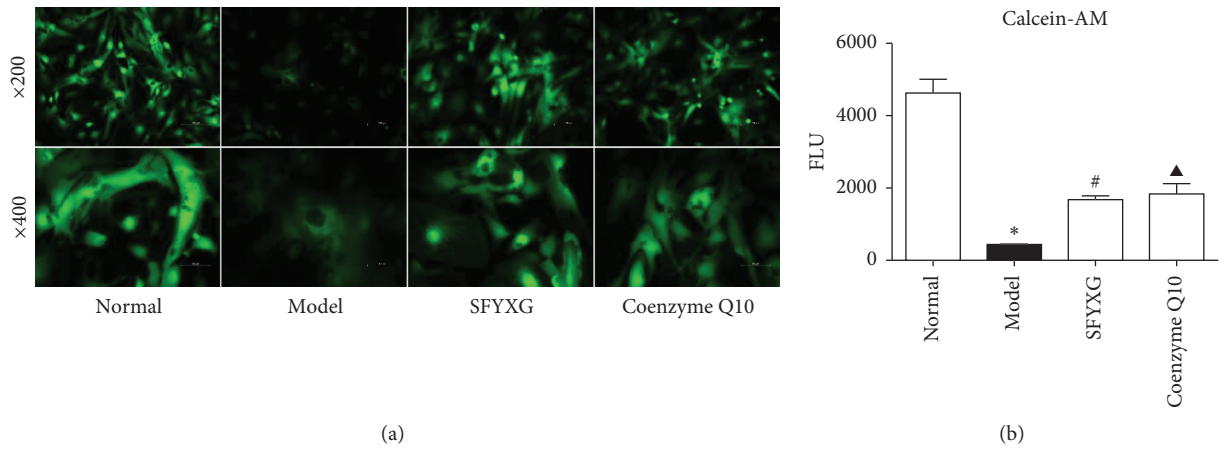

Figure 3: The activity of cardiomyocytes was determined by calcein-AM method. $P^{*}<0.05$ vs normal group (392.70 ± 32.84 vs 4626.11 ± 383.38); $P^{\#}<0.05$ vs model group (1645.30 ± 112.35 vs 392.70 ± 32.84); $P^{\blacktriangle}<0.05$ vs model group (1808.44 ± 289.77 vs 392.70 ± 32.84). The fluorescence excitation wavelength is 490 nm and the emission wavelength 515 nm.

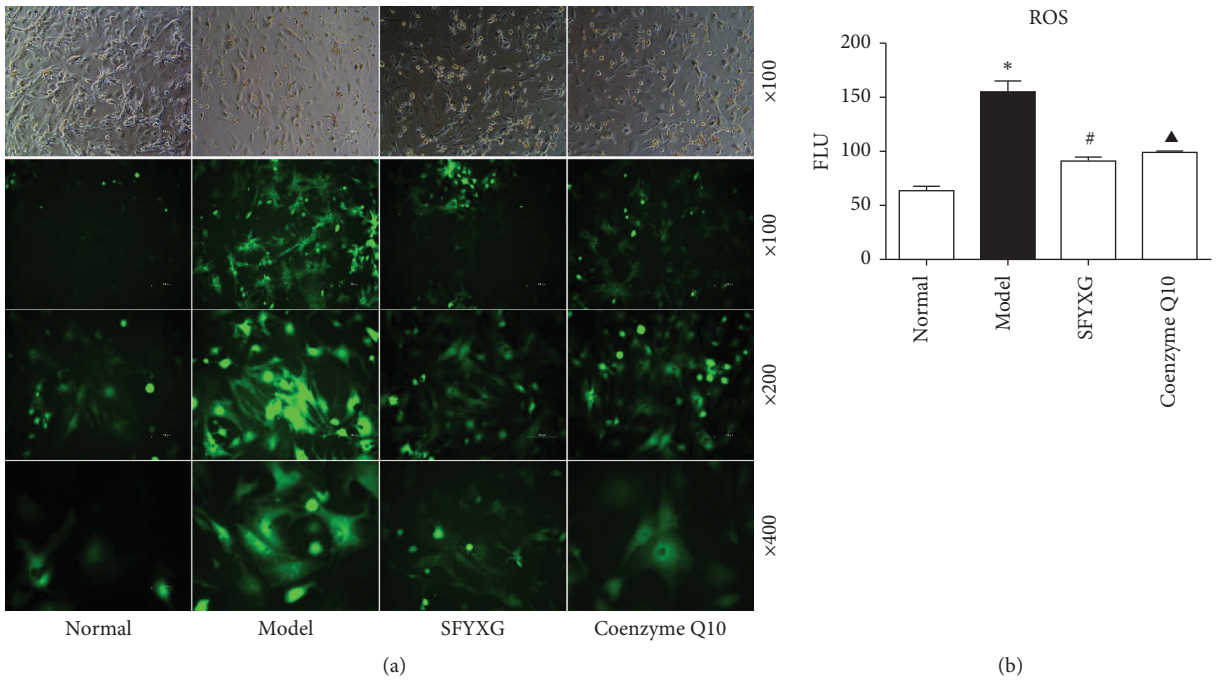

Figure 4: Effect of SFYXG on ROS level. Note: (a) the green fluorescence intensity is proportional to the ROS level. The fluorescence excitation wavelength is 488 nm and the emission wavelength 525 nm. ROS level was tested by microplate reader (b).

treatment with SFYXG and coenzyme Q10, Bax, caspase-3, caspase-8, and caspase-9 in cardiomyocytes decreased significantly, whereas Bcl-2 mRNA expression increased ($P < 0.05$; Figure 9).

3.9. Effect of SFYXG on Protein Expressions of Bax, Bcl-2, Caspase-3, Caspase-8, and Caspase-9. The results show that the proapoptosis proteins expressions of Bax, caspase-3, caspase-8, and caspase-9 notably increased, while the expression of the antiapoptosis Bcl-2 decreased in the apoptosis model. The expression levels of the related proteins were significantly reversed by SFYXG ($P < 0.05$; Figure 10), which demonstrates that SFYXG could regulate cardiomyocytes apoptosis through Bax, caspase-3, caspase-9, and Bcl-2. From the results, we also conclude that SFYXG decreased caspase-8 expression, but there were no statistical differences.

4. Discussion

In fact, "heart failure" has been found in traditional Chinese medicine literature. However, it refers to the deficiency of heart-qi and heart-blood, which is quite different from the "heart failure" in the western medicine. Based on the clinical characteristics, heart failure can be included in the diseases of "asthma," "palpitation," and "edema," which are now

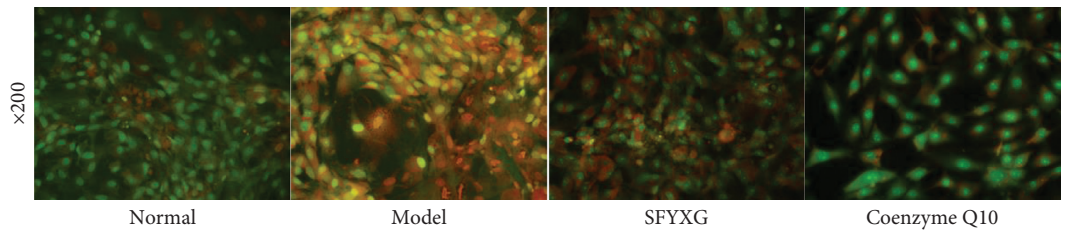

Figure 5: SFYXG influence on cardiomyocytes morphology.

