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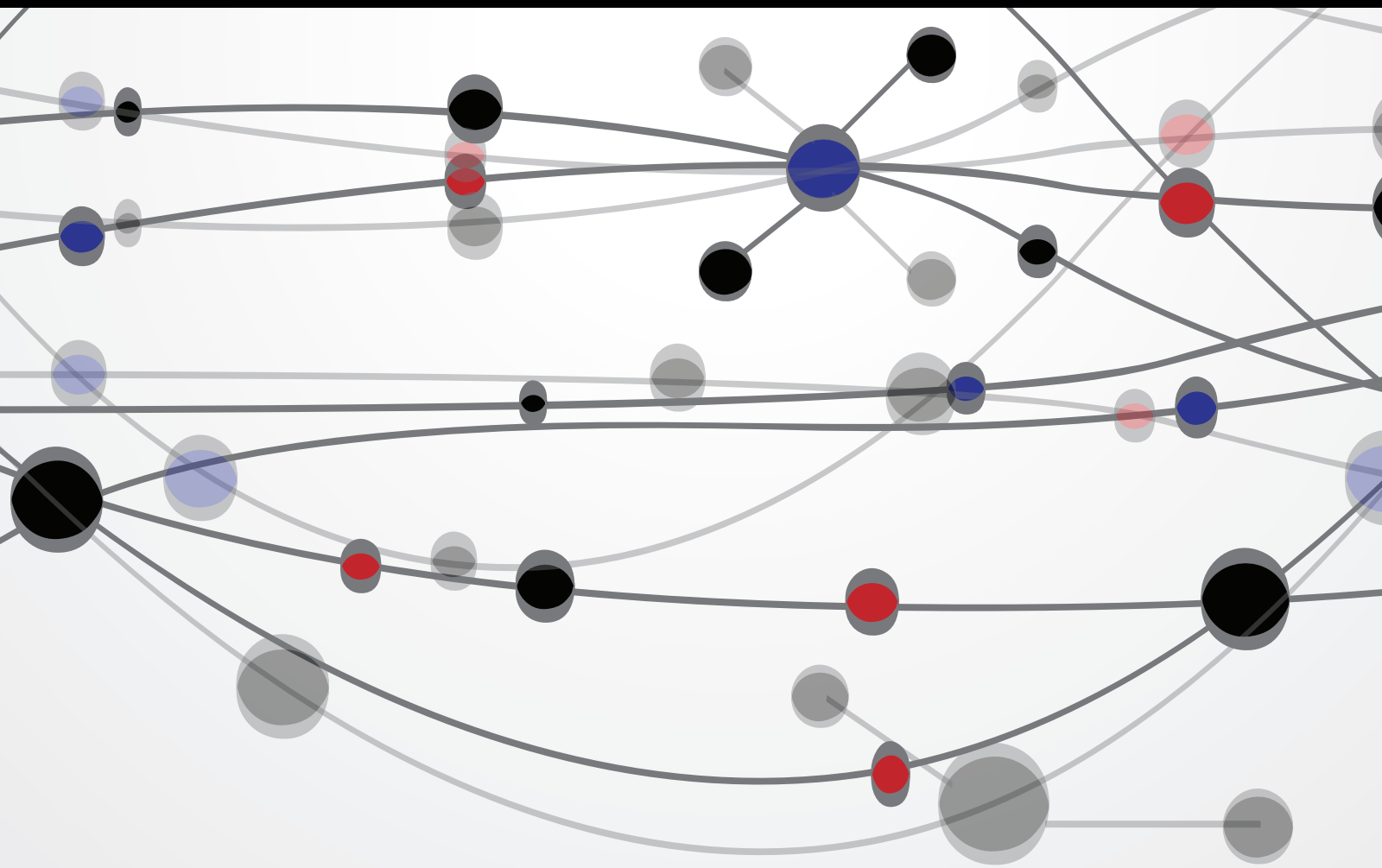
Novel Therapeutic Targets in Metabolic Disorders: From the Bench to the Bedside

Guest Editors: Maddalena Illario, Carolina Di Somma, Guido Iaccarino, Pietro Campiglia, Uma Sankar, and Nunzia Montuori



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Editorial

Novel Therapeutic Targets in Metabolic Disorders: From the Bench to the Bedside

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The aim of this special issue is to explore innovative approaches to bridge laboratory investigation and clinical research in endocrine-metabolic disorders. "From bench to bedside" refers to the concept of "translational medical research": within public health, translational medicine is indeed focused on ensuring that proven strategies for disease treatment and prevention are actually implemented within the community. Translational research also concerns how the process of transfer of innovations to daily use can be more effective and can speed up the impact of discoveries in the lives of patients. Another goal of this special issue was to encourage the multidisciplinarity efforts in health research: integration of evidence and knowledge from multiple sources and backgrounds enhances our understanding of health processes and carries the potential to generate breakthrough innovations to improve human health in the future.

It is an honor to invite you to enjoy reading this special issue.

One article was "*Possible potentiation by certain antioxidants of the anti-inflammatory effects of diclofenac in rats,*" where authors investigated the potential beneficial impact of the addition of antioxidant supplements to diclofenac regimen in a model of carrageenan-induced paw. In conclusion the combination of diclofenac and any of the anti-inflammatory agents tested appears to preserve the immunomodulating effect of the antioxidant alone. Thus, the authors conclude that the addition of antioxidants to any treatment regimen using this particular drug could have potential beneficial effects for the patients, albeit it does not build upon the effects from the diclofenac per se.

Another article was "*Detection of impaired cognitive function in rat with hepatosteatosis model and improving effect of GLP-1 analogs (exenatide) on cognitive function in hepatosteatosis.*" The aims of this study were to evaluate (1) detection of cognitive function changing in rat with hepatosteatosis model and (2) the effect of GLP-1 analog (exenatide) on cognitive function in hepatosteatosis. Authors concluded that memory performance falls off in rats with hepatosteatosis feeding with fructose (decreased latency time). However, GLP-1 ameliorated cognitive functions (increased latency time) in rats with hepatosteatosis and related metabolic syndrome. Indeed they also showed that exenatide treatment improved learning and memory performance in rats with hepatosteatosis and metabolic syndrome. So, this drug might be a candidate for alleviation of memory and cognitive dysfunctions in metabolic disorders.

In the article "*Evaluation of lung and bronchoalveolar lavage fluid oxidative stress indices for assessing the preventing*

effects of safranal on respiratory distress in diabetic rats" authors investigated the effects of antioxidant activity of safranal, a constituent of *Crocus sativus* L., against lung oxidative damage in diabetic rats, concluding that safranal treatment may be effective to prevent lung damage in diabetic rats by modulation of oxidative stress. These findings support the efficacy of safranal as natural antioxidant for diabetes and its complication management.

The aim of the paper "*A novel role of globular adiponectin in treatment with HFD/STZ induced T2DM combined with NAFLD rats*" was to evaluate the effects of globular adiponectin (gAd) on treatment of type 2 diabetic rats combined with NAFLD. Authors concluded that globular adiponectin could ameliorate the hepatic steatosis and vary the expressions of adiponectin receptors in liver and skeletal muscle by stimulating insulin secretion.

In the review article "*Endocrinopathies after allogeneic and autologous transplantation of hematopoietic stem cells,*" authors summarized (1) main endocrine disorders reported in literature and observed in their center (Hematology and Hematopoietic Stem Cell Transplant Center in the Department of Medicine and Surgery of University of Salerno,) as consequence of auto- and allo-HSCT and (2) an outline of current options for their management. Their analysis further provide evidence that the main recognized risk factors for endocrine complications after HSCT are the underlying disease, previous pretransplant therapies, the age at HSCT, gender, total body irradiation, posttransplant derangement of immune system, and, in the allogeneic setting, the presence of graft-versus-host disease requiring prolonged steroid treatment.

Last but not least, in this special issue was also published the review article "*Targeting mitochondria as therapeutic strategy for metabolic disorders*" where authors analyzed the critical role of mitochondria as key regulators of cell metabolism.

Acknowledgment

The editors thank the authors for their hard work and dedication.

Maddalena Illario
Carolina Di Somma
Guido Iaccarino
Pietro Campiglia
Uma Sankar
Nunzia Montuori

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Review Article

Endocrinopathies after Allogeneic and Autologous Transplantation of Hematopoietic Stem Cells

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Early and late endocrine disorders are among the most common complications in survivors after hematopoietic allogeneic- (allo-) and autologous- (auto-) stem cell transplant (HSCT). This review summarizes main endocrine disorders reported in literature and observed in our center as consequence of auto- and allo-HSCT and outlines current options for their management. Gonadal impairment has been found early in approximately two-thirds of auto- and allo-HSCT patients: 90–99% of women and 60–90% of men. Dysfunctions of the hypothalamus-pituitary-growth hormone/insulin growth factor-I axis, hypothalamus-pituitary-thyroid axis, and hypothalamus-pituitary-adrenal axis were documented as later complicances, occurring in about 10, 30, and 40–50% of transplanted patients, respectively. Moreover, overt or subclinical thyroid complications (including persistent low-T3 syndrome, chronic thyroiditis, subclinical hypo- or hyperthyroidism, and thyroid carcinoma), gonadal failure, and adrenal insufficiency may persist many years after HSCT. Our analysis further provides evidence that main recognized risk factors for endocrine complications after HSCT are the underlying disease, previous pretransplant therapies, the age at HSCT, gender, total body irradiation, posttransplant derangement of immune system, and in the allogeneic setting, the presence of graft-versus-host disease requiring prolonged steroid treatment. Early identification of endocrine complications can greatly improve the quality of life of long-term survivors after HSCT.

1. Introduction

Autologous- (auto-) and allogeneic- (allo-) hematopoietic stem cell transplant (HSCT) programs started in Europe about five decades ago and today their cumulative number exceeds 30.000 per year. Main underlying diseases leading to auto- and allo-HSCT are acute leukemias and myelodysplastic syndromes in the allogeneic setting and multiple myelomas, lymphomas, and leukemias in the autologous setting [1].

The progressive increase in the number of auto- and allo-HSCT performed in the last four decades for the treatment of malignant and nonmalignant hematological diseases has been accompanied by a parallel significant increase of long-term survivors due to a progressive decrease of transplant-related mortality. Given this progressive increase in the number of long-term survivors after auto- and allo-HSCT, ever greater attention by physicians is nowadays paid to prevention and to early diagnosis of early and late complications of HSCT procedure which may worsen the quality of life of transplant recipients [1–3].

The endocrine system is one of the most frequent targets of post-HSCT complications. However, the majority of

the data available on the early and late effects of HSCT on the endocrine system refers to the pediatric population, while still few are the prospective studies on adult transplanted patients. The underlying diseases, previous pretransplant therapies, the age at HSCT, the use of total body irradiation (TBI), its cumulative dose and administration schedule, and post-transplant treatments are the main risk factors for endocrine complications after HSCT [4, 5]. Both auto- and allo-HSCT procedures are preceded by conditioning regimens consisting of high-dose antiblastic treatments, associated or not with TBI; this is aimed at eradicating the underlying hematologic disease (mostly a malignancy) and suppression of the host immune system in the allogeneic setting. Most of the data concerning the functioning of the endocrine system is derived from the study of patients who underwent TBI as a part of pretransplantation treatments [6]. TBI has been reported to be responsible for a large part of posttransplant endocrinopathies. Only a few studies have been carried out in adult HSCT patients treated without TBI, so the impact of chemotherapy alone and/or of other possible risk factors on the endocrine system dysfunction has not been completely established in adult HSCT recipients [7, 8].

Allo-HSCT is associated with a more severe derangement of the immune system than auto-HSCT, due to the severe immunosuppressive effect of the conditioning regimens to avoid graft rejection, the cytokine storm occurring at time of transplantation, the prolonged administration of multiple immunosuppressive drugs to prevent graft-versus-host disease (GVHD), and the frequent development of acute or chronic GVHD resulting in additional relevant alterations of the immune system. Nevertheless, auto-SCT also is associated with a marked immunosuppression due to highly aggressive lympho- and myeloablative conditioning regimens that are likely followed by a resetting of immune system. Therefore, autologous HSCT has had an emerging role in the last decades as a promising treatment for several autoimmune diseases [9–11].

The aim of this review is to describe the most commonly observed endocrine complications after HSCT in our center, by integrating the experience of international scientific literature. Our experience is based on managing main endocrine dysfunctions in more than 300 long-term survivors after auto- ($n = 228$) and allo-HSCT ($n = 120$) up to 15 years after transplant.

2. Hypothalamic-Pituitary-Gonadal Axis in Women and Men

2.1. Women. Ovarian failure is the most frequent complication after auto- and allo-HSCT. It has been mainly considered a direct consequence of radiation therapy and high-dose chemotherapy. Posttransplant recovery of the ovarian function is rare and appears to be age-dependent, occurring more frequently in young girls than in adult women [4, 12–15]. It has been calculated that, with increasing of age at transplantation, the probability of recovery of ovarian function declines according to coefficient of 0.8 per year [16]. Although the effect of cytotoxic agents and TBI on the ovary is dose-dependent [16–19], the radiation dose responsible for the death of 50% of human oocytes (LD50) was estimated to be less than 4 Gy [20]. Also almost all antineoplastic drugs, and especially alkylating agents, exert toxic effects on the ovaries in a dose-dependent way; this is linked not only to a direct damage of oocytes but also to damage of the supporting granulosa cells of both proliferating and quiescent follicles [21, 22]. The known risk factors for gonadal damage after HSCT are summarized in Table 1.

Table 1: Risk factors for gonadal damage.

	Risk factor degree
Patient relating factor	
Pubertal stage	Postpuberal >prepuberal
Age of HSCT in women	>30 years
Sex	F > M
Underlying disease	ALL, lymphomas
Treatment relating factor	
Type of radiotherapy	TBI, pelvic, inverted Y, fractionated doses
Chemotherapy	Alkylating agents > other chemotherapy
Type of transplant	Previous TBI Allo-HSCT >auto-HSCT
HSCT complications	Presence of GVHD

ALL: acute lymphoblastic leukemia; allo: allogeneic; auto: autologous; GVHD: graft-versus-host disease; HSCT: hematopoietic stem cell transplant; F: female; M: male; TBI: "total body" irradiation.
Symbol (>) means more than.

In a large study of 144 women transplanted for leukemia after conditioning with cyclophosphamide and TBI, Sanders et al. reported the occurrence of hypergonadotropic amenorrhea in 100% of the women in the first years after SCT, with a delayed recovery of ovarian function in only 6% of cases [18]. In women receiving as conditioning regimen only chemotherapy with cyclophosphamide (CY), ovarian function recovered in 31% of patients transplanted at age < 26 years but in none of those over this age [16]. The addition of busulfan (BU: 16 mg/kg) to low or high doses of cyclophosphamide (CY: 120 and 200 mg/kg, resp.) caused permanent ovarian damage in everyone, with exception of a few cases [23–25]. Chatterjee et al., who investigated the acute effects of high-dose polychemotherapy with or without TBI on ovarian function in a large cohort of transplanted women, claim that the severity of acute injury is predictive of the probability of the later recovery of ovarian cycles [25].

In our experience, all women except one had experienced an ovarian insufficiency, regardless of the type of HSCT (auto- or allo-HSCT). Ovarian insufficiency manifested as secondary amenorrhea associated with hypergonadatrope hypogonadism and reduced volumes of ovaries and uterus. About one-third of women went into menopause several months before the transplant as the result of previous chemotherapy, while in the remaining patients ovarian cycles disappeared after the conditioning regimens. Only rare cases (<5%) of young women transplanted under 21 years, one of which had undergone an allo-HSCT for aplastic anemia

and therefore had not received antiblastic agents in pretransplant period, experienced spontaneous recovery of ovarian function with regular menstrual cycles after 10–18 months of amenorrhea [26, 27].

Although cycles recovered more frequently in auto-HSCT women, the difference when compared with the allo-HSCT group was not significant. However, recovery is not an early event and may occur long time after HSCT. Statistical analysis of predictive factors in our cohort of women undergoing allo- or auto-HSCT in their reproductive period showed that none of the following factors was directly related to the probability of spontaneous recovery of ovarian function: the type of transplant, bone mass index, and previous use of alkylating agents and/or steroids. In addition, we found that autotransplanted women had ovarian volumes similar to those observed in women after a corresponding interval from the physiological menopause, whereas the ovarian volumes of allo-HSCT were lower, suggesting greater damage to the ovarian tissue (Figure 1) [11].

Concerning hormonal pattern, serum 17beta-estradiol and Delta-4-androstenedione levels in our cohort of allo-HSCT patients were significantly decreased. In the group of allotransplanted women, signs of toxic effects on the ovaries were much more severe in those women affected by cGVHD. In contrast, in autotransplanted patients, only serum 17beta-estradiol levels were decreased, while Delta-4-androstenedione levels were found normal 12 ± 24 months after transplantation [11].

We have also documented reduced values of circulating androgens due to ovarian damage and adrenal suppression caused by immunosuppressive treatments, especially in women with cGVHD. Ovarian contribution to lower serum androgens was suggested by the correlation between ovarian volume and patients estradiol and androgen levels, although the lower dehydroepiandrosterone sulphate (DHEA-S) values observed in patients with cGVHD are also to be related to the prolonged use of corticosteroids [11, 26, 27].

A premature menopause for its clinical and psychological implications needs replacement treatment. The hormone replacement therapy (HRT) should be started after the complete hematologic recovery after transplantation, but it may be contraindicated for longer periods in women suffering from severe chronic liver GVHD. Indeed, estroprogestin may further worsen liver damage already caused by chronic GVHD [28, 29]. Moreover, HRT may not be fully effective if there is a simultaneous gastrointestinal or skin GVHD that interferes with the drug absorption; this was documented in 30% of women in a previous study [26]. In our experience, the cyclical sequential combination of estradiol (2 mg daily) plus dydrogesterone (10 mg for 14 days/month) was associated with excellent compliance, due to its simple administration and few adverse effects, allowing achieving a dramatic improvement of vasomotor, urogenital, and psychological symptoms mediated by estrogen deficiency. A withdrawal of hormone therapy for a period of 2 to 3 months per year together with monitoring of reproductive axis function is also suggested [27]. In fact, ultrasonographic evidence of ovarian follicles is often associated with a likelihood of cycles recovery, but there are no serum markers to predict the return of ovarian function in these patients. In our cohort of transplanted women, the cyclical sequential combination of estradiol (2 mg daily) plus dydrogesterone (10 mg for 14 days/month) was associated with excellent compliance because of its simple administration regimen and good safety and tolerability profile. In addition, the observed rate of ovarian function recovery in our center was 7% in the allo-HSCT setting and 25% in auto-HSCT setting [26, 27]. All the pieces of information, that is, ovarian ultrasonographic findings, hormonal pattern, and the recovery rates of ovarian function, are aligned in providing an evidence of more severe ovarian damage in the allogeneic HSCT setting (Figure 1).

A serious complication that may occur in the early posttransplant is polymenorrhea. Given that this complication can occur even before the full hematological recovery, especially when the platelet count is still low, the control of bleeding may be really difficult to manage [30]. The commonly used treatment for this complication is the norethisterone acetate that has been documented to be a risk factor for the development of liver venoocclusive disease [31]. Recently, the use of gonadotropin releasing hormone analogues (aGnRH), exerting suppressive effects on the hypothalamic-pituitary-gonadal axis, has been reported as being able to prevent peritransplant vaginal bleeding, without interfering with the hemostatic balance or inducing liver toxicity [32]. However, the administration of aGnRH should be started at least one month before the conditioning regimen due to the initial mild stimulatory effect on the hypothalamic-pituitary-gonad axis [31]. Although it has been established that hypogonadism induced by aGnRH is capable of preventing peritransplant bleeding, there is still insufficient evidence that it can be efficacious in preventing ovarian damage due to the antiblastic treatments [33].

Although the skin, the liver, and the gastrointestinal system are the sites most frequently involved in cGVHD, virtually any organ or tissue can be involved. The genital tract of women has been documented to be a potential target for cGVHD; in fact, variable degrees of vulvovaginal lesions related to gynecological cGVHD were described in 25% of cases by ourselves and by a large recent multicentric Italian study including 213 women [34, 35]. Milder forms of cGVHD were characterized by an increased frequency of developing vulvovaginal infections and inflammation, while the more severe forms have been found responsible for vaginal and cervical stenosis and malformations of the internal and external genitalia and sometimes associated even with perineal involvement. In addition, women with cGVHD may develop intrauterine adhesions that should be closely monitored by pelvic exam and ultrasonography during the first months of HRT, to avoid unpleasant complications such as hematocolpometra [36–38].

Therefore, female recipients of HSCT, and in particular those after allo-HSCT, require early surveillance and long-term follow-up of hormonal pattern and gynecological apparatus by skilled health care givers.

2.2. Men. Both alkylating agents and irradiation exert gonadotoxic effect on germ cell epithelium and Leydig cells of

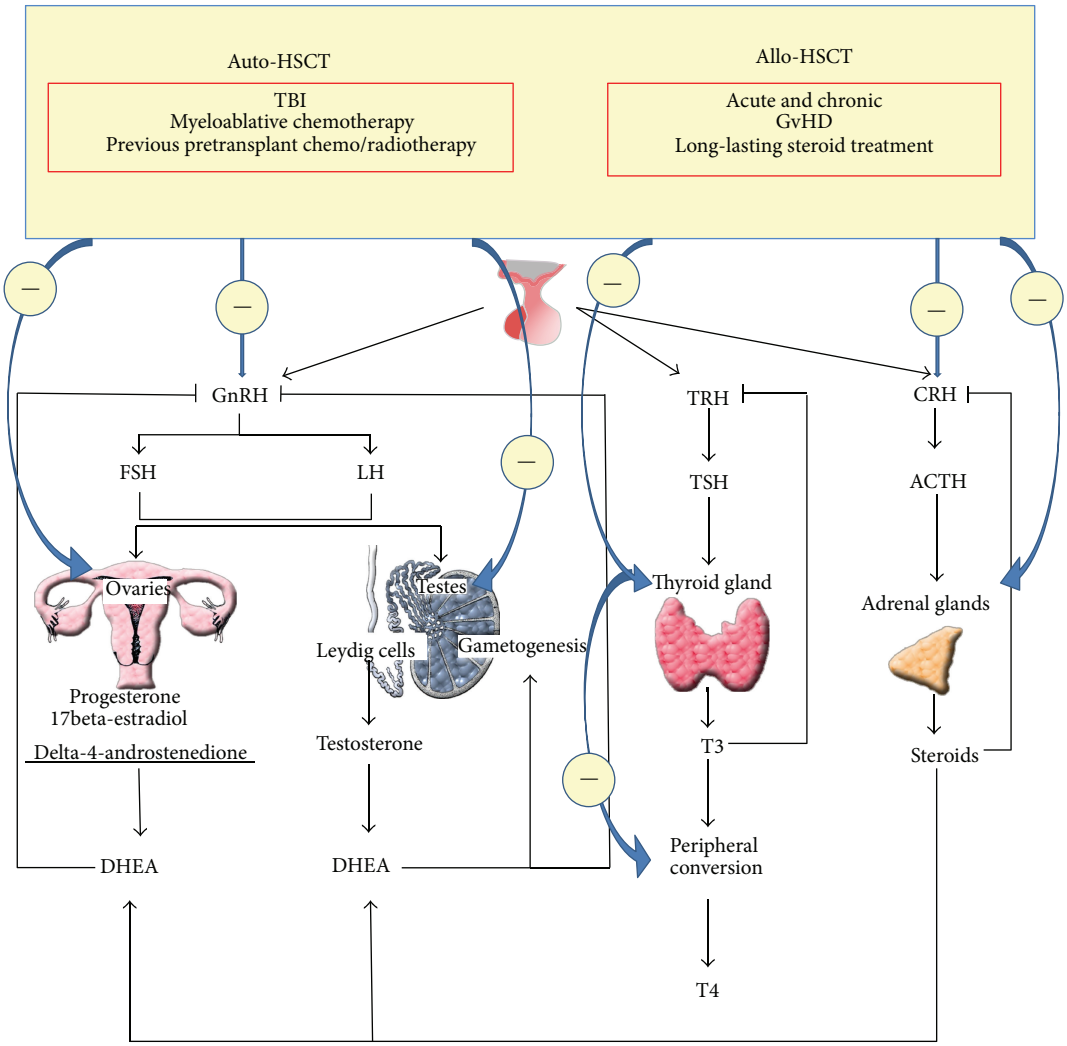

Figure 1: Main endocrine abnormalities after hematopoietic stem cell transplantation. Auto-HSCT and allo-HSCT: autologous- and allogeneic-hematopoietic stem cell transplantation; GnRH: gonadotropin releasing hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; DHEA: dehydroepiandrosterone; TBI: total body irradiation; cGvHD: chronic graft-versus-host disease; ACTH: adrenocorticotropic hormone; CRH: corticotropin-releasing hormone; T4: thyroxine; T3: triiodothyronine; TSH: thyroid-stimulating hormone; TRH: thyrotropin releasing hormone. Symbol (–) means inhibition.

testis, in either childhood or adulthood [5, 39–43]. Usually there is a good correlation between the degree of spermatogenesis impairment and the corresponding increase of follicle-stimulating hormone (FSH) levels in patients after auto- and allo-HSCT. A return to elevated FSH levels within the normal range has been occasionally observed in patients treated with high-dose chemotherapy and TBI single fraction of 7.5 Gy, suggesting in these cases an unexpected recovery of germline function [44]. By contrast, recovery of spermatogenesis has been described very rarely in adult transplanted patients conditioned with fractionated TBI [45]. Molassiotis et al. documented persistently increased levels of FSH and luteinizing hormone (LH) in the majority of transplanted males, regardless of use of TBI [46].

In our experience impaired spermatogenesis damage was observed in all transplanted patients who underwent seminal fluid analysis at 12 months, about 90% of them showing germinal aplasia with azoospermia [25]. The increase in FSH levels was found only in 85% of cases, indicating that spermatogenesis damage was not always associated with increased FSH levels. The finding of oligozoospermia/azoospermia in transplanted patients that is lacking a corresponding FSH increase may have several explanations: (1) a partial damage to the hypothalamic-pituitary axis mediated by chemotherapy/radiotherapy leading to impaired gonadotrophin release, or (2) a partial/complete arrest of spermatogenesis at the spermatid level and of a rarer partial/complete occlusion of the spermatic tract [47].

In our cohort of transplanted patients, radiotherapy was associated with finding of significantly higher levels of FSH; this can be suggestive of a greater testicular toxic effect in patients treated with irradiation of abdominal/pelvic area [27]. Similarly, a greater cumulative probability for injury of germinal cells was previously reported in patients treated

with radiation therapy when compared to those treated with chemotherapy only [17, 25, 41, 48, 49]. In addition, lower sperm counts were observed in long-term survivors affected by cGVHD when compared to unaffected patients, suggesting some possible effects of this chronic posttransplant complication on gonadal status [39, 50]. For men, the age at the time of treatment seems to be less important than for women in terms of susceptibility to gonadal failure. Conversely, the underlying disease, the type of antiblastic drugs, and/or the duration of their administration, all, affect spermatogenesis damage in men [5, 17].

Leydig cells are less vulnerable than germinal epithelium of the testis to the gonadotoxic effects of HSCT and/or treatments received prior to HSCT [40]. In fact, decrease of serum testosterone is mostly transient and recovers weeks-to-months after grafting (Figure 1) [45, 46, 51].

In our experience, testosterone was reduced in about 30% of patients up to 1 year after HSCT. However, testosterone production was unaffected in our long-term survivors out of treatments and none of the subjects reported a regression of secondary sexual characteristics. On the other hand, all men evaluated during acute and chronic GVHD had low testosterone levels, likely due to an inhibitory effect of the immunosuppressive treatments on the hypothalamic-pituitary-gonadal axis [27]. In fact, glucocorticoids are able to inhibit GnRH release and consequently the whole reproductive axis function. Moreover, glucocorticoids may also suppress the adrenal source of androgens [52]. The role of cyclosporine-A treatment in inducing Leydig cell damage cannot be excluded as it is known to exert a severe gonadotoxic effect on testicles in long-term treatments [53, 54]. The effects of new immunosuppressive drugs on gonadal function such as tacrolimus and mycophenolate mofetil have not yet been investigated exhaustively.

As sexual steroid hormone decrease in men after HSCT is mostly mild and transient; testosterone replacement is rarely required. There is one case report describing the failure of preventing TBI-induced testicular germinal cell damage by aGnRH administration. Currently, the higher chance to achieve a conceivement after HSCT, especially after allo-HSCT, is through sperm cryopreservation [55].

2.3. Fertility. The majority of auto- and allo-transplanted patients, as above described, experience different degree of gonadal damage that is responsible for their infertility. The ability to preserve fertility in these transplanted patients, mostly young people, is the subject of ongoing studies worldwide [56]. In the last two decades, aGnRH has been used to reduce and prevent chemotherapy-induced ovarian damage in small cohorts of young women with various types of cancer, and it has been reported that some patients receiving aGnRH in conjunction with gonadotoxic chemotherapy were able to spontaneously recover ovulation and becoming pregnant [57]. However, the ability of aGnRH to protect the ovarian function after HSCT is still debated. Although there are isolated case reports and a study on 30 women undergoing HSCT for hematological malignancy showing no benefit from aGnRH therapy in preventing the germ cell damage after HSCT [33], a more recent study in a cohort of 47 young transplanted women has reported that aGnRH administration in conjunction with conditioning chemotherapy before HSCT may significantly decrease the rate of premature ovarian failure from 82% to 33% in patients with lymphoma but not in those with leukemia [58–60].

Sperms and embryos conceived by *in vitro* fertilization can be well cryopreserved, but oocyte banking, in the absence of a male partner, is difficult and currently is still an experimental procedure. Not only the conservation but also oocyte retrieval is technically complex. For satisfactory oocyte retrieval, women need ovarian hyperstimulation for several weeks before starting high-dose chemotherapy, monitoring the follicular growth by ultrasonography. Following these procedures, a harvesting of the follicles should be performed. However, all these procedures before HSCT are often difficult to carry out, given the severity of the underlying malignant disease and the urgency to start antiblastic treatments as soon as possible [59, 61].