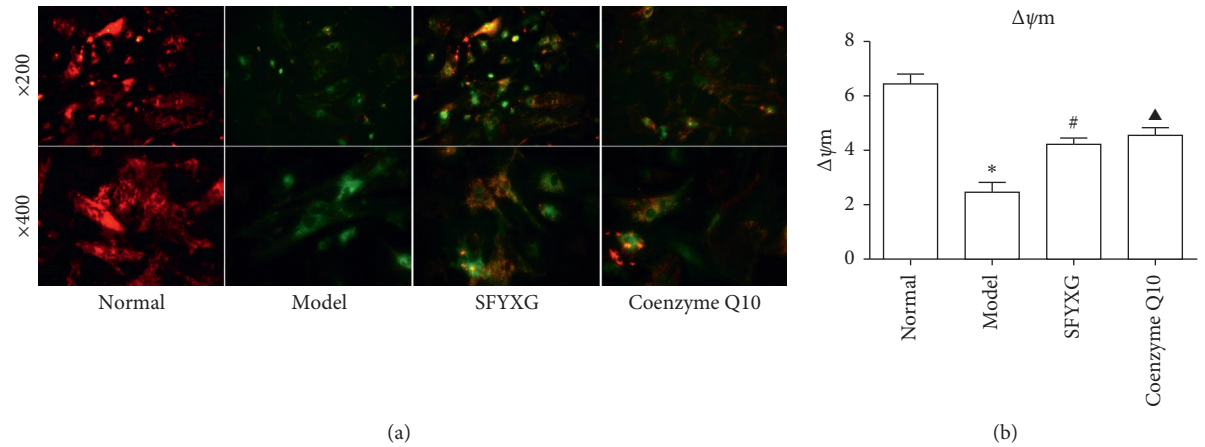

Figure 6: SFYXG influence on cardiomyocytes MMP. Note: mitochondrion membrane potential (MMP) changes were observed by fluorescence microscope (a) and the MMP level was tested by microplate reader (b).

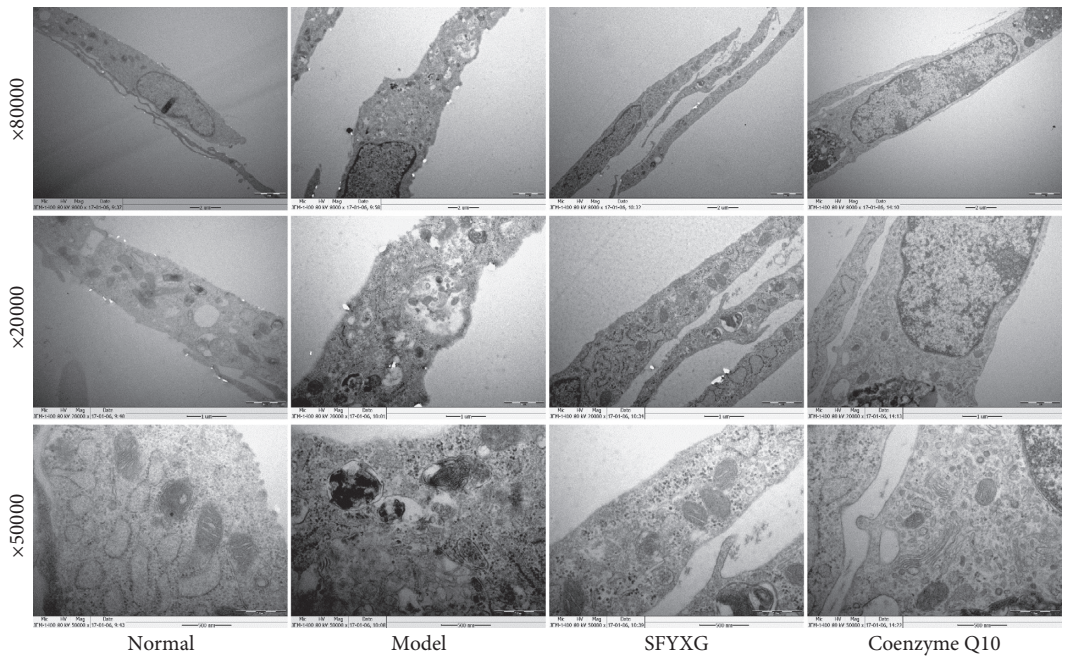

Figure 7: SFYXG effect on mitochondrion ultrastructure.

unanimously named "heart failure disease" (HFD). In the late stage of HFD, the insufficiency of heart-qi and heart-yang leads to blood stasis in the heart. SFYXG is usually used to benefit heart-qi, warm heart-yang, and promote blood circulation in order to remove blood stasis. Previous studies have demonstrated that SFYXG could downregulate Ang II and block the RASS system, so as to improve myocardial pathological morphology and myocardial ultrastructure to delay myocardial remodeling. The present research observed that SFYXG could improve the apoptotic myocardiocytes

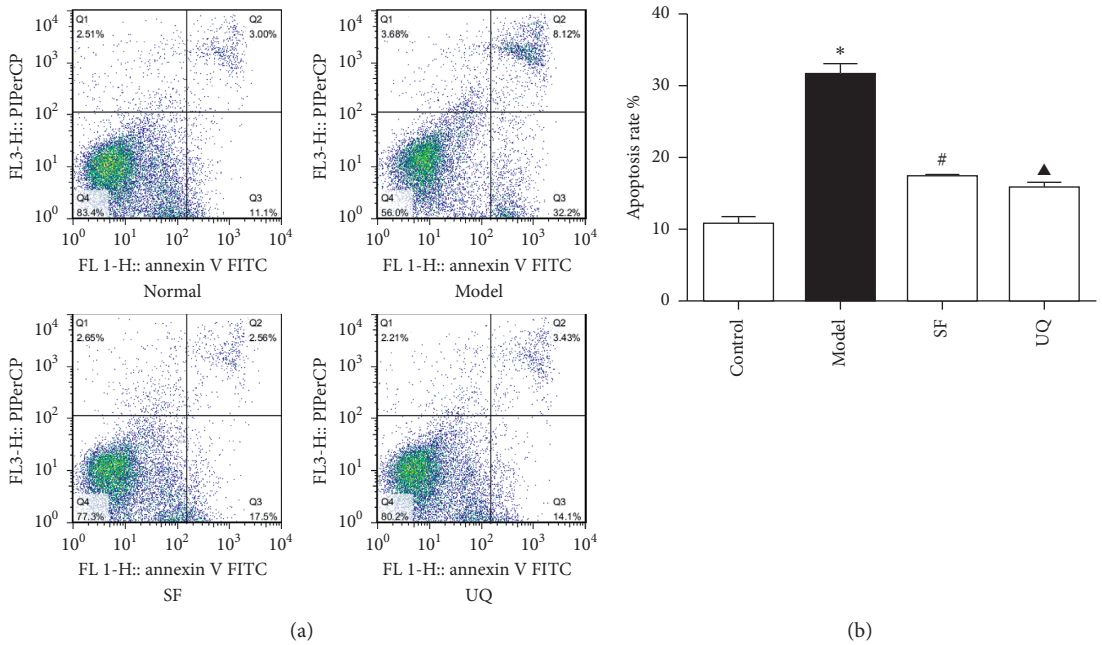

Figure 8: Effect of SFYXG on apoptosis rate. Note: apoptosis rate was detected by the flow cytometer (a) and the data were analyzed by FlowJo7.6 (b).