Occasional successful pregnancies have been reported in recent decades, especially in women who underwent autografting [62]. Most of data regarding this issue are derived from two large surveys performed in Europe and the USA. In both studies, partners of male patients had uncomplicated pregnancies and delivered normal children, whereas women after allografting showed a high incidence of miscarriage, preterm labor, and low birth weight babies, indicating the difficulty in completing their pregnancy due to damage of both the ovaries and gynecological tract or urogynecological apparatus. The rates of congenital malformations, developmental delays, and malignant diseases in the offspring of HSCT recipients were not different from those reported in the general population [24, 63].

In our experience of a total of 125 long-term survivors after allo-HSCT, only two pregnancies occurred: one was reported in a wife of a male patient who had received allograft 3 years earlier. One woman delivered two healthy twins 5 years after allo-SCT with hormonally fully assisted pregnancy obtained with oocytes donated by a sister [47].

Relapse of leukemia during or after pregnancy has been reported in various case reports. However, it cannot be excluded that these women have relapsed independently from pregnancy, and it is uncertain whether the natural history of the primary hematological disease can be influenced by pregnancy. Since the relationship between underlying diseases and pregnancy is still unclear, Salooja et al. recommended that pregnancy would be delayed by at least 2 years after HSCT [63].

3. Hypothalamus-Pituitary-Adrenal Axis

Secondary adrenal insufficiency due to the suppression of the hypothalamic-pituitary-adrenal axis is mainly related to the duration and cumulative dose of corticosteroid treatments received after HSCT. Only the initial report by Sanders et al. described high incidence of long-lasting adrenocortical insufficiency following TBI-based conditioning allo-HSCT [64], but subsequent studies have rarely documented

a permanent reduction of plasma cortisol levels after transplant [26, 65]. More recently acute adrenal insufficiency after TBI-based conditioning regimens has been reported in children [66, 67].

In agreement with a previous hypothesis suggesting a relative resistance to irradiation of the adrenal tissue [4], in our cohort of nonirradiated allo-transplanted recipients, the onset of adrenal insufficiency was always related to the duration (more than 100 days) and cumulative dose (greater than 10 gr/m^2) of corticosteroid treatment. However, though all our patients who developed a secondary adrenal insufficiency (a total of about 20% of cases in the auto- and allo-setting) were under treatment with corticosteroids, all of them having exceeded the cumulative dose and duration treatment above reported, we did not observe adrenal insufficiency in some age- and disease-matched patients which had assumed similar cumulative doses of steroids for similar periods, suggesting a variable-individual sensitivity of the hypothalamic-pituitary-adrenal axis to exogenous suppression. In our experience, corticoadrenal failure recovered in all patients after about 3–12 months of short acting steroid substitution therapy [26, 27]. Patients with chronic GVHD, in whom corticosteroid treatment is suddenly withdrawn because of the onset of serious infections, are at a high risk of developing an acute adrenal crisis that can further worsen their already compromised clinical condition [68–72]. As a stimulation test should always be carried out to rule out even a slight degree of dysfunction of the hypothalamic-pituitary-adrenal axis after high dose and/or chronic steroid treatment, the prevalence of nonovert adrenal insufficiency is likely an underestimated condition unless specifically investigated by endocrinologists. However, autoantibodies to the adrenal cortex (ACA) and to 21-hydroxylase assayed in our cohort of transplanted patients with adrenal insufficiency were absent (Figure 1) [27].

The use of replacement therapy with short acting steroids is recommended until the adrenal axis recovers in allo- and auto-HSCT recipients with a progressive reduction of the corticosteroid dose, to enable the adrenal axis to gradually recover [27, 73].

4. Hypothalamic-Pituitary-Insulin-Like Growth Factor-I Axis

Previous studies on GH secretion have documented that GH secreting pituitary cells are less vulnerable to irradiation damage in adulthood than in childhood. Littley et al. described a normal GH peak response to GH stimulation, 17–55 months after TBI-based conditioning regimen allo-HSCT [45]. Indeed, Kauppila et al. documented normal insulin-like growth factor-1 (IGF-1) levels in all transplanted patients, 20% of them showing impaired response to growth hormone-releasing hormone (GHRH) stimulation after TBI-based conditioning regimen allo-HSCT (<5 mcg/L with GHRH administration) [74]. In addition, decreased growth and GH secretion have previously been reported after cranial irradiation [75]. A less significant height reduction occurred after TBI [76–78].

Contradictory results were reported in children transplanted for leukemia after BU/CY conditioning regimen. Sanders showed a significant incidence of GH deficiency [79, 80], whereas Wingard et al. have documented a similar growth rate after BU/CY and CY/TBI in the first two years after HSCT [81]. Conversely, several other studies did not find any significant growth impairment up to 5 years after BU/CY-conditioning for allo-HSCT [82–86]. However, definitive conclusions about the conditioning BU/CY effect on the growth require further study.

Sanders et al. also found that cGVHD may result in impaired growth [64]. Glucocorticoids representing, alone and in combination with other immunosuppressive drugs, the first line therapy for cGVHD treatment exert acute growth-suppressive effects; however, decreased growth rates were also described in cGVHD patients treated without steroids, suggesting that immune system derangement underlying cGVHD physiopathology may contribute to their growth impairment [82]. Known risk factors for GH deficiency after transplant are summarized in Table 2.

Hypothalamus-pituitary-IGF-I axis function has not been systematically investigated in adult allo- and auto-HSCT recipients. In our cohort of allo-HSCT conditioned with BU/CY, we have found that, although the GH profile showed levels within the normal range in all subjects, IGF-I levels were lower in 38% of allo-HSCT recipients affected by cGVHD, whereas IGF-1 resulted in the normal range in only 7% of subjects cGVHD-free [26]. This finding may partially explain previous observations by Sanders et al. suggesting that cGVHD represents a condition of multiorgan injury associated with lower body mass index (BMI) [64]. However, the hypothesis of a possible influence of the general health conditions on the GH-IGF axis is also supported by le Roith et al. and Kami et al., who investigated the effects of complications after allo-HSCT conditioned with chemotherapy alone on the growth; they documented that children with any type of complications, especially those including starvation and malnutrition, showed decreased growth rates compared with normal growth of children without posttransplant complications [87, 88].

Similarly to allo-HSCT, we and others have reported that auto-HSCT recipients show serum IGF-I levels below the age-reference values (about 56% of patients in our cohort) within 3 months after transplant, with a subsequent return to normal range in about 20% of cases, while remaining low in the other 38% of cases up to 1 year after transplant. However, since IGF-I deficiency is often transient and less frequently persistent, the use of replacement therapy is not currently recommended. On the other hand, the follow-up of IGF-I levels is suggested after the recovery from transplant and adequate period from corticosteroid withdrawal [27].

5. Hypothalamus-Pituitary-Thyroid Axis

Frequent overt or subclinical thyroid dysfunctions, including persistent low T3 syndrome, chronic thyroiditis, subclinical hypo- or hyperthyroidism, and neoplastic transformation,

Table 2: Risk factors for GH deficiency.

	Risk factor degree
Patient relating factors	Pediatric age >adult age
Treatment relating factors	Radiotherapy >chemotherapy
	Single dose >fractioned dose TBI
	Intrathecal chemotherapy

TBI: "total body" irradiation. Symbol (>) means more than.

have been described after allo-HSCT [7, 26, 88, 89] and more rarely after auto-HSCT [17, 27, 90].

Permanent or transitory hypothyroidism following TBI or BU-CY regimens, occurring also years after allo-HSCT, has been reported in both children and adults [74, 75, 91, 92]. The incidence of thyroid dysfunctions after fractionated TBI (15-16%) [63, 72, 93] is significantly lower than after single-dose TBI (46–48%) [6, 94–96]. However, the long-term effects of TBI-induced thyroid dysfunctions are still unknown as well as its timing and peak incidence [97]. Subclinical hypothyroidism, characterized by mildly elevated TSH levels with thyroid hormone within the normal range, has been frequently reported after TBI doses of 10–12 Gy, with overt hypothyroidism rarely documented [65, 91, 94, 95]. Al-Fiar et al. have also described a gradual increase in the incidence of hypothyroidism with increasing TBI doses. Subclinical hypothyroidism, within 2 years after allo-HSCT, was also documented after BU-CY conditioning regimen with an incidence of 11% versus 16.7% after 12 Gy TBI [98]. Similar frequency was reported by Toubert et al. in a cohort of patients (14%) not receiving TBI-based conditioning regimen [7].

"Low T3 syndrome" (normal FT4 and TSH and FT3 below ranges of normality) has been reported by Toubert et al. in 48% of patients at 3 months, falling to 19% at 14 months after allo-SCT, whereas Schulte et al. documented this syndrome in 100% of patients at day 14 after allo-HSCT, claiming that its persistence up to day 28 after transplant was associated with a higher probability of fatal outcome [7, 99]. Indeed, although the availability of sensitive and specific assays for the evaluation of subclinical hypothyroidism has now replaced the use of thyrotropin releasing hormone (TRH) test, Siegert et al. reported an exaggerated TSH response to TRH, after a mean of 3.2 years following transplant, in 35% of allo-SCT recipients showing also normal values of TSH [72, 100].

In our experience, subclinical hypothyroidism was found also later after allo-SCT, up to 5 years after allo-HSCT. Substitutive treatment with levothyroxine was given to all patients [26, 90]. In addition, we have found persistence of the "low T3 syndrome" in patients with chronic extensive GVHD, even 12–48 months after allo-HSCT, likely due to decreased extrathyroidal conversion of thyroxin to 3,5,3′ triiodothyronine induced by both chronic disease and glucocorticoid therapy [101, 102]. However, in our experience, low T3 syndrome did not represent a negative prognostic factor for the general outcome.

In the auto-HSCT setting, we detected subclinical hypothyroidism in 9% and 12% of patients at 3 months and at 12 months, respectively. The "low T3 syndrome" was documented in about 30% of patients at 3 months after auto-HSCT, whereas no one showed this syndrome at 12 months. As expected, the incidence of hypothyroidism was higher in patients previously treated (15–36 months earlier) with neck/thoracic radiotherapy than in untreated patients (50% versus 1.3%, resp.) [27, 90]. Transient "low T3 syndrome" may partly be the result of adverse nutrition and metabolic conditions that may persist for several months after transplantation, particularly in the allogeneic setting, and disappear thereafter; indeed, corticosteroids and antiblastic treatments may also contribute to this thyroid dysfunction, in particular in autologous setting [99]. The "low T3 syndrome" was usually asymptomatic, especially in auto-HSCT recipients, and did not require any treatment. Nevertheless, these patients should be monitored every 3 months until their endocrine values do not return into the normal range [103]. However, none of our allo- or auto-HSCT patients developed overt hypothyroidism; the possible explanation can consist in the fact that no one did receive TBI (Figure 1) [27, 90].

Increased frequency of transient subclinical hyperthyroidism (normal FT3 and FT4 levels and TSH values below the normal range) has been reported early after allo-HSCT (peak incidence, about 100 days), mainly within the period of immunologic reconstitution suggesting that the major pathogenetic factor of thyroid damage is the immune system derangement occurring within the first 6 months after transplant [26, 88, 90]. We diagnosed transient subclinical hyperthyroidism in 15% of patients 12–18 months after allo-HSCT, in agreement with data from a larger longitudinal study by Kami et al. (12.3%) [88]. A similar disorder may also occur after auto-HSCT; however, in auto-HSCT recipients the transient subclinical hyperthyroidism was documented to be less severe, perhaps due to a milder degree of immune system derangement occurring in the auto-HSCT setting. Posttransplant thyroid ultrasound showed a nonhomogeneous hypoechoic pattern in 30% of patients with subclinical hyperthyroidism and was sometimes associated with a mild increase in autoantibodies, suggesting chronic autoimmune thyroiditis [104–106].

On the other hand, biochemical and ultrasound evidence of chronic thyroiditis associated with normal thyroid function has been documented in patients assessed also 2–10 years after transplant. Discrepancies between functional and ultrasound results and the appearance or absence of thyroid autoantibodies, particularly in allo-HSCT recipients, may be related to the particular immunological conditions of these patients as well as to immunologic effects of immunosuppressive therapies. Transient hyperthyroidism was usually nonsymptomatic, especially in auto-HSCT recipients, and did not require any treatment [26, 27, 90]. However, both transplanted patients with transient hyperthyroidism and those with evidence of thyroiditis and normal thyroid function for their risk of developing hypothyroidism should be monitored every 3 to 4 months after transplant until their endocrine parameters are normalized [107, 108]. Known risk factors for thyroid dysfunction are summarized in Table 3.

Only a few studies have investigated the development of thyroid carcinoma in long-term survivors after HSCT

Table 3: Risk factors for thyroid function.

Disease	Risk factor degree	Timing from HSCT
Hypothyroidism	Radiotherapy to the neck >TBI Single dose of TBI >fractionated TBI Allo-HSCT >auto-HSCT Chronic GVHD	Late effect (years from HSCT)
Subclinical hyperthyroidism	Allo-HSCT	Early effect (within 12 months after HSCT)
Low T3 syndrome	Infections Nutritional status Chronic GVHD Immunosuppressive therapies	Variable up to years after HSCT
Thyroid carcinoma	Radiochemotherapy >chemotherapy Pediatric age >adult age	Late effect (years after HSCT)

Allo: allogeneic; auto: autologous; GVHD: graft-versus-host disease; HSCT: hematopoietic stem cell transplant; TBI: "total body" irradiation.
Symbol (>) means more than.

[1, 109–114]. Increased incidences of secondary follicular and papillar thyroid carcinoma have been recently documented after HSCT recipients in the largest retrospective multicenter study (68.936 patients receiving allo- or auto-HSCT) that was carried out on this specific issue by the European Group for Blood and Marrow Transplantation (EBMT) registry [109]. In this large cohort of transplanted patients, a higher risk of secondary thyroid carcinoma (32 cases) has been documented compared to the general European population, with a ratio of observed to expected cases of 3.26. Median interval between HSCT and the diagnosis of secondary thyroid carcinoma was 8.5 years, similar only to that observed in patients with Hodgkin's disease [115]. Statistically significant risk factors for secondary thyroid carcinoma documented in this population were younger age (<10 years), TBI, dose of TBI, female sex, and chronic GVHD [109].

As there are several evidences that TSH increase may be a risk factor for thyroid tumorigenesis, generally the patients with subclinical hypothyroidism are treated with a substitutive levothyroxin dose in order to normalize TSH levels [116]. However, in the EBMT cohort of HSCT recipients developing thyroid carcinoma reported above, thyroid function abnormalities (serum TSH and thyroxin levels and thyroid specific antibodies) were found in only few cases, suggesting that thyroid laboratory tests do not help to suspect and diagnose the thyroid carcinoma in HSCT patients [109]. With the increasing use of ultrasound scans and fine-needle aspiration biopsy (FNAB), thyroid carcinoma tends to be diagnosed very accurately at early stages. Recently, Vivanco et al. reported an incidence of 8% for thyroid cancer after ultrasound examination in transplanted patients receiving TBI-based conditioning regimen HSCT during childhood, claiming the need to use for thyroid investigation in transplanted patients not only functional tests but also ultrasound scans, followed by FNAB of thyroid nodules when required, every 1–3 years after transplant [114].

Periodical yearly monitoring of thyroid function and morphology is recommended in HSCT recipients.

6. Conclusions

Autologous- (auto-) as well as allogeneic- (allo-) hematopoietic stem cell transplantation (HSCT) is nowadays an essential part of treatment strategy of several malignant and nonmalignant hematologic diseases as well as some non-hematologic diseases including inherited metabolic disorders. Often this is lifesaving therapeutic intervention. Over the past four decades, the transplant-related mortality progressively decreased, thanks to the development of less toxic pretransplant conditioning regimens and the improvement of prophylaxis and therapy of infections and GVHD as well as of other supportive care. These achievements resulted in parallel increase in the numbers of long-term HSCT survivors, a proportion of which is affected by early and late HSCT complications that warrant an appropriate management as they may significantly worsen the quality of life and cause long-term morbidity and mortality. Indeed, currently estimated increase in morbidity of long-term survivors after transplantation is five- to ten-fold versus matched general population over 30 years of observation and the estimated increase of mortality is about 30% in the same time frame. Both morbidity and mortality seem to be higher in the allogeneic setting. This is the reason why ever greater attention by physicians is now focused on how to better prevent, detect, and treat early and late complications and effects of HSCT.

The relatively high proportion of growing cells contained in the endocrine glands realize why early and late endocrine complications, varying from minimal subclinical symptoms to life-threatening disorders, are among the most common complications observed in survivors after auto- and allo-HSCT.

Gonadal failure has been found in approximately two-thirds of auto- and allo-HSCT patients, 90–99% of women and 60–90% of men, in both prevalently related to TBI- and high dose chemotherapy-based conditioning regimens. Impairments of hypothalamus-pituitary-GH/IGF-I and adrenal axis have been more frequently documented later and occurred in approximately 10% and 40–50% of patients,

respectively, being related to antiblastic treatments, immune system derangement, and immunosuppressive treatments. Overt or subclinical thyroid dysfunctions, including persistent low T3 syndrome, chronic thyroiditis, subclinical hypo- or hyperthyroidism, and thyroid carcinoma, occurring in about 30% of HSCT recipients prevalently as late events may persist for many years, more frequently after allo-HSCT than auto-HSCT. Although it has been well established that TBI may predispose to thyroid disorders and neoplastic transformation, high-dose antiblastic treatments, alkylants agents, corticosteroid and acute and, especially, chronic GVHD development may also contribute to thyroid dysfunctions after transplant.

The underlying diseases, previous pretransplant therapies, the age at HSCT, TBI, and its dose and administration schedule, and posttransplant development of acute and chronic GVHD requiring prolonged high doses of steroids are the main risk factors for endocrine complications after HSCT.

Since the early identification of endocrine complications can greatly improve the quality of life of long-term survivors after HSCT and as it can be difficult for physicians who operate outside of specialized centers, it is mandatory to set up a multidisciplinary program consisting of hematologists, endocrinologists, and primary care physicians in order to decide the most appropriate investigating timing, prevention, diagnosis, and monitoring of multiple early and late endocrine disorders after auto- and allo-SCT. Personalized approach to each patient is preferable, including identification of endocrine disorders requiring treatment and those which need to be followed up.

Conflict of Interests

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

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Review Article

Targeting Mitochondria as Therapeutic Strategy for Metabolic Disorders

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Mitochondria are critical regulator of cell metabolism; thus, mitochondrial dysfunction is associated with many metabolic disorders. Defects in oxidative phosphorylation, ROS production, or mtDNA mutations are the main causes of mitochondrial dysfunction in many pathological conditions such as IR/diabetes, metabolic syndrome, cardiovascular diseases, and cancer. Thus, targeting mitochondria has been proposed as therapeutic approach for these conditions, leading to the development of small molecules to be tested in the clinical scenario. Here we discuss therapeutic interventions to treat mitochondrial dysfunction associated with two major metabolic disorders, metabolic syndrome, and cancer. Finally, novel mechanisms of regulation of mitochondrial function are discussed, which open new scenarios for mitochondria targeting.

1. Mitochondria and Cellular Energy

Mitochondria are membrane-bound, cytoplasmic organelles, mainly involved in oxidative energy metabolism by regulating energy homeostasis through the metabolization of nutrients, producing ATP and generating heat [1]. Mitochondria produce more than 90% of our cellular energy by oxidative phosphorylation [2]. Energy production is the result of two metabolic processes—the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). The TCA cycle uses carbohydrates and fats as substrates for the synthesis of ATP leading to production of the coenzymes NADH and FADH which enter into the ETC in the inner mitochondrion membrane. Mitochondria constantly metabolize oxygen, thereby producing reactive oxygen species (ROS) as a byproduct. Indeed, mitochondria are the most important source of ROS in most mammalian cells. During normal oxidative phosphorylation, in mitochondria, 0.4–4.0% of all oxygen consumed is converted to the superoxide (O_2^-) radical [3, 4]. Superoxide is transformed to hydrogen peroxide (H_2O_2) by a class of enzymes called superoxide dismutases [5] and then to water by glutathione peroxidase (GPX) or peroxiredoxin III (PRX III) [6]. These organelles have their own ROS scavenging mechanisms that are required for cell survival [7]. Indeed, under normal conditions, the effects of ROS are counteracted by a variety of antioxidants, by both enzymatic and nonenzymatic mechanisms. Oxidative stress is considered as the result of an imbalance of two opposing and antagonistic forces, ROS and antioxidants, in which the effects of ROS are more potent than the compensatory capacity of antioxidants. In turn, excessive ROS production contributes to mitochondrial damage in several conditions and is also important in redox signalling from the organelle to the rest of the cell [8, 9].

2. Mitochondrial Dysfunction and Metabolic Disorders

The most important function of mitochondria is the synthesis of ATP by oxidative phosphorylation. Thus, mitochondria generate energy through oxidation of nutrients, such as free

fatty acids, to create a proton gradient across the mitochondrial inner membrane used as a source of potential energy to generate ATP, transport substrates or ions, or produce heat [5]. Oxygen radicals are also generated during oxidative phosphorylation which could cause damage of the mitochondrial and cellular DNA, proteins, lipids, and other molecules and leading to oxidative stress and mitochondrial dysfunction. Mitochondrial dysfunction is characterized by inhibition of mitochondrial O_2 consumption, changes in the mitochondrial membrane potential, and a reduction of ATP levels due to an imbalance between energy intake and expenditure [10]. Damage to mitochondria is primarily caused by ROS generated by the mitochondria themselves [11, 12], in particular by complexes I and III of the electron respiratory chain [13]. Direct damage to mitochondrial proteins decreases their affinity for substrates or coenzymes and, thereby, decreases their function [14]. ROS represented the mechanism of mitochondrial dysfunction during inflammation. Stimulation of cultured cardiac myocytes with tumor necrosis factor (TNF-α) or angiotensin II increased ROS generation and myocyte hypertrophy and treatment with antioxidants inhibited both effects [15]. Also TNF-α induces mitochondrial dysfunction by reducing complex III activity in the ETC, increasing ROS production, and causing damage to mtDNA [16]. Also the nutrition status and availability of nutrients can cause mitochondrial dysfunction. Indeed, optimal metabolic function of mitochondria depends on the availability of many essential vitamins, minerals, and other metabolites [17, 18]. These micronutrients are critical cofactors that support basic metabolic functions of the mitochondria including ATP and heme synthesis, building electron transport complexes, and detoxification of oxygen. Inadequate amounts of these micronutrients inhibit critical enzymatic activities of the electron transport complexes, thus increasing the production of reactive oxidants and impairing mitochondrial function [17, 18]. For example, several micronutrients (biotin, pantothenate, pyridoxine, riboflavin, copper, iron, and zinc) are required for heme synthesis in mitochondria. A severe deficiency of these micronutrients will cause a deficit of heme and therefore of complex IV, of which heme-a is an essential component [18–20]. The deficits of complex IV result in oxidant leakage, DNA damage, accelerated mitochondrial decay, and cellular aging [18–20].

Mitochondrial dysfunction is closely associated with metabolic disorders [21]. Indeed, it has been demonstrated in various target tissues of metabolic syndrome and insulin resistance including skeletal muscle, liver, fat, heart, and pancreas [22–27]. In skeletal muscle, decreased mitochondrial respiration capacity, reduced ATP production rate, and increased ROS levels lead to reduced fatty acid oxidation and increased cytosolic free acid levels that result in insulin resistance and obesity/diabetes [28–30]. It remains to be established whether mitochondrial dysfunction is the consequence rather than the cause of insulin resistance [31, 32]. Impaired mitochondrial β-oxidation is found in patients with nonalcoholic fatty liver disease (NAFLD), a potential cause of hepatic steatosis, and liver injury [33–35], playing an important role in the early stages of liver fibrosis [36]. In adipose tissue, mitochondria provide key intermediates for the synthesis of triglycerides (TGs) and are critical for lipogenesis [37]. Adipose mitochondria are also important for lipolysis through β-oxidation of fatty acids, which constitutes an important source of energy for ATP production to supply the energy requirement of the cell. The sirtuins (SIRT) are a class of Nicotinamide Adenine Dinucleotide (NAD)-dependent deacetylase which regulate cellular metabolism. Among them, SIRT3-5 are localized in mitochondria to deacetylate several crucial enzymes involved in mitochondrial functions [38]. SIRT3 deacetylates various key enzymes, such as long-chain acyl-CoA dehydrogenase, leading to an increase of mitochondrial fatty acid oxidation in liver and its deficiency causes metabolic syndrome [39, 40]. In this review, we will deal with the effect of mitochondrial dysfunction in the development of two widespread metabolic disorders, metabolic syndrome and cancer, and the established therapeutic approaches for these conditions.

2.1. Mitochondrial Dysfunction in the Metabolic Syndrome. The metabolic syndrome is described as a group of various abnormal metabolic risk factors such as obesity, dyslipidemia, increased blood pressure, increased plasma glucose (prediabetes) levels, prothrombotic condition, and proinflammatory condition [41, 42]. This group of abnormalities recognizes insulin resistance as the intrinsic and common mechanism [41, 43]. Most of the patients with metabolic syndrome gradually develop type 2 diabetes and its complications, like cardiovascular diseases (heart failure, thrombosis, and cardiac arrhythmias). Defective cell metabolism is considered as the main culprits of the syndrome [42] due to the imbalance between nutrient intake and its utilization for energy. Decreased fatty acid oxidation increases the intracellular accumulation of fatty acyl-CoAs and other fat-derived molecules in various organs (adipocytes, skeletal muscle, and liver). This causes the inhibition of insulin signaling leading to hyperinsulinemia which on turn damages various organs in metabolic syndrome [42]. Genetic factors, oxidative stress, mitochondrial biogenesis, and aging affect mitochondrial function, leading to insulin resistance and associated pathological conditions [44–46] (metabolic syndrome, T2DM, and attendant cardiovascular complications) [47–49]. However, it is still not clear whether mitochondrial dysfunction is the primary cause or it is the secondary effect of the metabolic syndrome.

2.1.1. Genetic Factors. Genetic mutations in mitochondrial DNA lead to the so-called mitochondrial diabetes. The most common mutation leading to mitochondrial diabetes is the A3243G mutation in the mitochondrial encoded tRNA (*Leu, UUR*) gene [44, 50]. This mutation leads to impaired synthesis of multiple mitochondrial proteins and overall mitochondrial dysfunction. The A3243G variant of mitochondrial diabetes is characterized by decreased glucose-induced insulin release but not insulin resistance, suggesting that the major pathology occurs within mitochondria of pancreatic β cells [44, 50].

2.1.2. Mitochondrial Morphology. Mitochondrial dysfunction could depend on defects in mitochondrial morphology, fission, and fusion. In particular, biopsies of skeletal muscle from subjects with type 2 diabetes and obesity show mitochondria of smaller size and number compared to controls and size appears to correlate with insulin sensitivity [23]. Obesity in both humans and rodents is associated with reduced levels of mitofusin, involved in docking to initiate fusion [51], and polymorphisms of presenillin-associated rhomboid-like (PARL) protein, important for morphologic integrity [52].

2.1.3. Oxidative Phosphorylation and ROS. Impaired mitochondrial biogenesis has been suggested as the cause for reduced mitochondrial number and capacity for oxidative phosphorylation in diabetes [53–55]. Studies of human subjects and rodents provide evidence for impaired oxidative phosphorylation in muscle mitochondria in insulin-resistant states in which there are reduced levels of NADH oxidoreductase and reduced citrate synthase activity [23]. Moreover, in diabetic subjects, there is a decreased mRNA expression of several genes associated with oxidative phosphorylation, including genes coordinately regulated by PGC-1α and nuclear respiratory factors [55]. Mitochondrial ROS is involved in both the pathogenesis and long-term complications of diabetes. Indeed, elevated glucose or free fatty acids drive the formation of ROS [56, 57], impairing both β-cell insulin release and insulin sensitivity and contributing to the complications of diabetes [6, 58].

2.1.4. Mitochondrial Dysfunction and Insulin Signaling. It has been demonstrated that mitochondrial dysfunction inhibits insulin signaling [59]. Insulin interacts with α-subunits of its receptor (IR) on cell membrane. In response to stimuli, tyrosine residues undergo autophosphorylation, and the IR acquires tyrosine kinase activity. This leads to phosphorylation of insulin-receptor substrate-1 (IRS-1), activating a downstream cascade leading to the activation of Akt and translocation of the glucose transporter type 4 (GLUT4) to the cell membrane. GLUT-4 fusion with the membrane results in glucose uptake by facilitated diffusion. Mitochondrial dysfunction is depicted to oppose insulin signaling in two ways: interfering with oxidation of fatty acyl-CoA and consequent accumulation of intracellular lipid and diacylglycerol with consequent activation of protein kinase C [28] and through the generation of ROS [60] (Figure 1). Both processes activate serine kinase reactions, leading to serine phosphorylation of IRS-1, thus interfering with insulin signal transduction. Furthermore, mitochondrial dysfunction seems to play a central role in metabolic and cardiovascular disorders. Cardiovascular diseases, including coronary artery disease, hypertension, heart failure, and stroke, are associated with insulin resistance and diabetes [61, 62]. Free fatty acids (FFAs) contribute to insulin resistance and reduce mitochondrial oxidative capacity, cardiac efficiency, and ATP production and increase myocardial oxygen consumption in obese and insulin-resistant *ob/ob* mice [63]. In addition, intramyocardial lipid accumulation induces lipotoxic injury and cardiac dysfunction, including diastolic dysfunction, left ventricular hypertrophy, and impaired septal contractility in rodent and human obesity [64, 65]. Thus, the reduced mitochondrial oxidative capacity contributes to cardiac dysfunction.