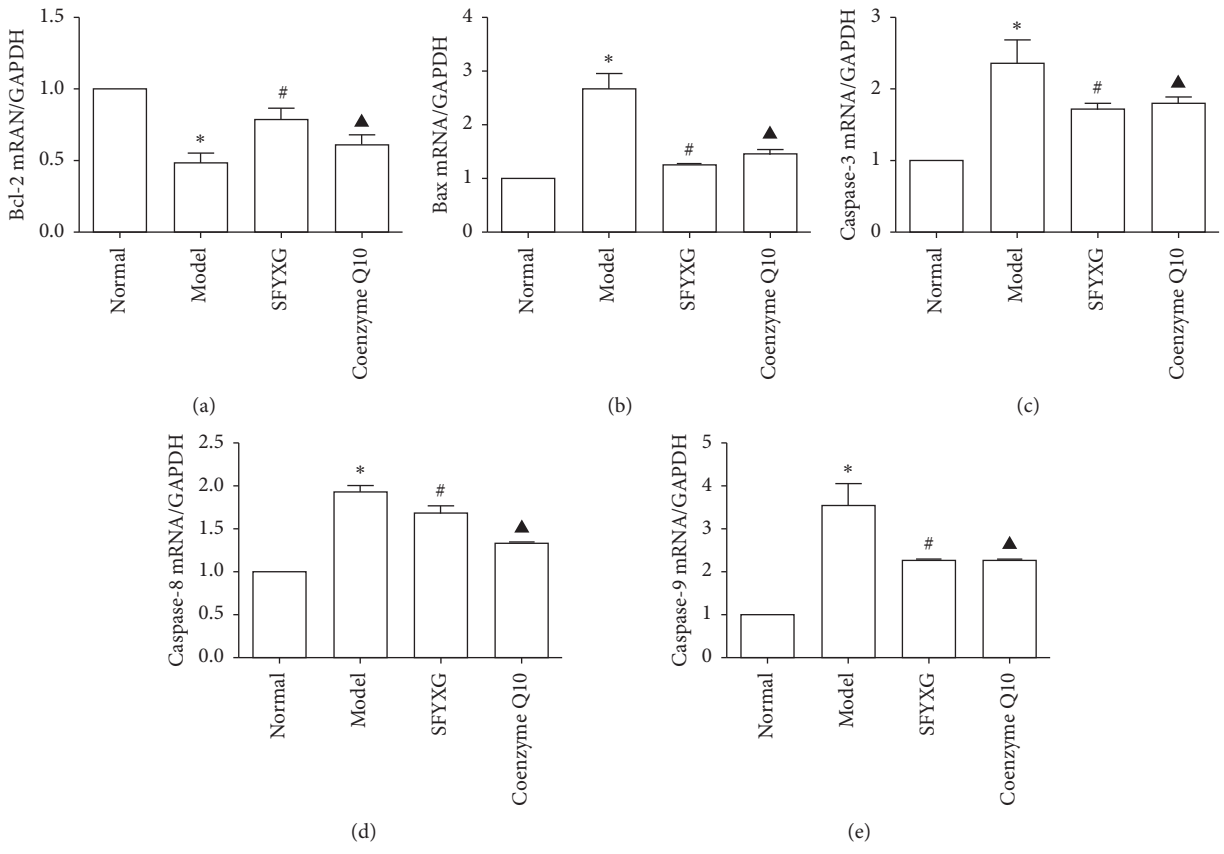

Figure 9: Effect of SFYXG on mRNA expressions of the corresponding genes. Note: $P^{*}<0.05$ was compared with the normal group; $P^{\#}<0.05$ and $P^{\blacktriangle}<0.05$ were compared with the model group.

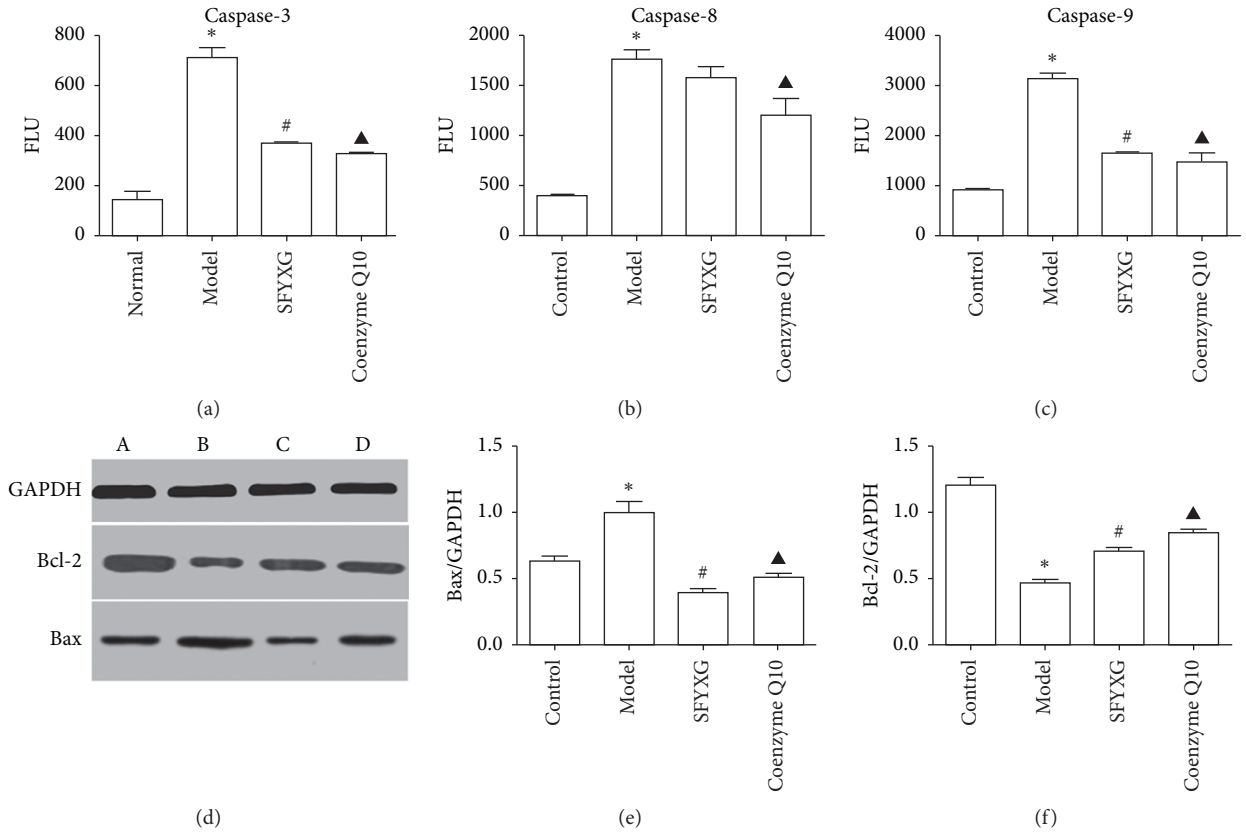

Figure 10: Effect of SFYXG on protein expressions of the corresponding factors. Note: caspase-3, caspase-8, and caspase-9 were detected by Caspase-Glo®3 Assay, Caspase-Glo®8 Assay, and Caspase-Glo®9 Assay, respectively; Bcl-2 and Bax were detected by Western blot. $P^{*}<0.05$ was compared with the normal group; $P^{\#}<0.05$ and $P^{\blacktriangle}<0.05$ were compared with the model group.

caused by H2O2 through activating myocardial cells under oxidative stress, and weakening the excessive ROS. Then, the apoptosis rate decreases. In addition, SFYXG reduces the number of apoptotic cardiomyocytes and improves the cell morphology. Furthermore, it is found that SFYXG could improve the morphology of mitochondrion, protect the integrity of mitochondrion structure, reduce vacuolar change, and decrease the dissolution of mitochondrion crista and myocardial fibers.

As is well known, mitochondrion is where life activities take place, including cell respiratory chain, oxidative phosphorylation, and cell apoptosis. The electron transfer chain of the mitochondrion is the main place for the production of ROS [10, 11]. Normally, a small amount of ROS produced in mitochondrion is essential for the cell functions, including the defense, detoxification, and synthesis of some important substances. However, when HF occurs, the activity of antioxidant enzymes such as superoxide dismutase (SOD) decreases, and the scavenging of ROS declines, which result in the excessive accumulation of ROS. However, the excessive ROS damages the mitochondrion, decreases the MMP, changes the permeability of mitochondrion membrane, and induces cardiomyocyte apoptosis through mitochondrion signal transduction pathway [12–14].

It is noteworthy that the Bcl-2 family and the caspase family are closely related to cardiomyocyte apoptosis [15, 16]. The Bcl-2 family contains more than 20 homologous proteins, which can be divided into anti-apoptotic protein and proapoptotic protein. The typical antiapoptotic members, such as Bcl-2, Bcl-xL, Mcl-1, and Bcl-W, are mainly found in the mitochondrion outer membrane. The major proapoptotic protein, Bax, for example, is homologous to the amino acid sequence of Bcl-2. And Bax exists in the cytoplasm in the form of monomer in the normal cells. When the cells are stimulated by the associated apoptotic signal, they will be transferred to the mitochondrion to change the MMP and mitochondrion permeability, and to promote the release of cytochrome c (apoptotic active substance). Additionally, Bcl-2 is found to interact with Bax to form heterodimer, and thus prevent Bax release and inhibit cell apoptosis [17–19]. This study examines the changes in MMP, the mRNA, and protein expression of Bcl-2 and Bax under the cardiomyocytes under oxidative stress induced by H2O2. The results show that the MMP of the cells in the model group deceases significantly, whereas there is recovery after treatment by SFYXG. In the model group, the mRNA and protein expression level of Bcl-2 decreases and Bax increases. The results present that the changes are both corrected by SFYXG. The results suggest that the effect

of SFYXG on apoptotic cardiomyocyte induced by H_2O_2 could be attributed to its ability to recover the stability of MMP and the expression of Bax and Bcl-2.

Caspase belongs to cysteine protease. Once activated by the signal transduction pathway, caspase could degrade the protein and cause irreversible cell death. The caspase family, involved in cell apoptosis, is divided into two categories: one is the executioner, such as caspase-3, which could degrade the structural and functional protein and lead to direct apoptosis; the other is the initiator, such as caspase-8 and caspase-9, which could be activated by self-shearing and causes the cascade reaction. It is generally believed that caspase-8 mediates apoptosis in the death receptor pathway [20]. Caspase-3 is activated accordingly followed by the activating caspase-9, and they are both involved in mitochondrion apoptosis pathway [21, 22]. Overexpression of Bcl-2 on mitochondrion membrane could avoid cell apoptosis by inhibiting the changes of mitochondrion permeability, reducing the release of cytochrome C, and inhibiting caspase activation. Furthermore, overexpression of Bcl-2 causes the accumulation of glutathione (GSH) in the nucleus, the change of redox equilibrium, and weakens caspase activity [23]. When cells are subjected to oxidative stress, ROS and caspase interact with each other to promote cell apoptosis. Our results show that the expression of caspase-9 and caspase-3 increases significantly after the treatment of H_2O_2, but decreases significantly after the treatment of SFYXG, which indicates that oxidative stress could activate not only the Bcl-2 in the upstream of the mitochondrion, but also caspase. However, no remarkable change is found in caspase-8, which may be related to intermediation of the death receptor pathway.

5. Conclusion

SFYXG could inhibit cardiomyocytes apoptosis. It may raise cell viability by clearing the excessive ROS, protecting the morphology and structure of mitochondrion, stabilizing MMP, downregulating mRNA and protein expression of Bax, caspase-3, and caspase-9, and upregulating Bcl-2. This study may provide a theoretical and experimental basis for the clinical application of SFYXG in the prevention and treatment of heart failure.

Data Availability

All data were acquired from the Center Lab of the First Affiliated Hospital of Henan University of CM.

Ethical Approval

All procedures of this study performed on the animals were in accordance with welfare law.

Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Authors' Contributions

Zhu Mingjun, Wang Xinlu, and Hao Xuanxuan conceived and designed the experiments; Wang Xinlu, Hao Xuanxuan, Wang Youping, Li Bin, Cui Lin, and Xie Shiyang performed the experiments and analyzed the data; Wang Xinlu and Hao Xuanxuan wrote the paper. Xinlu Wang and Xuanxuan Hao contributed equally to this work.

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Review Article

Effects of Tai Chi Chuan on Cognitive Function in Older Adults with Cognitive Impairment: A Systematic and Meta-Analytic Review

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This systematic and meta-analytic review aimed to investigate the effects of Tai Chi Chuan (TCC) on the cognitive function of the elderly with cognitive impairment and to analyze the moderators of these effects. We searched eight electronic databases for randomized controlled trials on the effects of TCC on cognitive function, published up to June 14, 2020. The PEDro scale was used to evaluate the methodological quality of the included literature. Stata14.0 software was used for meta-analysis, subgroup analysis, and publication bias testing. A total of 19 studies and 1,970 samples were included. The methodological quality of the included literature was fair to good, and there was no publication bias. Overall, the research shows that the effect of TCC on the elderly with cognitive impairment is statistically significant (SMD = 0.31, $p < 0.0001$). Five of the cognitive function subdomains were significant moderators [Q (5) = 15.66, $p = 0.008$], and the effect size (ES) was the largest for global cognitive function (SMD = 0.41), followed by executive function (SMD = 0.33), memory (SMD = 0.31), and verbal fluency (SMD = 0.27). Regarding the exercise prescription variables, results were significantly moderated by the length of exercise training [Q (2) = 6.00, $p = 0.05$], with ESs largest for moderate length (SMD = 0.41), followed by short length (SMD = 0.40) and long length (SMD = 0.29). However, the results were not moderated by session time or frequency. TCC can improve multiple cognitive functions of the elderly with cognitive impairment. The intervention effects are moderated by exercise length, but not by exercise session time and frequency.

1. Introduction

As the aged population continuously grows, age-related cognitive decline has become a global public health problem; in particular, the numbers of people with mild cognitive impairment (MCI) and dementia are increasing. MCI is an intermediate stage between normal aging and dementia, and people with MCI are at high risk of developing dementia. The average prevalence of MCI is 16% in the old population [1], and the risk of developing dementia in patients with MCI (10–15%) is much higher than that in healthy old people (1-2%) [2]. The 2015 World Alzheimer's Disease Report predicts that the number of people with dementia worldwide will increase from 46 million to 131.3 million by 2050 [3]. So far, there is no effective drug to delay the cognitive decline caused by MCI and dementia. How to delay the further decline of cognitive function in people with MCI and dementia and how to improve their quality of life in later years have become urgent problems to be solved in aging societies.

A growing number of studies have proved that physical exercise can improve cognitive function. Animal experiments have found that exercise can stimulate neuronal regeneration and reduce the deposition of β-amyloid protein, thus alleviating the symptoms of Alzheimer's disease in mice [4]. Research using two-photon in vivo imaging technology revealed that exercise can activate the brain-derived neurotrophic factor (BDNF) pathway in the mouse brain, reduce the loss of apical dendritic spines of neurons, and thereby improve the neural network and the learning ability for

motor skills and cognitive function [5]. A large number of clinical experimental studies and systematic reviews have confirmed that physical exercise can improve cognitive function, effectively delay cognitive decline [6], and reduce the risk of dementia [7–10]. In December 2017, the American Academy of Neurology recommended physical exercise to MCI patients [11]. In summary, it is increasingly accepted that physical exercise is an effective non-pharmacological treatment to delay the cognitive decline of the elderly.

Research on exercise interventions to improve cognitive function has mostly used, and proven the effects of, aerobic exercise, but the effect of TCC on cognitive function has received little research attention. TCC perfectly integrates traditional philosophy, the theory of traditional Chinese medicine, and the five-element theory; it also combines physical movement with respiration, mind with consciousness, consciousness with the body, and qi with the body. It strives to achieve a high degree of unity of mind, consciousness, strength, qi, and shape, while constantly adjusting the direction, range, power, and speed of movement. The practice requires not only memory but also a variety of higher-level cognitive functions (such as perceptual speed, visual-spatial ability, attention, multitasking, and planning) to maintain postural stability. Accordingly, the process of movement activates the relevant brain areas and stimulates the excitability of brain cells, which is helpful to strengthen the brain, maintain its perceptual functions, and improve the memory of the elderly [12].

In recent years, many researchers have focused on TCC, and a growing body of empirical studies [13–15] and systematic reviews [16–18] has demonstrated that TCC can effectively improve the cognitive function of the elderly, although some studies have found no relationship between TCC and cognitive decline [19]. One previous meta-analysis only analyzed a few studies and included both healthy individuals and individuals with cognitive impairment in the sample range [20]. Other reviews have included non-randomized controlled trial (RCT) studies [18, 21], thereby introducing a variety of confounding factors that may lead to inaccurate results. Additionally, previous studies analyzing cognitive function [22] have not considered the moderating effects of different aspects of physical exercise, so the dose-response relationship remains unclear. To address these gaps, this study analyzes the effects of TCC intervention on various cognitive domains and how these are moderated by variation in the dose of physical exercise, aiming to provide a theoretical basis for accurate exercise prescription.

2. Methods

This study was performed and reported according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [23].

2.1. Literature Search Strategy. We searched eight electronic databases (PubMed, Embase, The Cochrane Library, WOS, PsycINFO, CNKI, Wanfang, and China Biology Medicine) from inception to June 14, 2020. Two researchers (C.Z.D and J.W.T) independently used the following search terms (among others) for retrieval: Tai Chi Chuan (TCC), Mild Cognitive Impairment, Alzheimer's disease, dementia, cognitive performance, cognitive function, cognitive processes, executive function, memory, attention, inhibition, shifting, working memory, and randomized controlled trial (RCT). The retrieval strategy adopted the combination of subject words and free words and was determined after repeated prechecking. It was supplemented by manual retrieval of the gray literature and the tracing of previous systematic reviews and references where necessary. Language and publication types were not limited to literature retrieval.