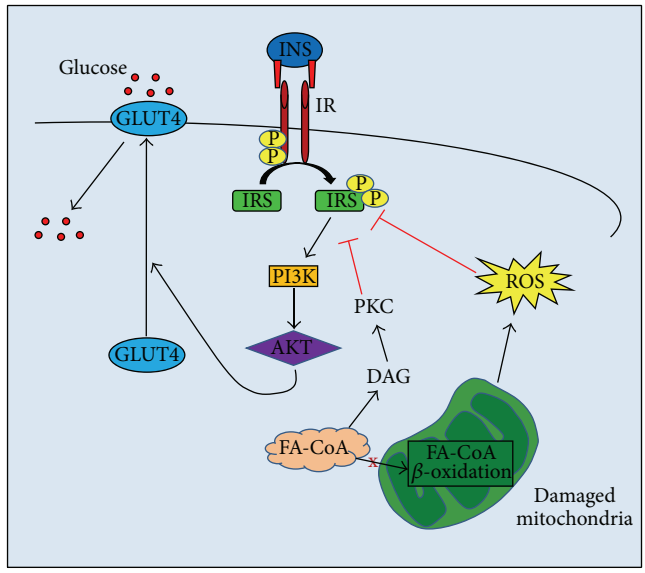

Figure 1: Mitochondrial dysfunction regulates insulin signaling. Insulin interacts with α-subunits of its receptor (IR) on cell membrane. In response to stimuli, tyrosine residues undergo autophosphorylation, and the IR phosphorylates the insulin-receptor substrate-1 (IRS-1) in serine residues, activating Akt, with phosphorylation of the type 4 glucose transporter (GLUT4) to the cell membrane and facilitation of glucose uptake. Mitochondrial dysfunction inhibits insulin signaling, leading to insulin resistance, by (1) interfering with oxidation of fatty acyl-CoA and consequent accumulation of diacylglycerol, with consequent activation of protein kinase C and phosphorylation of IRS-1 in tyrosine residues preventing its activation, and (2) through the generation of ROS, which inhibits IRS phosphorylation in serine residues.

2.2. Mitochondrial Dysfunction in Cancer. Several lines of evidence support the hypothesis that cancer is primarily a disease of energy metabolism [66]. Indeed, the mitochondrial dysfunction has been found to be associated with the development of several human cancers [67, 68]. Numerous studies show that tumor mitochondria have impaired morphology and function and are not able to generate normal levels of energy [69–73]. It has been reported that mitochondrial dysfunction in tumors could be caused by inhibitors of mitochondrial electron transport chain [74], pathogenic mutations in mitochondrial DNA (mtDNA), and mutations in nuclear gene coded electron transport chain proteins [75], oncogenic stress, loss of p53 tumor suppressor, and aberrant expression of metabolic enzymes.

2.2.1. Warburg Effect. A prominent alteration in energy metabolism in cancer cells is the increase in aerobic glycolysis, a phenomenon known as the Warburg effect [76, 77]. Recent studies suggest that upregulation of glucose

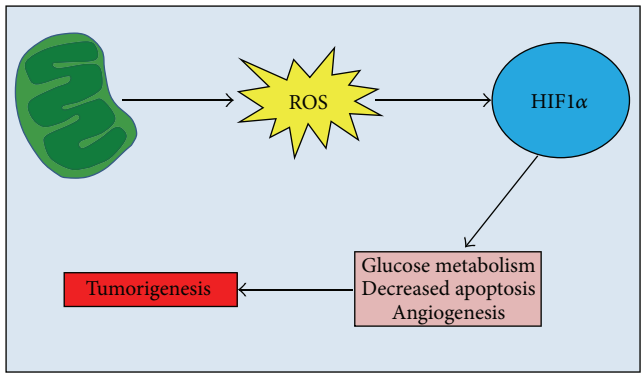

Figure 2: Mitochondrial dysfunction and hypoxia in cancer. Schematic representation of the role of mitochondrial dysfunction in tumorigenesis. Damaged mitochondria release ROS which on turn activates HIF1α. Finally, HIF1α activates the tumorigenic hypoxia pathway by initiating transcription of genes involved in glucose metabolism, angiogenesis, and survival.

transporters and hexokinases may be involved in promoting the Warburg effect. Elevated expression of glucose transporters (GLUTs) especially GLUT1, which has been correlated with tumor invasiveness and metastasis, is induced by oncogenic transformation caused by c-Myc [78], ras, or scr [79]. C-Myc also activates lactate dehydrogenase A (LDH-A) overexpression, which seems required for c-Myc-mediated transformation [80].

2.2.2. Hypoxia. Mitochondrial dysfunction and hypoxia in the tumor microenvironment are considered as two major factors contributing to the Warburg effect [81–83]. Hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates the cellular response to hypoxia, induces several genes that mediate tumorigenesis and the development of resistance to chemotherapy [84, 85]. It is known that HIF-1 is a heterodimer that consists of the oxygen sensitive HIF-1α subunit and the constitutively expressed HIF-1β subunit [86, 87]. Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases on the proline residues in the oxygen-dependent degradation domain [88, 89]. In hypoxic conditions, low oxygen leads to HIF-1α stabilization due to the inhibition of prolyl-hydroxylation and subsequent reduction in HIF-1α ubiquitination and degradation [89]. Mitochondrial dysfunction promotes cancer cell motility partly through HIF1α accumulation mediated via increased production of ROS (Figure 2) [90].

2.2.3. The Tumor Suppressor p53. The tumor suppressor p53 has been shown to be an important molecule that affects glucose metabolism, and loss of p53 function in cancer cells, induced by mitochondrial dysfunction [91], may contribute to the glycolytic phenotype. Wild-type p53 represses GLUT1 and GLUT4 gene transcription, while mutations within the DNA binding domain of p53 impair the repressive effect on GLUT transcription, leading to increased glucose metabolism [92].

2.2.4. ROS Production. Compelling evidence suggests that cancer cells tend to have elevated levels of ROS, compared to the normal cells of the same tissue origins [93]. Cancer cells exhibit increased levels of reactive oxygen species (ROS) partly due to the impaired mitochondrial function [94, 95]. The increased ROS in cancer cells may in turn affect certain redox sensitive molecules and further lead to stimulation of cellular proliferation, cell migration, and invasion, contributing to carcinogenesis [96, 97].

2.2.5. Mitochondrial DNA Mutations. Mitochondrial DNA (mtDNA) mutations correlate with increased ROS levels in solid tumors and leukemia cells [97–99]. Several mtDNA mutations have been identified in various types of human cancer which are present in both the noncoding region and coding regions of the mtDNA [100–104].

2.2.6. Apoptotic Signaling. Proper balance between cell proliferation and cell death is essential to maintain tissue homeostasis, and the failure to eliminate cells by apoptosis may play an important role in carcinogenesis. Abnormal decrease in apoptosis has been considered as a mechanism responsible for the accumulation of cancer cells, especially in certain malignancies such as chronic lymphocytic leukemia [105]. Mitochondria play a pivotal role in regulating apoptosis. Among the important molecules that affect the intrinsic apoptotic pathway through mitochondria, the Bcl-2 family proteins play a major role in cell survival and drug sensitivity since dysregulation of Bcl-2 family is often observed in various types of human cancer, including renal, ovarian, stomach, and brain tumors and leukemia [106–108]. It has been shown that increased expression of prosurvival Bcl-2 homologues [109] or lack of BH3-only protein expression and/or function (e.g., caused by loss of p53) [110] contributed to tumorigenesis and anticancer drug resistance.

3. Therapeutic Implications

Giving the main role of mitochondrial dysfunction in the development of several metabolic disorders, new therapeutic strategies have been developed during the last years to regulate mitochondrial function and biogenesis. These approaches could be useful to decrease insulin action and pancreatic beta-cell production, lipid accumulation in liver, skeletal muscle impairments, endothelial-mediated vasorelaxation, and both systolic and diastolic myocardial function. Pharmacologic interventions are focused on mechanisms regulating mitochondrial biogenesis, ROS, and respiration thus to restore mitochondrial function as well as mitochondrial ROS production.

3.1. Pharmacological Interventions. Newer pharmacologic approaches have been proposed to improve mitochondrial function. Resveratrol, an ingredient of red wines, is a polyphenolic SIRT1 activator that, like calorie restriction, has antiaging effects in lower organisms [111], reduces signs of aging in mice, and extends survival [112]. In mice, resveratrol

improves insulin resistance, protects against diet-induced obesity, induces genes for oxidative phosphorylation, and activates PGC-1α [113]. Other related small molecules are more potent than resveratrol to enhance the action of SIRT1 on substrates for deacetylation [114]. Similar to resveratrol, these compounds bind directly to the SIRT1-acetylated peptide complex at the same site and lower the K_m for peptide substrate resulting in a more productive catalytic complex [114]. Other potential targets for pharmacologic manipulation include AMPK [115], which enhances both glucose and fat oxidation [116, 117], pyruvate dehydrogenase [118], or the various shuttle mechanisms regulating uptake of TCA intermediates. Moreover, as recently showed [119], mitochondria targeted antioxidants may alter intact-cell fuel selectivity. Various vitamins and chemical compounds with antioxidant properties have been developed, including coenzyme Q [120], vitamin E [121], a-lipoic acid [122], N-acetylcysteine (NAC) [123], vitamin C, and inducers of heme oxygenase [124], which are able to reduce ROS production. Successively, antioxidant compounds specifically targeted to mitochondria have been synthesized, incorporating ubiquinone (mitoQ) or vitamin E (mitoVit E) [125]. Oral administration of mitoQ (500 mM in drinking water administered ad libitum) to normal male rats protected heart muscle function, prevented myocardial cell death, and improved the respiratory-control ratio (state 3 to state 4 respiration) in rats subject to ischemia/reperfusion injury [126]. Mitochondrial-targeted antioxidants protected Friedreich ataxia fibroblasts, in which glutathione synthesis was blocked, from oxidative stress [127] and reduced telomere shortening [128]. In bovine aortic endothelial cells, mitoQ reduced oxidative damage in cells stressed by 25 mM glucose and glucose oxidase [129]. Moreover, mitoQ also reduced ROS and reduced activation of the mitogen-activated protein kinase, p42-ERK2, in endothelial cells after hypoxic stress [130].

3.2. Exercise and Diet. Lifestyle modification, including exercise and diet, decreases the risk for developing type 2 diabetes [131], whereas physical activity improves glucose tolerance [132]. Exercise offers several benefits, including increased electron-transport activity in muscle, stimulation of mitochondrial biogenesis through effects on PGC-1α, and improved sensitivity to insulin [133, 134]. Moreover, it has been shown that it also activates AMPK, which improves both glucose and fat oxidation [133].

3.3. Therapeutic Approaches for Cancer. The primary strategic problem in cancer therapy is how to selectively activate apoptosis in transformed cells. Despite the heterogeneity of tumors and a consequent need of an individual approach for anticancer treatment, many tumor cells demonstrate enhanced uptake and utilization of glucose which leads to the stabilization of the mitochondria and an increased resistance to outer mitochondrial membrane (OMM) permeabilization and apoptotic cell death. Thus, a successful therapy should be based on the activation of apoptotic pathways, which are suppressed in tumor cells. Targeting mitochondria might be a promising strategy to increase the sensitivity of tumor cells to apoptotic stimuli [135, 136]. Suppression of pyruvate dehydrogenase kinase (PDK1) and LDH activities decreased mitochondrial membrane potential and increased mitochondrial production of ROS in cancer cells, but not in normal cells [137]. Similarly, overexpression of frataxin, a protein associated with Friedreich ataxia, stimulated oxidative metabolism and elevated mitochondrial membrane potential and ATP content in several colon cancer cell lines [138]. The Bcl-2 homology 3 (BH3) domain is crucial for the death-inducing and dimerization properties of proapoptotic members of the Bcl-2 protein family. It has been demonstrated that synthetic peptides corresponding to the BH3 domain of Bak bind to Bcl-x_L, antagonize its anti-apoptotic function, and rapidly induce apoptosis when delivered into intact cells via fusion to the Antennapedia homeoprotein internalization domain [139]. Treatment of HeLa cells with the Antennapedia-BH3 fusion peptide resulted in peptide internalization and induction of apoptosis within 2-3 hours [139].

4. Conclusions and Perspectives

Mitochondria are vital for cell function and survival; thus, it is not surprising that the loss of integrity of these organelles is associated with several pathological conditions. To date, great advances have been made to improve the knowledge of the link between mitochondrial dysfunction and metabolic diseases and different therapeutic approaches have been developed to reestablish normal function of the organelles and restore cellular homeostasis. However, an important question remains to be answered: is mitochondrial dysfunction a contributing factor or a consequence of metabolic diseases? Further studies are needed to solve this issue and to provide new insights for the development of specific and effective therapeutic treatments of metabolic diseases.

Conflict of Interests

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

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Research Article

Possible Potentiation by Certain Antioxidants of the Anti-Inflammatory Effects of Diclofenac in Rats

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In the present study, we investigated the potential beneficial impact of the addition of antioxidant supplements to diclofenac regimen in a model of carrageenan-induced paw. Rats were treated daily with antioxidants, that is, a-lipoic acid (50 mg/kg), selenium (2.5 mg/kg), vitamin C (1 g/kg), vitamin E (300 mg/kg), or zinc (25 mg/kg) on seven successive days and then received a single treatment with diclofenac or saline before carrageenan was injected to induce paw inflammation. The results indicated that these combinations did not significantly affect the percentage inhibition of paw edema caused by diclofenac alone; however, some combination treatments ameliorated signs of concomitant oxidative stress (such as alterations in plasma malondialdehyde (MDA) levels, hemolysate reduced glutathione levels, and erythrocytic superoxide dismutase enzyme activities) imparted by diclofenac alone. In some cases, few tested antioxidants in combination with diclofenac resulted in increased plasma levels of interleukin- (IL-) 6 and C-reactive protein (CRP). In conclusion, the results of these studies suggested to us that the added presence of natural antioxidants could be beneficial as standard anti-inflammatory therapeutics for a patient under diclofenac treatment, albeit that these effects do not appear to significantly build upon those that could be obtained from this common anti-inflammatory agent *per se*.

1. Introduction

During the last decade, great advances have been made for understanding the pathophysiology of inflammation and the involvement of reactive oxygen species (ROS) in its pathogenesis. Inflammation is a complex defense mechanism in which leukocytes migrate from the vasculature into damaged tissues to destroy the agents that can potentially cause tissue injury [1]. Millions of people all over the world are suffering from inflammatory disorders, making them use huge amounts of anti-inflammatory agents (i.e., diclofenac, a nonselective cyclooxygenase [COX] inhibitor) for many years in their lives. However, the serious side effects and the induced intolerance of the anti-inflammatory drugs have led to the search for methods in order to decrease their effective doses and improve their safety patterns.

A plethora of evidence has shown that overproduction of ROS occurs at sites of inflammation and this contributes to overall tissue damage. A subsequent oxidative stress (OS) predominates when production of ROS exceeds the capacity of cellular antioxidant defenses to remove these toxic species [2]. Due to their high reactivity, ROS are potentially causing damage to biomolecules such as DNA, lipids, and proteins. Thus, there is increasing interest in examining the potential benefits from providing patients antioxidants, such as α-lipoic acid (α-LA), selenium (Se), Vitamin C (Vit C), Vitamin E (Vit E), and zinc (Zn)-supplements, as "add-ons" to their diclofenac regimen.

Accordingly, the aim of this study was to investigate the ability of several vitamins and elements (commonly taken as food supplements) to reduce any deleterious side effects from diclofenac or to potentially bolster the desired effects from

the drug. While our main interest was to screen the potential efficacy of these vitamins as stand-alone agents in place of diclofenac or as "add-ons" to diclofenac against an induced inflammation *in situ*, we also hoped to begin to define any potential mechanisms of action that these agents might utilize in bringing about the observed effects. This included analyses of effects of each agent alone/in combination with diclofenac on some key cytokines\inflammatory mediators (i.e., interleukin [IL]-6 and C-reactive protein [CRP]) released during an inflammatory event). While we focused on the potential for these agents to reduce any undesired toxicities from the chronic use of diclofenac, we were also mindful that these studies could provide information that would allow for an unintended benefit potentially allowing for a decrease in the effective dose of diclofenac needed by a patient and a subsequent decrease in the risk of development of adverse effects.

2. Materials and Methods

2.1. Animals. Male Sprague Dawley rats (National Research Center, Giza, Egypt) weighing 100–120 g were used in this study. Rats were housed in a room with controlled temperature (18–22°C), humidity (60 ± 10%), and light/dark (12 hr/d) cycles for at least 1 week before being randomized into various experimental groups. Throughout the study, rats were provided *ad libitum* access to standard pellet chow (El-Nasr chemical Co., Cairo, Egypt) and filtered water. All experiments were conducted in accordance with the principles and procedures outlined in the International Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Faculty of Pharmacy Cairo University.

2.2. Drugs. Diclofenac (Diclofenac K; Novartis, Cairo, Egypt) was dissolved in distilled water to a concentration that would assure that a rat would receive *per os* a dose of 5 mg diclofenac/kg body weight. In these studies, the single diclofenac treatment occurred alone or in combination with (i.e., 2 hr after the final of the 7 daily doses) the test antioxidants. α-Lipoic acid (α-LA; EVA Pharma, Giza, Egypt; in normal saline) was delivered by intraperitoneal injection at a dose of 50 mg α-LA/kg [3]. Each daily *per os* delivery of sodium selenite (Sigma, St. Louis, MO; in water) at 2.5 mg sodium selenite/kg [4]; Vit C (ADWIC, Al Qalyubiyah, Egypt; in water) at 1 g/kg [5], Vit E (MP Biomedicals, Bas-Rhin, France; in olive oil) at 300 mg/kg [6], and zinc sulfate (ADWIC; in water) at 25 mg ZnSO4/kg [7] was performed without anesthesia and with a delivery volume of 1 mL/rat. The diclofenac volume (alone or after the final antioxidant dosing) was 1 mL/rat as well.

2.3. Experimental Design. Rats were randomly divided into 12 groups (n = 8/group): a carrageenan control, rats given diclofenac and then carrageenan, rats given Vit C, Zn, α-LA, Vit E, or Se daily for 7 d and then carrageenan, and rats given Vit C, Zn, α-LA, Vit E, or Se for 7 d, followed by diclofenac and then carrageenan. In the combination studies, treatment with diclofenac was always 2 hr after the final antioxidant/vehicle treatment; rats in the carrageenan-only and antioxidant-only groups received saline at this timepoint. The carrageenan injection was performed 2 hr later (i.e., 4 hr after the final daily administration with the test antioxidants (or vehicle)).

2.4. Induction of Inflammation. Rats were injected subcutaneously in the plantar side of their left hind paws with 0.05 mL of a 1% carrageenan solution. Footpad swelling was then assessed (by monitoring changes in pad thickness) using vernier calipers (Mitutoyo, Tokyo, Japan). Paw thickness was measured just before the injection and 1, 2, 3, and 4 hr after injection. Based on preliminary studies that showed that maximum paw thickness increase occurred at ≈3 hr after injection of carrageenan; the values obtained at that timepoint were used here to calculate values of percentage inhibition of paw thickness increase.

2.5. Preparation of Blood Samples. Four hours after the carrageenan injection, 4 mL blood was collected from the retroorbital plexus into heparinized tubes. A 0.5 mL aliquot was immediately transferred to another heparinized tube for erythrocyte separation and for determination of superoxide dismutase (SOD) activity. A 100 μL aliquot of the original blood was hemolyzed for determination of reduced glutathione (GSH) content. The remaining original blood was centrifuged (3000 rpm, 4°C, 15 min) to isolate plasma; this was stored at −20°C for subsequent determinations of levels of malondialdehyde (MDA), C-reactive protein (CRP), and IL-6.

2.6. Erythrocyte Separation. The aliquot spared for determination of SOD activity was centrifuged (3000 rpm, 4°C, 15 min); the precipitated RBC were washed with 3 mL cold saline and then centrifuged again at 3000 rpm for 10 min. After the supernatant was discarded, lysing of the RBC was performed by resuspension of the cells in 1.75 mL ice-cold distilled water and then vigorous shaking; the cells were then left to stand for 15 min at 4°C. The resulting hemolysate was used for determination of SOD activity.

2.7. Determination of Plasma Lipid Peroxides (MDA), CRP, and IL-6, Erythrocyte Superoxide Dismutase (SOD) Activity, and Blood Reduced Glutathione (GSH) Levels. The determination of lipid peroxide levels (as reflected in amounts of measurable MDA) was done as prescribed before [8]. In brief, to 0.2 mL plasma, 1.2 mL of 1% (w/v) *o*-phosphoric acid and 0.4 mL of 0.67% (w/v) thiobarbituric acid were added and mixed, and then the mixture was heated for 45 min in a boiling water bath. After cooling, 1.6 mL *n*-butanol was added and the sample was mixed vigorously. The butanol layer was separated by centrifugation at 3000 rpm for 15 min. The absorbance of the pink product in the butanol fraction was then measured at 535 and 520 nm in a Shimadzu double beam spectrophotometer (UV-150-02); all samples were read against a blank processed in parallel containing 0.2 mL distilled water instead of sample. The difference in

absorbance between the two readings (i.e., $\Delta A_{535-520}$) was taken as a reflection of the level of MDA (nmol/mL) in the sample.

The pyrogallol autoxidation method was adopted for determination of erythrocyte SOD activity [9]. In brief, from the previously separated hemolysate of erythrocytes, 250 μL was mixed vigorously with 0.75 mL chloroform-ethanol mixture (3 : 5 v/v) to precipitate hemoglobin in the sample. In a microcuvette, 1 mL Tris-HCl buffer (pH 8.2) was added to 30 μL of 10 μM pyrogallol solution and 100 μL distilled water; the absorbance at 420 nm was then measured 30 and 90 sec thereafter. The difference in absorbance (ΔA) was used to reflect the rate of pyrogallol autoxidation (in 1 min) and was considered the experiment blank. The same procedure was then carried out using the prepared blood samples or standards containing SOD in the place of distilled water. The reduction or inhibition of rate of autoxidation in 1 min (compared to the blank) was used as an index of the SOD activity. The percentage change in pyrogallol autoxidation was calculated as follows: % change in pyrogallol autoxidation $= 100 - 100 \times (\Delta A_{T \text{ or } S}/\text{min})/\Delta A_B/\text{min}$, where $\Delta A_T/\text{min}$ = change in absorbance of the test sample in 1 min. $\Delta A_S/\text{min}$= change in absorbance of the standard sample in 1 min, and $\Delta A_B/\text{min}$ = change in absorbance of the blank sample in 1 min.

Hemoglobin (Hb) was measured via the cyanomethemoglobin [10]. Hemoglobin in a sample aliquot (20 μL) was converted to cyanomethemoglobin by addition of 5 mL Drabkin's reagent (0.6 mM potassium ferricyanide\0.77 mM potassium cyanide); absorbance of the cyanomethemoglobin was then monitored at 540 nm. From these latter values, the concentration of Hb was calculated as follows: Hb concentration = $A_{\text{sample}} \times 36.77$ (g/dL), where A_{sample} is sample absorbance at 540 nm, and 36.77 is a unitless constant factor. Based on these values, the SOD activities were then reexpressed as (U SOD/g Hb) in blood sample) according to the following equation: SOD activity in blood sample (U/g Hb) = SOD conc. (U/mL)/g Hb in sample.

GSH levels in blood were determined as prescribed before [11]. In brief, 1 mL of hemolysate (0.1 mL original blood + 0.9 mL distilled water) was combined with 1.5 mL of a protein precipitating solution (1.67 g *m*-phosphoric acid, 0.2 g EDTA, and 30 g NaCl in 100 mL distilled water) and then placed at room temperature for 5 min. The sample was then centrifuged (3000 rpm, 15 min) and the resultant supernatant assayed for GSH, that is, 1 mL supernatant (or standard GSH solution) was combined with 4 mL of the phosphate solution and 0.5 mL Ellman's reagent (40 mg 5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB] in 100 mL 1% sodium citrate solution). The absorbance of the resulting yellow solution was then measured at 412 nm (within 5 min) in a spectrophotometer. From the absorbance values of standard GSH solutions, a standard curve was prepared and levels of GSH in each sample were then extrapolated (mg %).

Plasma CRP and IL-6 levels were measured according to the procedures described in IMMULITE CRP and IL-6 assay kits, respectively (Diagnostic Product Corporation (DPC), Los Angeles, CA). The sensitivity of the CRP and IL-6 assay kits—defined as the concentration two standard deviations above the response at zero dose—was ≈1 pg/mL.

Table 1: Effects of diclofenac, α-LA, selenite, Vit C, Vit E, or zinc on paw thickness increase after 3 hr in a rat carrageenan-induced paw edema model.

Groups	Paw edema (thickness increase [cm] after 3 hr)	Inhibition %
Carrageenan (50 μL of 1% solution)	0.26 ± 0.063	
Diclofenac (5 mg/kg, per os)	0.16 ± 0.001[#]	38%
α-LA (50 mg/kg, IP)	0.20 ± 0.047[#a]	23%
Se (2.5 mg/kg, po)	0.21 ± 0.043[#a]	19%
Vit C (1 g/kg, po)	0.21 ± 0.038[#a]	19%
Vit E (300 mg/kg, po)	0.18 ± 0.031[#a]	31%
$ZnSO_4$ (25 mg/kg, po)	0.20 ± 0.044[#a]	23%

Values are means of 8 rats (±SD).
[#]Value significantly different from carrageenan control ($P < 0.05$).
[a]Value significantly different from diclofenac group ($P < 0.05$).

2.8. Statistical Analysis. Values were expressed as mean ± SD. Results were analyzed using one-way analysis of variance (ANOVA) followed by a least significant difference test (LSD) to compare between the different groups. A P value < 0.05 was accepted as significant in these tests. SPSS software (Chicago, IL) was used to carry out all analyses.

3. Results

3.1. Effects of Various Agents on the Rat Paw Edemas. Normal rats injected subcutaneously with *carrageenan* had an average 0.26 cm increase in paw thickness. Administration of diclofenac 2 hr prior to the carrageenan markedly inhibited induced edema by ≈39%; that is, increase in paw thickness was 0.16 cm (Table 1). Daily administration of α-LA, Se, Vit C, Vit E, or Zn for 7 d prior to the injection of carrageenan significantly inhibited the induced paw edema by 23, 19, 19, 31, and 23%, respectively (i.e., thickness values were 0.20, 0.21, 0.21, 0.18, and 0.20 cm, resp.).

Administration of the following treatment combinations, diclofenac + α-LA, diclofenac + Se, diclofenac + Vit C, diclofenac + Vit E, or diclofenac + Zn, prior to injection of carrageenan markedly inhibited induced paw edema by 19, 27, 31, 27, and 46%, respectively, relative to those in hosts that had received no drug treatment (i.e., Group I). Increases in paw thicknesses in these hosts were 0.21, 0.19, 0.18, 0.19, and 0.14 cm, respectively (Table 2). The inhibitory effects imparted by the combinations were significantly no better than the diclofenac alone; only in the case of combination with Zn the inflammation was reduced better than the outcome induced by diclofenac alone.

3.2. Effects of Test Agents on Plasma MDA Levels. Injection of a 1% carrageenan solution into the paws of naive rats induced an oxidative stress reflected as a significant increase

Table 2: Effects of combination treatments of diclofenac and the test antioxidants on paw thickness increase after 3 hr in a rat carrageenan-induced paw edema model.

Groups	Paw edema (thickness increase [cm] after 3 hr)	Inhibition %
Carrageenan (50 μL of 1% solution)	0.26 ± 0.06	
Diclofenac (5 mg/kg, per os)	0.16 ± 0.001$^{\#}$	38%
Diclofenac + α-LA (50 mg/kg, IP)	0.21 ± 0.001$^{\#a}$	19%
Diclofenac + Se (2.5 mg/kg, po)	0.19 ± 0.03$^{\#a}$	27%
Diclofenac + Vit C (1 g/kg, per os)	0.18 ± 0.01$^{\#a}$	31%
Diclofenac + Vit E (300 mg/kg, po)	0.19 ± 0.04$^{\#a}$	27%
Diclofenac + Zn (25 mg/kg, po)	0.14 ± 0.001$^{\#a}$	46%

Values are means of 8 rats (±SD).
$^{\#}$Value is significantly different from carrageenan control ($P < 0.05$).
^{a}Value is significantly different from diclofenac group ($P < 0.05$).

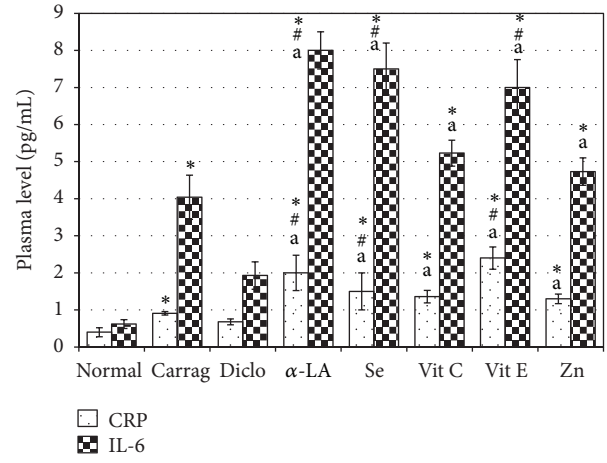

Figure 1: Effect of treatments on plasma CRP and IL-6 levels.All rats were provided daily oral doses of diclofenac (5 mg/kg), selenite (2.5 mg/kg), Vit C (1 g/kg), Vit E (300 mg/kg), or $ZnSO_4$(25 mg/kg); α-LA (50 mg/kg) was administered IP. $^{*\#}$Value is significantly different from normal control and carrageenan-only rats, respectively ($P < 0.05$). ^{a}Value is significantly different from that of diclofenac-treated hosts ($P < 0.05$).

in plasma MDA; values in these rats were 2.77-fold above background (Tables 3 and 4). Rats that received diclofenac 2 hr before the carrageenan injection had a marked decrease in plasma MDA, that is, 56% relative to values in saline-treated carrageenan-injected rats. Daily injection of α-LA for 7 d prior to the carrageenan also caused a significant decrease in plasma MDA levels (47.6%) induced by the carrageenan itself. In rats that received a diclofenac + α-LA combination, the decrease in MDA levels (relative to carrageenan-only rat levels) reached 69.4%. Selenite administration for 7 d prior to induction of inflammation resulted in MDA levels being significantly lower (51.4%) than those in nondrug-treated counterparts; in comparison, the diclofenac + selenite combination significantly decreased MDA levels by just 40.7%. Treatment with Vit C for 7 d before induction of acute inflammation caused a significant 55.9% decrease in MDA levels; the diclofenac + Vit C combination significantly reduced (by 67.8%) MDA levels to values approximating those in normal rats. Vit E given for 7 d prior to inflammation induction also resulted in a significant 67.8% decrease in plasma MDA levels compared to the level in the carrageenan-only rats. Unlike with Vit C, the diclofenac + Vit E treatment was less effective than the antioxidant alone; that is, the change compared to the inflamed rat values was now only 40.0%. Lastly, dosing with Zn for 7 d before injection of carrageenan significantly decreased plasma levels of MDA (43.0%) relative to that in the nondrug-treated hosts; in this case, the additional presence of diclofenac resulted in a 69.5% reduction in MDA values.