2.2. Eligibility Criteria. Two researchers (C.Z.D and J.W.T) independently screened the literature according to the inclusion and exclusion criteria. After the screening, any discrepancy between the two researchers was resolved through consulting the other two researchers (W.N and Y.J.L) until consensus was reached.

The inclusion criteria were as follows: (1) the subjects were elderly with MCI or dementia; (2) the intervention was TCC; (3) all or some of the outcome indicators were cognitive functions; and (4) the study was an RCT.

We set the following exclusion criteria: (1) the subjects were elderly with normal cognition or mental disorders; (2) the intervention program contains confounding factors other than exercise, such as cognitive training, vitamin supplements, and drugs; (3) literature data cannot be extracted, even after contacting the authors; and (4) publications that are qualitative studies, case studies, reviews, nonintervention studies, or conference papers.

2.3. Data Extraction. Two researchers (C.Z.D and J.W.T) independently extracted the relevant information using a standardized form. Where data were missing or could not be extracted due to insufficient statistical reporting, we contacted the author(s) to request the missing data.

Extraction contents and coding were as follows. First, we captured the basic details of the literature, including the names and nationalities of authors and the year of publication. Second, we collated and processed the basic details of the subjects, including cognitive status, sample size, age, and education level. Third, we captured data on three exercise prescription variables: frequency, session time, and length [6]. Exercise frequency was classified by the number of exercise sessions per week: low frequency: ≤2 times; moderate frequency: 3-4 times; and high frequency: ≥5 times. Exercise session time was classified as follows: short: ≤45 minutes; moderate: >45 minutes to ≤60 minutes; and long: >60 minutes. Exercise length was classified according to the length of the intervention period: short: 4–12 weeks; mid-length: 13–24 weeks; and long: >24 weeks. We did not consider intensity as a moderator because there was a lack of consistent intensity measurement criteria in the included literature. Finally, regarding outcome indicators, all

behavioral indicators reflecting cognitive functions were extracted in the form of mean and standard deviation.

2.4. Assessment of Study Quality. Methodological quality was evaluated by two researchers (C.Z.D and J.W.T) independently using the Physiotherapy Evidence Database (PEDro) scale [24]. The PEDro scale comprises 11 items: eligibility criteria, randomization, concealed allocation, similar baseline, blinding of subjects, blinding of therapists, blinding of assessors, more than 85% retention, intent-to-treat analysis, between-group comparison, point measure, and measures of variability. The "eligibility criteria" are not scored. One point is scored for each item on which relevant information is explicitly presented, and the maximum score for a study is 10 (9-10 = excellent, 6–8 = good, 4-5 = fair, and <4 = poor).

2.5. Statistical Analysis. Stata14.0 software was utilized for data analysis. Extracted data included the mean (M) and standard deviation (SD) of each group at postintervention and the sample size. The standardized mean difference (SMD) was selected as the magnitude of effect sizes (ESs). ESs were calculated by Cohen's d, taking 0.2, 0.5, and 0.8 as the respective thresholds for small, medium, and large effects [25]. Heterogeneity was calculated by Higgins's I^2 statistics, taking 75%, 50%, and 25% as the respective thresholds for high, medium, and low ratios of interstudy heterogeneity [26]. Publication bias was tested using the Egger test in Stata14.0.

After calculating the overall ES for cognitive function, subgroup analyzes were conducted for the cognitive function domains (global cognitive function, memory, executive function, attention, verbal fluency, and visual-spatial function), exercise prescription variables (frequency, session time, and length), and cognitive status.

3. Results

3.1. Literature Search. Figure 1 summarizes the flow of the literature search and study selection. The initial search returned 1,705 articles. After removing 113 duplicate articles and 1,527 articles according to the inclusion/exclusion criteria and abstract screening, 19 articles were finally included in the review.

3.2. Study Characteristics. Table 1 presents the characteristics of all 19 studies included in this review. The sample size ranged from 36 to 398. The overall sample size was 1,970, including 871 in the experimental groups and 1,099 in the control groups. Among the 19 studies included, 16 focused on MCI elderly and three focused on the elderly with dementia[28, 29, 31]. Participants ranged in age from 66 to 82 years. There were no gender restrictions in any studies, although most participants were women. The included studies were conducted in five countries: eleven in China (57.9%), four in the United States (21.1%), two in Thailand (10.5%), one in Vietnam, and one in France (5.3%).

Sixteen of the 19 studies used Yang-style TCC; of the others, one used Sun-style TCC [44], one used the Westernized version [35], and one did not report this information [36]. Exercise frequency varied from one to five times/week, with three times being most common; exercise session time varied from 20 minutes to 120 minutes, with 60 minutes being most common; and exercise program length varied from 10 weeks to 52 weeks, with 24 weeks being most common. Among the 19 studies, TCC was compared with health education by seven studies [19, 27, 28, 35, 39, 40, 45], no intervention by five studies [31, 37, 41, 43, 46], stretching exercises by three studies [32–34], social activities by three studies [29, 36, 38], and physical training by one study [44].

The main outcomes were six cognitive function areas: global cognitive function, memory, executive function, attention, verbal fluency, and visual-spatial function. The specific test indicators are shown in Table 1. The postintervention mean and standard deviation were compared between the experimental group and the control group. In addition to neurocognitive tasks, some studies explored the cognitive mechanism of TCC through biological and electrophysiological indicators, such as the level of plasma BDNF and magnetic resonance imaging (MRI).

3.3. Methodological Quality. The methodological quality of the included studies is reported in Table 2. The PEDro scores of the included studies range from 4 to 10 points, with an average of 6.9 points. The overall methodological quality is fair to good, with PEDro scores ≥6 for 11 studies (good) and PEDro scores of 4-5 for three studies (fair). All the included studies carried out randomization, between-group comparison, point measure, and measures of variability, and 11 achieved more than 85% retention. Eight studies used concealed allocation, blinding of assessors, and intent-to-treat analysis, while three studies used blinding of subjects and blindness of therapists.

3.4. Meta-Analysis. A total of 89 effects are included in the meta-analysis, and the overall ES is 0.31, 95% CI (0.28, 0.35), $p < 0.001$, with a statistically significant difference between the experiment and control groups. This indicates that TCC intervention can significantly improve the cognitive function of patients with cognitive impairment. The results of the heterogeneity test revealed a high degree of heterogeneity in the included studies (Table 3), so a random effect model was used to synthesize the data. The funnel plot in Figure 2 is basically symmetrical, which indicates that there is no publication bias. Egger's test shows that there is no publication bias in this study, indicating that the small sample size study does not affect the results ($t = 1.15$, $p > |t| = 0.252 > 0.05$) (Table 4) [47, 48].

3.5. Subgroup Analysis

3.5.1. Cognitive Function Domain. Subgroup analysis revealed that five subdomains of cognitive function significantly moderated the effect of TCC on cognitive function

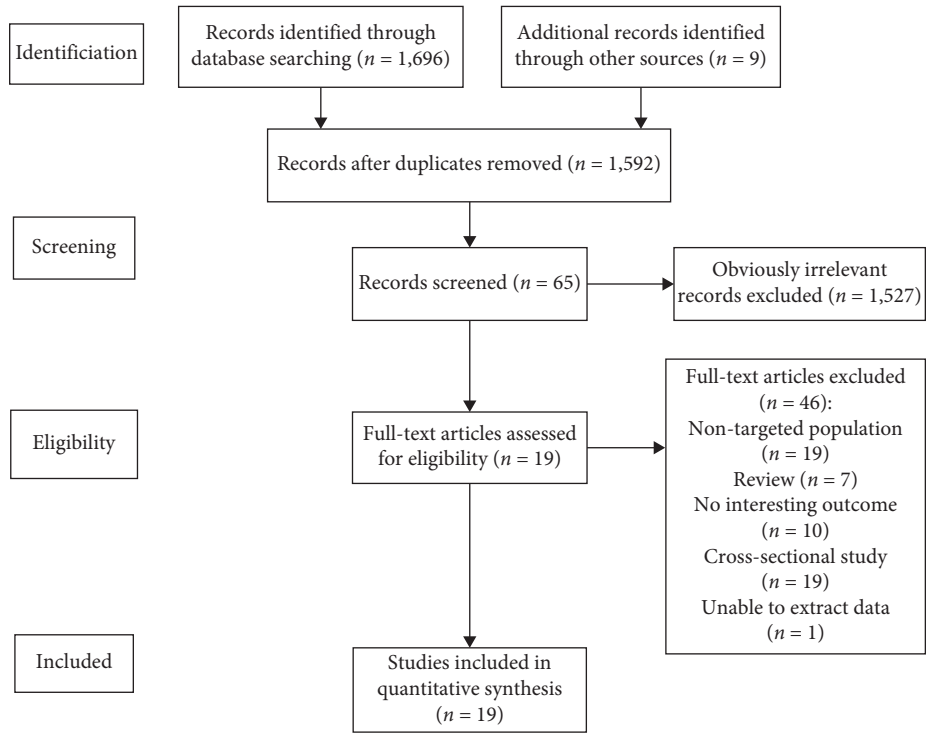

Figure 1: Literature selection flow diagram.

Table 1: Basic characteristics of the included literature.

Author, year	Country	Sample characteristics	Mean age	Cognitive status	Outcome measures	Exercise prescription variables ($F \times T \times L$)
Bao, 2019 [27]	China	TCC ($n = 31$)/health education ($n = 31$) Male ($n = 29$)/female ($n = 33$)	67	MCI	Global cognition: MMSE, MoCA Memory: MIC	Yang style $3 \times 60 \times 24$
Chan et al., 2016 [28]	China	TCC ($n = 27$)/health talk ($n = 25$) Male ($n = 8$)/female ($n = 44$)	80	DE	Global cognition: MMSE Memory: MIC	Yang style $2 \times 60 \times 12$
Cheng et al., 2014 [29]	China	TCC ($n = 35$)/handicraft ($n = 39$) Gender unreported	80	DE	Global cognition: MMSE Memory: DSF	Yang style $3 \times 60 \times 12$
Deschamps et al., 2009 [19]	France	TCC ($n = 15$)/health education ($n = 21$) Male ($n = 22$)/female ($n = 30$) (baseline)	81	DE	Global cognition: MMSE	Yang style $4 \times 30 \times 24$
Fogart et al., 2016 [30]	Britain	TCC ($n = 19$)/memory training ($n = 21$) Male ($n = 19$)/female ($n = 21$) (baseline)	72	MCI	Memory: HVLT Executive function: TMT-B	$2 \times 90 \times 10$
Huang et al., 2019 [31]	China	TCC ($n = 36$)/routine treatments ($n = 38$) Male ($n = 24$)/female ($n = 50$)	82	MCI	Global cognition: MoCA, MMSE Memory: IR, DR	Yang style $3 \times 20 \times 40$
Lam et al., 2011 [32]	China	TCC ($n = 171$)/stretching ($n = 218$) Male ($n = 97$)/female ($n = 297$)	78	MCI	Global cognition: MMSE, DRC, ADAS-Cog Memory: DR, DSF, MIC Executive function: DSB, CTB Attention: VS, CTA Language: VF	Yang style $3 \times 30 \times 52$

Table 1: Continued.

Author, year	Country	Sample characteristics	Mean age	Cognitive status	Outcome measures	Exercise prescription variables ($F \times T \times L$)
Lam et al., 2012 [33]	China	TCC ($n = 92$)/stretching ($n = 169$) Male ($n = 92$)/female ($n = 297$) (baseline)	78	MCI	Global cognition: MMSE, DRC, ADAS-Cog Memory: DR, DSF, MIC Executive function: DSB, CTB Attention: VS, CTA Language: VF	Yang style $3 \times 30 \times 52$
Lam et al., 2014 [34]	China	TCC ($n = 96$)/ stretching ($n = 169$) Male ($n = 92$)/female ($n = 297$) (baseline)	78	MCI	Global cognition: MMSE, DRC, ADAS-Cog Memory: DR, DSF, MIC Executive function: DSB, CTB Attention: VS, CTA Language: VF	Yang style $3 \times 30 \times 52$
Lavretsky et al., 2011 [35]	USA	TCC ($n = 33$)/health education ($n = 35$) Male ($n = 28$)/female ($n = 45$) (baseline)	71	MCI	Global cognition: MMSE Memory: CVLT Attention: TMT	Yang style $1 \times 120 \times 10$
Mortimer et al., 2012 [36]	USA	TCC ($n = 30$)/no intervention ($n = 30$) Male ($n = 20$)/female ($n = 40$)	68	MCI	Global cognition: MDRS Memory: CAVLT Executive function: SCWT, DSB, TMT-B Language: BNT Visuospatial ability: CDT	Westernized version $3 \times 50 \times 40$
Nguyen and Kruse, 2012 [37]	Vietnam	TCC ($n = 39$)/routine daily activities ($n = 34$) Male ($n = 48$)/female ($n = 48$) (baseline)	69	MCI	Executive function: TMT-B Attention: TMT-A	Yang style $2 \times 60 \times 24$
Sun et al., 2015 [38]	China	TCC ($n = 72$)/playing cards or singing ($n = 66$) Male ($n = 34$)/female ($n = 104$)	69	MCI	Global cognition: MMSE Attention: FAB	Yang style $2 \times 60 \times 24$
Sungkarat et al., 2017 [39]	Thailand	TCC ($n = 33$)/health education ($n = 33$) Male ($n = 9$)/female ($n = 57$)	66	MCI	Memory: DR Executive function: DSB Visuospatial ability: BDT	Yang style $3 \times 50 \times 15$
Sungkarat et al., 2018 [40]	Thailand	TCC ($n = 33$)/health education ($n = 33$) Male ($n = 9$)/female ($n = 57$)	66	MCI	Memory: DR Executive function: DSB Visuospatial ability: BDT	Yang style $3 \times 50 \times 24$
Tao et al., 2016 [41]	China	TCC ($n = 21$)/no intervention ($n = 25$) Male ($n = 14$)/female ($n = 32$)	60	MCI	Memory: WMS	Yang style $5 \times 60 \times 12$
Tao et al., 2017 [42]	China	TCC ($n = 21$)/no intervention ($n = 25$)	60	MCI	Memory: WMS	Yang style $5 \times 60 \times 12$
Tao et al., 2017 [43]	China	Male ($n = 14$)/female ($n = 32$)	60	MCI	Memory: WMS	Yang style $5 \times 60 \times 12$
Taylar-Piliae et al., 2010 [44]	USA	TCC ($n = 37$)/health education ($n = 56$) Male ($n = 28$)/female ($n = 65$)	69	MCI	Memory: SDF Executive function: SDB Language: BNT	Yang style $5 \times 45 \times 24$
Tsai et al., 2013 [45]	USA	TCC ($n = 28$)/health education ($n = 27$) Male ($n = 15$)/female ($n = 40$)	55	MCI	Global cognition: MMSE	Sun style 3×30–40×20

Note: DE, Dementia; MMSE, Mini-Mental-State Examination; MoCA, Montreal Cognitive Assessment Scale; MIC, Memory Inventory for Chinese; ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; DRC, Chinese Dementia Rating Scale; MDRS, Mattis Dementia Rating Scale; IVR, immediate verbal recall; DVR, delayed verbal recall; DSF, digit span forward; DSB, digit span backward; HVLT, Hopkins Verbal Learning Test; DR, delayed recall; IR, immediate recall; WMS, Wechsler Memory Scale; TMT-B, Trail Making Test B; CTB, Chinese Trail B; VS, visual span; CTA, Chinese Trail A; FAB, Frontal Assessment Battery; VF, verbal fluency; BNT, Boston Naming Test; CDT, Clock-Drawing Test; BDT, Block Design Test; ($F \times T \times L$), frequency × time × length; I/C, Intervention/Control.

Table 2: Methodological quality assessment for the included studies.