3.3. Effects of Test Agents on Hemolysate GSH Levels and Erythrocyte SOD Activity. Significant decreases in hemolysate GSH levels and erythrocyte (RBC) SOD activity (72 and 86%, respectively, were noted in rats that received the carrageenan injection compared to values associated with the blood/cells of naïve [normal] rat) (Tables 3 and 4). Rats that received diclofenac 2 hr before injection of carrageenan had GSH levels and SOD activity that were significantly greater (5.70- and 2.90-fold, resp.) compared to those in the saline-injected inflamed rats. The daily α-LA regimen led to significant increases in the hemolysate GSH levels (2.15-fold versus control) and SOD activity (7.20-fold versus control) as well; the diclofenac + α-LA combination led to significant increases in hemolysate GSH and RBC SOD of 3.95- and 2.44-fold, respectively. Selenite treatments prior to induction of inflammation resulted in significant increases in the GSH (2.17-fold) and SOD activity (4.00-fold) levels; the diclofenac + selenite regimen led to a significant increase in hemolysate GSH (3.99 fold) levels, but the change in RBC SOD activity was insignificant as compared to the values for the carrageenan-only rats. Treatments with Vit C or Vit E for 7 d before induction of inflammation, each caused significant increases in hemolysate GSH (3.00- and 2.43-fold, resp.) and RBC SOD activity (4.75- and 2.60-fold, resp.) compared to values in the rats injected with carrageenan only. The additional presence of diclofenac resulted in significant increases in hemolysate GSH of ≈4- and 2.45-fold for Vit C and Vit E, respectively; RBC SOD activity was increased by ≈4- and 4.28-fold, respectively, as compared to levels in nondrug-treated inflamed rats. Lastly, Zn treatment prior to the carrageenan injection resulted in significant increases in hemolysate GSH levels (≈2-fold) and RBC SOD activity (4-fold) versus those in the carrageenan-only rats (Table 3). The diclofenac + Zn combination also led to significant increases in hemolysate GSH (≈3-fold) and RBC SOD activities (≈5-fold).

3.4. Effects of Test Agents on Plasma CRP and IL-6 Levels. Levels of CRP and IL-6 in the plasma were also significantly increased (by 2.30- and 6.50-fold above naive level, resp.)

Table 3: Effects of diclofenac, α-LA, selenite, Vit C, Vit E, or zinc on plasma levels of MDA, hemolysate GSH, and erythrocyte SOD activity in a rat carrageenan-induced paw edema model.

Groups	MDA (nmol/mL)	GSH (mg %)	SOD (U/g Hb)
Normal (50 μL saline)	0.21 ± 0.038	2.03 ± 0.21	3.95 ± 0.91
Carrageenan (50 μL of 1% solution)	$0.59 \pm 0.06^{*}$	$0.55 \pm 0.26^{*}$	$0.55 \pm 0.21^{*}$
Diclofenac (5 mg/kg, per os)	$0.26 \pm 0.05^{\#}$	$3.16 \pm 0.34^{*\#}$	$1.61 \pm 0.16^{*\#}$
α-LA (50 mg/kg, IP)	$0.31 \pm 0.09^{*\#}$	$1.19 \pm 0.26^{*\#a}$	$3.93 \pm 0.87^{\#a}$
Se (2.5 mg/kg, po)	$0.29 \pm 0.02^{*\#}$	$1.59 \pm 0.12^{*\#a}$	$2.10 \pm 0.44^{*\#a}$
Vit C (1 g/kg, po)	$0.26 \pm 0.03^{\#}$	$1.69 \pm 0.30^{*\#a}$	$2.59 \pm 0.32^{*\#a}$
Vit E (300 mg/kg, po)	$0.19 \pm 0.03^{\#a}$	$1.35 \pm 0.07^{*\#a}$	$1.44 \pm 0.25^{*\#}$
$ZnSO_4$ (25 mg/kg, po)	$0.33 \pm 0.05^{*\#a}$	$1.20 \pm 0.16^{*\#a}$	$2.23 \pm 0.57^{*\#a}$

Values are means of 8 rats (±SD).
*Value is significantly different from normal control ($P < 0.05$).
$^{\#}$Value is significantly different from carrageenan control ($P < 0.05$).
^{a}Value is significantly different from diclofenac group ($P < 0.05$).

Table 4: Effects of combination treatments of diclofenac and the test antioxidants on plasma levels of MDA, hemolysate GSH, and erythrocyte SOD activity in a rat carrageenan-induced paw edema model.

Groups	MDA (nmol/mL)	GSH (mg %)	SOD (U/g Hb)
Normal (0.05 mL, 1% saline)	0.21 ± 0.05	2.03 ± 0.21	3.95 ± 0.91
Carrageenan (50 μL of 1% solution)	$0.59 \pm 0.06^{*}$	$0.55 \pm 0.26^{*}$	$0.55 \pm 0.21^{*}$
Diclofenac (5 mg/kg, po)	$0.26 \pm 0.048^{\#}$	$3.16 \pm 0.34^{*\#}$	$1.61 \pm 0.16^{*\#}$
Diclofenac + α-LA (50 mg/kg, IP)	$0.18 \pm 0.03^{\#a}$	$2.19 \pm 0.18^{\#a}$	$1.33 \pm 0.27^{*\#}$
Diclofenac + Se (2.5 mg/kg, po)	$0.35 \pm 0.08^{*\#a}$	$2.21 \pm 0.17^{*\#a}$	$0.51 \pm 0.28^{*a}$
Diclofenac + Vit C (1 g/kg, po)	$0.19 \pm 0.02^{\#a}$	$2.20 \pm 0.30^{\#a}$	$2.12 \pm 0.55^{*\#a}$
Diclofenac + Vit E (300 mg/kg, po)	$0.36 \pm 0.09^{*\#a}$	$1.36 \pm 0.22^{*\#a}$	$2.33 \pm 0.39^{*\#a}$
Diclofenac + Zn (25 mg/kg, po)	$0.18 \pm 0.03^{\#a}$	$1.62 \pm 0.20^{*\#a}$	$2.65 \pm 0.21^{*\#a}$

Values are means of 8 rats (±SD).
*Value is significantly different from normal control ($P < 0.05$).
$^{\#}$Value is significantly different from carrageenan control ($P < 0.05$).
^{a}Value is significantly different from diclofenac group ($P < 0.05$).

as a result of carrageenan injection (Figures 1 [single agent treatments] and 2 [combination treatments]). Rats that received diclofenac 2 hr before injection of carrageenan had insignificant decreases in the "now-elevated" plasma levels of CRP and IL-6 (25.27 and 52.35% decrease, resp.) noted in the carrageenan-only-treated rats; interestingly, these latter levels were increased 2.30- and 6.50-fold, respectively, above naïve rat values. Daily α-LA injections prior to the carrageenan caused a significant increase in plasma levels of CRP (5.0- and 2.2-fold) and IL-6 (12.90- and 1.98-fold) compared to the levels in naive and carrageenan-only-treated rats, respectively (Figure 1). Rats that received the diclofenac + α-LAcombination also had significant increases in plasma CRP (3.77- and 1.65 fold) and IL-6 (8.30- and 1.28-fold) levels compared, respectively, to levels in naive and carrageenan-only rats (Figure 2). Selenite treatments also caused significant increases in plasma CRP (3.75- and 1.50-fold) and IL-6 (12.00- and 1.86-fold) levels; the diclofenac + selenite combination resulted in slightly greater significant increases in CRP (5.00- and 2.20-fold) and IL-6 (10.50- and 1.60-fold) levels. Vit C administration (alone or in combination with diclofenac) did not significantly affect plasma levels of CRP and IL-6 as compared to the levels in carrageenan-only rats. In contrast, Vit E given for 7 d prior to induction of inflammation resulted in significant increases in plasma CRP (6.00- and 2.60-fold) and IL-6 (12.00- and 1.86-fold) levels relative to levels in normal and carrageenan-only rats, respectively; the diclofenac + Vit E regimen caused slightly lower, albeit still significant, increases in CRP (5.5- and 2.4-fold) and IL-6 (9.7- and 1.5-fold) levels. Administration of Zn (alone or in combination with diclofenac) before the carrageenan did not significantly affect plasma CRP and IL-6 levels relative to those in the inflamed nondrug-treated rats.

4. Discussion

Interest in the relationship between inflammation and oxidative stress has risen in recent years as they share a common role in the etiology of a variety of chronic diseases. Many of these disorders share a common pathophysiological link in terms of chronic low-grade inflammation and overproduction of reactive oxygen and nitrogen species (ROS and RON).

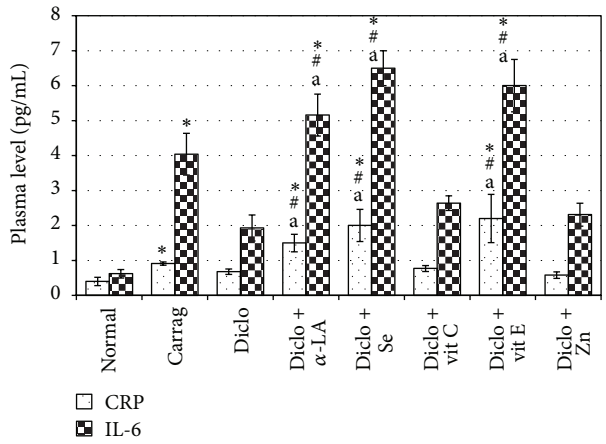

Figure 2: Effect of combinational treatments on plasma CRP and IL-6 levels. All rats were provided daily oral doses of diclofenac in combination with selenite, Vit C, Vit E, or $ZnSO_4$; α-LA (50 mg/kg) was administered IP. Doses were the same as outlined in Figure 1 legend. $^{*\#}$Value is significantly different from normal control and carrageenan-only rats, respectively ($P < 0.05$). ^{a}Value is significantly different from that of diclofenac-treated hosts ($P < 0.05$).

Based on this, studies were initiated to investigate the efficacy of several antioxidants to potentially reduce inflammation and cytokine mediators that occur in a model of acute inflammation (i.e., carrageenan-induced rat paw edema) [2].

In the study here, treating the acutely inflamed rats with diclofenac caused a significant inhibition in paw thickness increases, a finding previously noted using an oral dose dissimilar to that here [12]. Another study also showed that diclofenac was effective in reducing paw edema in both irradiated and nonirradiated rats using celecoxib [13]. The assessment here of the anti-inflammatory efficacy of antioxidants α-LA, Se, Vit C, Vit E, and Zn revealed a significant inhibition in paw thickness increases to different extents, that is, Vit E- > α-LA, Zn > Se\Vit C. In all cases, these agents (alone or in combination with diclofenac) yielded changes in thickness increases significantly no better than the diclofenac alone; only in the case of combination with Zn the inflammation was reduced better than the outcome induced by diclofenac alone. This finding gave us a pause as to the potential significance of use of these antioxidants as *adjunctive* therapeutics. However, on their own, these agents did induce significant reductions in paw thickness; as such, our interest in their potential use as *stand-alone* anti-inflammatory agents remained intact.

Our findings with the antioxidants alone were in accordance with the study that investigated the anti-inflammatory effects of α-LA in carrageenan-paw edema model [3] and another study, which showed that administration of Vit C reduced expected increases in hind paw inflammatory edema in a model of adjuvant-induced arthritis in rats [5]. Abou-Mohamed et al. concluded that ZnSO4 supplementation reduced carrageenan-paw edema in rats as well [7].

An association between oxidative stress and inflammatory responses induced by carrageenan in our study was reflected in significant increases in plasma levels of MDA and decreases in both hemolysate GSH levels and RBC SOD activities. Such outcomes are similar to those reported by other studies [3, 14]. Another evidence indicated that diclofenac induced amelioration of elevated plasma MDA levels and increased the activity of total plasma SOD in a rat adjuvant arthritis model [15]. However, Khayyal et al. presented contrasting results; that is, diclofenac failed to alter plasma levels of MDA or affect GSH levels or SOD activity; it has been suggested that this contrasting outcome may be attributed to the small dose of diclofenac that these investigators used [13]. The antioxidant efficacy of the studied agents in terms of reduction of elevated MDA levels and elevation in both hemolysate GSH and erythrocyte SOD activity reiterates the findings of several previous studies [16–18]. Nevertheless, though the adjunctive therapy of the test agent with diclofenac failed to present any significant increased efficacy against changes in paw thickness edema measure, the findings still proved a significant promising in regard to antioxidant activity.

In general, plasma CRP levels correlate with severity of inflammatory disease or tissue injury [19]. In vitro studies have shown that control of this response is primarily regulated by IL-6 [20]. However, Jones et al. showed that the relationship between IL-6 and CRP is more complex than previously thought, since IL-6R shedding in response to CRP likely contributes to the formation of an agonistic sIL-6R/IL-6 complex [21]. Thus, CRP not only acts as an acute phase reactant but it also may have a profound effect on distal IL-6–mediated events that occur during the inflammatory process. Indeed, CRP levels in several diseases have been found to correlate with those of sIL-6R [22].

The consensus that the increase in plasma CRP and IL-6 levels correlates with severity of inflammatory disease was confirmed in the current study. Another study demonstrated an increase in IL-6 levels in blood 3 hr after injection of carrageenan in a model of carrageenan-induced hyperalgesia. Such outcomes are suggestive of a possibility that circulating IL-6 could act as a messenger of information from peripheral inflammatory sites to the CNS [23]. Furthermore, another study demonstrated that IL-6 serum levels were significantly increased at 24 hr following edema induction, but not after 3 hr, in a model of carrageenan-induced rat paw edema [24]. However, contradictory results indicated that LPS-induced inflammatory paw edema in rats, but not the type induced by carrageenan, resulted in measurable levels of IL-6 in serum within 3 hr of induction [25].

Oral administration of diclofenac here lowered the elevated plasma levels of CRP and IL-6, but the changes were not significant. Similarly, other investigators noted that circulating IL-6 levels remained unaffected after intra-arterial or peritoneal injection of diclofenac [26]. Moreover, investigating the modulatory effects of diclofenac on IL-6 and prostaglandin (PG) levels showed that diclofenac significantly decreased PGE2 production but had no significant effect on IL-6 levels [27]. As the anti-inflammatory effect of diclofenac was reflected only in the inhibition of the paw thickness increase as compared to that in the untreated inflamed rats but not in the decreases in plasma IL-6, it could be concluded that the cytokine inhibition does not

completely explain the efficacy of cyclooxygenase (COX) inhibitors (like diclofenac) in downregulating acute inflammation (i.e., suggesting another mechanism independent on COX inhibition). This observation appears to confirm earlier observations [28, 29].