Reference	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Item 7	Item 8	Item 9	Item 10	Sum score
Bao [27]	1	0	1	0	0	0	1	1	1	1	6
Chan et al. [28]	1	1	1	0	0	1	1	1	1	1	8
Cheng et al. [29]	1	0	1	0	0	0	1	1	1	1	6
Deschamps et al. [19]	1	0	0	0	0	1	0	0	1	1	4
Huang et al. [31]	1	1	1	1	1	1	1	1	1	1	10
Lam et al. [32]	1	1	1	0	1	1	1	1	1	1	9
Lavretsky et al. [35]	1	1	1	1	0	1	1	0	1	1	8
Mortimer et al. [36]	1	0	1	0	0	0	1	0	1	1	5
Nguyen and Kruse [37]	1	0	1	0	0	0	1	0	1	1	5
Sun et al. [38]	1	1	1	0	0	0	0	1	1	1	6
Sungkarat et al. [39]	1	1	1	0	0	1	1	1	1	1	8
Tao et al. [43]	1	1	1	1	0	1	0	0	1	1	7
Taylar-Piliae et al. [44]	1	0	1	0	0	0	1	1	1	1	6
Tsai et al. [45]	1	1	1	0	1	1	1	0	1	1	8

Note: Item 1, randomization; Item 2, concealed allocation; Item 3, similar baseline; Item4, blinding of subjects; Item 5, blinding of therapists; Item 6, blinding of assessors; Item 7, more than 85% retention; Item 8, intent-to-treat analysis; Item 9, between-group comparison; Item 10, point measure and measures of variability; 1, explicitly described and present in details; 0, absent, inadequately described, or unclear.

Table 3: Summary of meta-analysis and subgroup analysis results.

	Q (df)	I^2 (%)	n (ES)	ES (95% CI)	p
Overall	**572.48 (88)**, $p < 0.001$	**84.6**	**89**	**SMD = 0.31 (0.28, 0.35)**	**<0.001**
Session time (minutes)	**1.82 (2)**, $p = 0.402$				
Short (≤45 min)		86.2	38	SMD = 0.30 (0.25, 0.34)	<0.001
Moderate (45–60 min)		84.3	48	SMD = 0.35 (0.28, 0.42)	<0.001
Long (>60 min)		52.6	3	SMD = 0.30 (0.02, 0.58)	0.03
Frequency (week/times)	**1.08 (2)**, $p = 0.583$				
Low (1-2 times)		92.7	12	SMD = 0.32 (0.28, 0.36)	<0.001
Moderate (3-4 times)		82.2	75	SMD = 0.26 (0.13, 0.39)	<0.001
High (≥5 times)		88.4	5	SMD = 0.34 (0.16, 0.62)	0.001
Length	**6.00 (2)**, $p = 0.05$				
Short (≤12 week)		73.5	12	SMD = 0.40 (0.30, 0.50)	<0.001
Moderate (12–24 week)		85.4	21	SMD = 0.41 (0.26, 0.55)	<0.001
Long (>24 week)		85.8	56	SMD = 0.29 (0.24, 0.33)	<0.001
Cognitive function domains	**15.66 (5)**, $p = 0.008$				
Global cognitive function		67.2	23	SMD = 0.41 (0.33, 0.48)	<0.001
Memory function		68.9	22	SMD = 0.31 (0.22, 0.39)	<0.001
Executive function		77.4	18	SMD = 0.33 (0.25, 0.42)	<0.001
Verbal fluency		0	5	SMD = 0.27 (0.13, 0.41)	<0.001
Attention		96	14	SMD = 0.25 (0.17, 0.34)	<0.001
Visual space function		54.7	7	SMD = 0.03 (−0.28, 0.33)	0.7
Cognitive status	**0.04 (1)**, $p = 0.843$				
MCI		85.7	80	SMD = 0.33 (0.17, 0.50)	<0.001
DE		56.0	9	SMD = 0.31 (0.27, 0.35)	<0.001

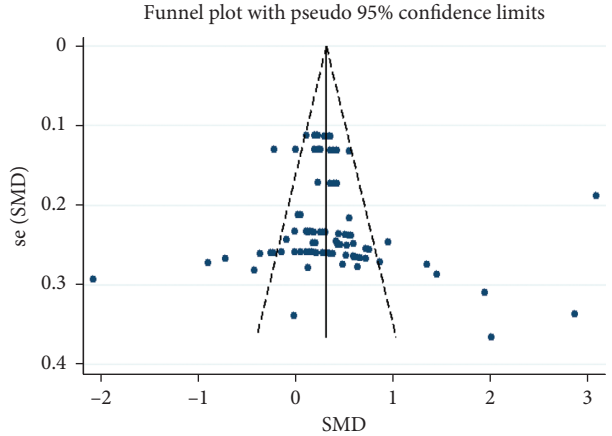

Figure 2: Funnel plot of exercise on inhibition.

Table 4: Results of Egger's test.

Std_EFF	Coef.	Std. err.	t	$p > \|t\|$	95% CI
Slope	0.1606565	0.1419637	1.13	0.261	−0.1215116, 0.4428247
Bias	0.8837072	0.7670769	1.15	0.252	−0.6409412, 2.408356

[Q (5) = 15.66, p = 0.008]. Only visual-spatial function was not a significant moderator. The ES of global cognitive function (Cohen's d = 0.41) was greater than that of memory function (Cohen's d = 0.31), executive function (Cohen's d = 0.33), attention (Cohen's d = 0.25), and verbal fluency (Cohen's d = 0.27).

3.5.2. Exercise Prescription Variables. The length of the exercise intervention significantly moderated the effect of TCC on cognitive function [Q (2) = 6.00, p = 0.05]. The results of the subgroup analysis indicated that the ES for older adults engaged in a moderate-length TCC intervention (12–24 weeks) (Cohen's d = 0.41) was larger than that for TCC interventions that were short (<12 weeks) (Cohen's d = 0.40) or long (>24 weeks) (Cohen's d = 0.29).

There were no significant differences among the ESs based upon exercise frequency [Q (2) = 1.08, p = 0.58] or exercise session time [Q (2) = 0.21, p = 0.42].

3.5.3. Cognitive Status. There were no significant differences among the ESs based upon cognitive status [Q (1) = 0.04, p = 0.40].

4. Discussion

4.1. Overall Analysis of TCC Intervention Effects. To the best of our knowledge, this is the first meta-analysis of RCTs investigating the effects of TCC exercise prescription on cognitive function. It is very important to further understanding of how the exercise prescription potentially moderates the intervention effect. Additionally, no previous meta-analysis has investigated whether cognitive status influences the effect of TCC on cognitive function in the elderly with cognitive impairment.

This meta-analysis included 19 studies and synthesized 89 ESs. The results demonstrate that TCC improves the cognitive function of the elderly with cognitive impairment, with a positive, statistically significant, yet small ES. Based on the results of this review, we believe that TCC is an effective way to improve the cognitive function of elderly with cognitive impairment, which is generally consistent with the results of previous meta-analyses [16, 18, 22]. Techniques such as functional MRI (fMRI) and event-related potential provide further evidence that TCC improves cognitive function. The reviewed studies also provide evidence that TCC improves cognitive function by changing the brain structure [49] and enhancing brain functional connectivity [41, 43], neural activity, and brain electrical activity [50].

4.2. Subgroup Analysis of TCC Intervention Effects

4.2.1. Cognitive Function Domains. Although previous studies have shown that TCC has a positive effect on the cognitive function of the elderly, this meta-analysis provides an important extension to the literature by exploring TCC's effects on subdomains of cognitive function. The results of subgroup analysis indicate that TCC has different effects on different cognitive function domains of elderly with cognitive impairment. The results indicate that TCC improves five specific domains of cognitive function: global cognitive function, memory, executive function, attention, and verbal fluency. Conflicting with previous studies, there was no significant effect on visual-spatial function [22]. A cross-sectional study found that open movement can significantly improve visual-spatial ability [51]. The insignificant effect on visual-spatial function in this meta-analysis may be explained by the small number of ESs included (only six), which may not reveal the real effect of the intervention. Although some outcome measures, such as Rey Figure, Clock Drawing, and Block Design, do not show significant improvement, the performance trend in experimental groups was better than in control groups. The findings overall suggest that TCC influences multiple aspects of cognitive function.

Twelve studies analyzed global cognitive function, and they include 23 ESs: 20 positive and three negative. The results of the meta-analysis revealed a significant effect on global cognitive function (Cohen's d = 0.40), similar to the findings of previous studies [22]. The main tools used for measuring cognitive function were MMSE, MoCA, ADAS-Cog, CDRS, and MDRS, which are commonly used in cognitive testing. MMSE is the most commonly used cognitive impairment screening scale in clinical practice. It is effective in screening between normal elderly and those with dementia, but not between normal elderly and those with MCI. MoCA has higher sensitivity in the diagnosis of MCI, whereas ADAS-Cog, CDRS, and MDRS have better sensitivity to dementia [52]. In addition to neuropsychological testing tools, Mortimer et al. also used fMRI and reported that 40 weeks of TCC exercise increased brain capacity, thereby improving cognitive function [36]. Sungkarat et al. argued that TCC exercise can significantly improve the executive function and memory of elderly with MCI by upregulating the level of serum BDNF [40]. On closer inspection of the three negative effects reported in the literature, we found that the subjects of Deschamps et al.'s study [19] were frail elderly under long-term nursing, and the control group participated in cognitive action exercises, such as strength training of the upper and lower limbs and breathing exercises. The MMSE scores increased post-intervention for both the experimental group and control group, indicating that both TCC and cognitive action exercise can improve cognitive performance. The main purpose of Lavretsky's study was to relieve depression, while the main purpose of Chan et al.'s study [28] was to improve sleep. Although neither study reported significant results, they both showed a trend of improvement. Different research purposes and different disease groups may interfere with the actual effects of TCC intervention.

Memory is an important domain of cognitive function. The results of this study showed that the ES on memory was 0.31. Thirteen included studies analyzed the effect of TCC on memory function, and all 22 ESs showed a positive impact. Although Huang et al. [31] found no difference in immediate recall between the experimental group and control group after 5 months of intervention, there was a significant difference after 10 months of intervention. The intervention effect of TCC on memory function is generally considered substantive. Pesce and Audiffren believes that, for physical exercise interventions on cognitive function, there should be focus not only on the quantitative aspects of exercise prescription (e.g., duration and intensity) but also on the cognitive needs of tasks [53]. TCC contains a rich action structure and relatively fixed sequence, so it requires many cognitive resources to maintain attention, memory, judgment, and decision-making. Among the elderly, these motor learning and coordinated movements may lead to an increase in the volume of gray matter in the middle temporal region, including the hippocampus and frontal parietal lobe network, which may improve memory function [54].

There is a phenomenon of cross overlap in the measures of executive function, attention, and visual-spatial function. Executive function is a high-level cognitive function, including shifting, updating, and inhibition [55]. There are currently 29 methods for testing executive function, each of which focuses on different subcomponents of executive function: examples include the Wisconsin test, Trail Making Test, and Stroop test [56]. A test can also reflect different cognitive areas: for instance, TMT can be regarded as a test of both attention and executive function; CDT can test not only executive function but also visual-spatial function; and DST can test attention, short-term memory, and working memory. Therefore, the differences in the division of cognitive domains lead to differences in the results of meta-analyses. Wayne et al. combined TMT, DST, Stroop, and other indicators and found a large ES (SMD = 0.9) of TCC intervention on executive function [18]; Yang combined attention and processing speed and found a medium ES (SMD = 0.51) and combined abstraction, neural flexibility, and reasoning combine effect and found a small ES (SMD = 0.29). In sum, there is a significant improvement in executive function, attention, and visual-spatial function, but different classifications across meta-analyses lead to substantial differences in ESs.

For verbal fluency, only five ESs (Cohen's $d = 0.27$) were included in this meta-analysis, so the effect of TCC intervention on this domain needs to be confirmed by more studies.

4.2.2. Exercise Prescription Variables. The current meta-analytic takes an important step by evaluating the effects of another group of moderators (exercise prescription) on the effects of TCC on cognitive function. The findings indicate that only exercise length moderates the influence on cognitive function. Among the 19 studies reviewed, four used 4–12-week programs (21.1%), eight used 13–24-week programs (42.1%), and six used programs of more than 24 weeks (31.6%). Most of the included studies adopted the length of an exercise program as 12 weeks or 24 weeks.

This study revealed an inverted U-shaped relationship between cognitive function and exercise program length. The ES of exercise length was the highest for moderate length, followed by short length and finally long length. This result may be due to the "ceiling effect" of TCC on the cognitive function of the elderly with cognitive impairment, such that the effect gradually weakens after 24 weeks, or it may be due to the cognitive decline of patients. In addition, as exercise length increases, the compliance rate may decrease, which may also influence the effect. Previous studies have not reached consensus on the most effective exercise length. Some studies contend that the effect of long programs is greater [57], others argue that short programs are more effective [58], and still others assert that exercise length does not influence the intervention's effectiveness [59]. We suggest that future research should extend the exercise length, increase the number of follow-ups, evaluate whether the cognitive difference between the intervention and control groups increases with age, and encourage the elderly to maintain their exercise habits to improve the compliance rate.

This study's subgroup analysis indicated that exercise session time was not a moderator. This implies that TCC interventions of any exercise session time positively affect cognitive function in older adults with cognitive impairment. However, there were only 3 ESs for 60-minute sessions, so this tentative conclusion should be treated with caution. Many previous studies have revealed that 20 minutes' exercise can significantly improve the cognitive function of the elderly [60, 61]. Very short exercise sessions cannot trigger changes in brain arousal and structure and in body functions, whereas overly long exercise may lead to excessive fatigue in the elderly and no obvious change in brain plasticity. Therefore, a specific causal relationship appears more likely for sessions of moderate duration [62].

The subgroup analysis also indicated that exercise frequency was not a moderator, which suggests that TCC interventions of any exercise frequency result in positive effects for cognitive function in older adults with cognitive impairment. This finding is similar to previously reported results: for instance, Northey et al. found that both low-frequency and moderate-frequency exercise can improve the cognitive function of the elderly [6]. However, because our study only included four articles with high-frequency exercise, our conclusion on this relationship may be unreliable and should be treated with caution.

4.2.3. Cognitive Status. Regarding cognitive status, this study's results show that TCC can significantly improve the cognitive function of people with MCI or dementia, but cognitive state does not moderate the intervention effect. This finding is consistent with those of previous studies [63–65]. A large number of studies have shown that physical exercise can significantly improve the cognitive function of the healthy elderly, elderly with MCI, and dementia patients [66–68].

5. Strengths and Limitations

This study's primary strength was the inclusion of RCT studies. In previous studies, the inclusion of cross-sectional studies introduced confounding variables that affect the authenticity of the research results. Another strength of this study is that it analyzed exercise prescription as a set of potential moderators. The results provide a theoretical basis for accurate exercise prescription.

Conversely, our meta-analysis had several limitations, which should be considered in the future research. First, the number of included studies is relatively small, albeit much larger than those of previous meta-analyses. Research on how TCC can influence cognitive function is a relatively recent development, and few experimental studies have been conducted so far, so more research is needed to confirm the nature of the relationship. Second, the included studies have methodological defects, especially the absence of blinding. Third, we only searched Chinese and English databases. Fourth, although most studies use Yang's simplified 24-style TCC, many do not describe the prescription, teaching content, and time allocation, which increases the difficulty of judging the dose-response relationship. It should be noted that TCC is a form of body-mind exercise, which emphasizes not only physical exercise but also psychological training, so the teaching idea, level, and methods of coaches may influence the effect of intervention. Finally, the positive effect of TCC on the cognitive function of the elderly may be moderated by subjects' cardiorespiratory fitness. However, there are not enough data in the included studies to analyze this variable.

6. Conclusions

The results of this systematic and meta-analytic review demonstrate that TCC is a promising way to improve the global cognitive function, memory, executive function, attention, and verbal fluency of the elderly with cognitive impairment. Additionally, we observed that exercise length moderated the intervention's influence. However, as neither exercise frequency nor session time appeared to have a moderating effect, the optimal TCC prescription remains unclear.

Data Availability

The raw data supporting this manuscript are from previously reported studies and datasets, which have been cited. The processed data are available in the supplementary files.

Conflicts of Interest

All authors have no conflicts of interest relevant to the content of this review.

Acknowledgments

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Supplementary Materials

Supplementary description: the raw data supporting this manuscript are from previously reported studies and datasets, which have been cited. The processed data are available in the supplementary files. (*Supplementary Materials*)

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