To our best understanding, the study here was the first attempt to test the additive anti-inflammatory impact on the studied test agents on diclofenac through assessment of CRP and IL-6. Interestingly, α-LA, Vit E, sodium selenite, and their combinations with diclofenac (contrary to with diclofenac monotherapy) caused significant increases in plasma levels of IL-6 and CRP as compared to levels measured in normal rats as well as in carrageenan-only-treated rats. Such immunostimulatory effects of these antioxidants, in particular, Vit E, have been demonstrated previously [30, 31].

A plethora of evidence indicates that CRP also performs anti-inflammatory functions *in situ* [32–37]. Moreover, it has been documented that IL-6 exhibits two contrasting features; it acts as a proinflammatory cytokine in models of chronic inflammatory diseases, that is, collagen-induced arthritis, murine colitis, or experimental autoimmune encephalomyelitis [38]. In contrast, in models of acute inflammation, IL-6 exhibits an anti-inflammatory profile [39]. It has also been reported that IL-6 is involved in T-cell activation and represents an essential competence factor that synergizes with IL-1 to control initial steps of T-cell activation, including induction of IL-2 and enhancement of responsiveness to IL-2 [40, 41]. As IL-2 production is dependent on the release of IL-6, and (of the agents tested here) Vit E supplementation increased IL-2 plasma levels [30, 31], this could explain the current interesting finding that Vit E caused a significant rise in plasma IL-6 levels in our inflamed hosts.

In conclusion, the results of the present study (when taken in the context of the above-noted studies) showed that the immunostimulatory effects of antioxidants, namely, α-LA, Vit E, and selenite, might be related to an induced release of IL-6 and subsequent induction of CRP release. The current results showed that the administration of other antioxidants, namely, Vit C, Zn, and their combinations with diclofenac, did not significantly affect plasma IL-6 and CRP levels. It is worth mentioning that the administration of diclofenac and α-LA, Se, and Vit E caused significant increases in plasma CRP and IL-6 levels as compared to values seen in untreated carrageenan-only injected rats. However, these levels were lower than those caused by administration of the each antioxidant individually.

In summary, the combination of diclofenac and any of the anti-inflammatory agents tested here appears to preserve any immunomodulating effect of the antioxidant alone. Thus, we conclude that the addition of antioxidants to any treatment regimen using this particular drug could have potential beneficial effects for the patient under treatment, albeit that it is not one that builds upon the effects from the diclofenac *per se*.

The presented findings are complementary to the conclusion drawn from the previously published systematic review of Canter et al. on evidenced randomized clinical trials (RCTs) for the effectiveness of the antioxidant Vitamins A, C, E, or selenium or their combination in the treatment of arthritis. Clinical trials testing the efficacy of Vitamin E in the treatment of arthritis have been methodologically weak and have produced contradictory findings. There is presently no convincing evidence that selenium, Vitamin A, Vitamin C, or the combination product selenium ACE are effective in the treatment of any type of arthritis [42].

Conflict of Interests

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

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Research Article

Detection of Impaired Cognitive Function in Rat with Hepatosteatosis Model and Improving Effect of GLP-1 Analogs (Exenatide) on Cognitive Function in Hepatosteatosis

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The aims of the study were to evaluate (1) detection of cognitive function changing in rat with hepatosteatosis model and (2) evaluate the effect of GLP-1 analog (exenatide) on cognitive function in hepatosteatosis. In the study group, 30% fructose was given in nutrition water to perform hepatosteatosis for 8 weeks to 18 male rats. Six male rats were chosen as control group and had normal nutrition. Fructose nutrition group were stratified into 3 groups. In first group ($n = 6$), intracerebroventricular (ICV) infusion of exenatide ($n = 6$) was given. ICV infusion of NaCl ($n = 6$) was given to second group. And also, the third group had no treatment. And also, rats were evaluated for passive avoidance learning (PAL) and liver histopathology. Mean levels of latency time were statistically significantly decreased in rats with hepatosteatosis than those of normal rats ($P < 0.00001$). However, mean level of latency time in rats with hepatosteatosis treated with ICV exenatide was statistically significantly increased than that of rats treated with ICV NaCl ($P < 0.001$). Memory performance falls off in rats with hepatosteatosis feeding on fructose (decreased latency time). However, GLP-1 ameliorates cognitive functions (increased latency time) in rats with hepatosteatosis and releated metabolic syndrome.

1. Introduction

The metabolic syndrome (MetS), a disease arising from the worldwide epidemic of obesity, is manifested as severe insulin resistance, hyperlipidaemia, hepatic steatosis, and diabetes [1]. And also MetS has been shown to predispose cognitive impairment [2–4]. Recent studies addressing the association of MetS with cognitive performance and risk for dementia report mixed results [5–7]. Potential explanatory models include impaired vascular reactivity, neuroinflammation, oxidative stress, and abnormal brain lipid metabolism [8]. In a study, Su et al. [9] suggested that rats with the metabolic syndrome have ineffective inflammation-resolving mechanisms that represent plausible reasons for the exaggerated and persistent postoperative cognitive decline. And also inflammation induces deficits in neurotransmitter-releasing neurons such as cholinergic, noradrenergic, and serotonergic neurons [10–12]. In previous studies [13–15], glucagon-like peptide-1 (GLP-1) and its receptor (GLP-1R) modulated neuronal activity and protected against neuronal damage induced by various insults in the brain. And they were involved in learning and memory. However, there were not many studies related to investigating the showing impaired cognitive function in rat with hepatosteatosis and GLP-1 effect on cognitive function. Therefore, the aims of the study were to evaluate (1) detection of cognitive function

changing in rat with hepatosteatosis model and (2) evaluate the effect of GLP-1 analog (exenatide) on cognitive function in hepatosteatosis.

2. Materials and Methods

2.1. Animals. In this study 24 male Sprague Dawley albino mature rats at 8 weeks, weighing 200–220 g, were used. Animals were fed ad libitum and housed in pairs in steel cages having a temperature-controlled environment (22 ± 2°C) with 12 h light/dark cycles. The experimental procedures were approved by the Committee for Animal Research of Ege University. All animal studies are strictly conformed to the animal experiment guidelines of the Committee for Human Care.

2.2. Experimental Protocol. In the study group, 30% fructose was given in nutrition water to perform hepatosteatosis for 8 weeks to 18 male rats. Six male rats were chosen as control group and had normal nutrition.

Fructose nutrition group were stratified into 3 groups. In the first group ($n = 6$), intracerebroventricular (ICV) infusion of exenatide ($n = 6$) was given. ICV infusion of NaCl ($n = 6$) was given to the second group. And also the third group had no treatment. ICV injection was performed under anesthetized. Rats were deeply anesthetized by the mixture of ketamine hydrochloride (40 mg/kg, Alfamine, Ege Vet, Alfasan International B.V., Holland) and xylazine hydrochloric (4 mg/kg, Alfazyne, Ege Vet, Alfasan International B.V., Holland), i.p., and placed in a stereotaxic frame (Figure 1). Exenatide 10 μg/kg (Byetta, Lilly) was infused 10 μL into the left lateral ventricle (AP = −0.8 mm; ML = ±1.6 mm; DV = −4.2 mm) with a 28-gauge Hamilton syringe in. Sham-operated rats received vehicle (10 μL isotonic NaCl). The needle was left in place for an additional 2 min for complete diffusion of the drug. All animals were given penicillin intraperitoneally to prevent postsurgical infection. After surgery, rats were weighed regularly and monitored daily for behavior and health conditions.

After 5-day recovery phase, passive avoidance task evaluating the memory was performed to study and control groups. This passive avoidance learning (PAL) is a one trial fear-motivated avoidance task in which the rat learns to refrain from stepping through a door to an apparently safer but previously punished dark compartment. The PAL box which is equal to the size of dark and light sections used. This box has a grid system which performs electric shock in dark compartment. Normally, rats when placed in the light compartment prefer to enter dark compartment. After 10 sec of habituation, the guillotine door separating the light and dark chambers was opened. When the rat passed to dark compartment, door between the light and dark compartments was closed. Then, 1.5 mA electric shock was applied for 3 seconds and the rat was removed from the dark chamber and returned to its home cage. After 24 hours, rats were placed in the same mechanism. The time (latency) to switch from light compartment to dark compartment of rats was recorded but foot shock was not delivered, and the latency time was recorded up to a maximum of 300 sec. The latency to refrain from crossing into the punished compartment serves as an index of the ability to avoid, and allows memory to be assessed. Then, the animals were euthanized and hepatectomy was performed for histopathological examination.

2.3. Histopathological Examination of Liver. For histological and immunohistochemical studies, all animals were anesthetized by an i.p. of ketamine (40 mg/kg)/xylazine (4 mg/kg) and perfused with 200 mL of 4% formaldehyde in 0.1 M phosphate-buffer saline (PBS). Formalin-fixed liver sections (4 μm) were stained with hematoxylin-eosin (H&E). All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope.

Morphological analysis was assessed by computerized image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc., USA) on ten microscopic fields per section examined at a magnification of ×40, with the observer blind to the study group. The liver pathologic findings were scored as follows [16] steatosis (the percentage of liver cells containing fat). The total liver pathology score was calculated by adding the scores from each parameter:

1+, less than 25% of cells containing fat;

2+, 26% to 50%;

3 + 51% to 75%; and

4+, more than 75%;

inflammation and necrosis: one focus/lobul 1+;

Two or more foci/lobule 2+.

2.4. Statistical Analysis. Statistical evaluation was performed using SPSS version 15.0 for Windows. The groups of parametric variables were compared using the Student's t-test and analysis of variance. Also, the groups of nonparametric variables were compared using the Mann-Whitney U test. In addition, the Shapiro-Wilk test was used for parametric-nonparametric differentiation. Results are presented as mean + SEM. A $P < 0.05$ was accepted as statistically significant.

3. Results

Macrovesicular hepatosteatosis was shown in rat who had received fructose. It was found that latency time was statistically significantly decreased in rat with hepatosteatosis ($P < 0.00001$) (Figure 2).

Mean levels of latency time in rats with hepatosteatosis treated with ICV GLP-1 analog (exenatide) was statistically significantly increased (memory improvement) than those of rats with hepatosteatosis treated with ICV isotonic NaCl ($P < 0.001$) (Table 1).

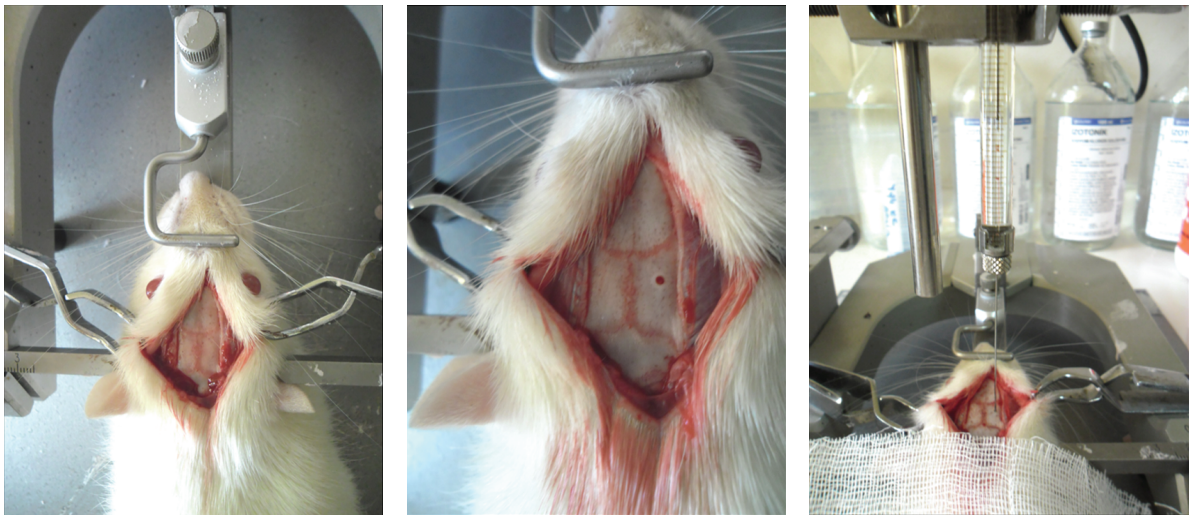
Mean levels of histopatologic score of liver were not significantly statistically different in rat with hepatosteatosis treated with ICV NaCl or ICV exenatide or anything ($P > 0.05$).

Mean levels of latency time were statistically significantly decreased in rats with hepatosteatosis than those of normal

Table 1: Mean levels of latency time and histopathologic score were shown in study and control groups.

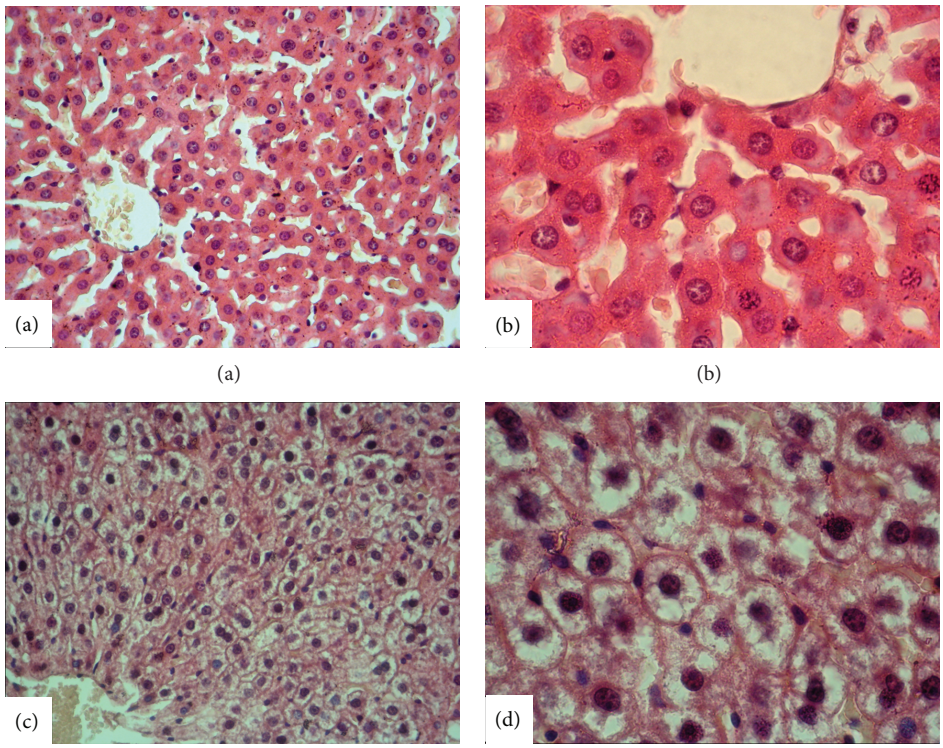
	Normal	Rat with hepatosteatosis (no treatment)	Rat with hepatosteatosis and ICV NaCl	Rat with hepatosteatosis and ICV exenatide
Latency time (sec)	161.5 ± 14.9	16.3 ± 1.8*	14.1 ± 0.9*	73.8 ± 6.4#
Histopathologic score	1.14 ± 0.12	5.38 ± 0.35**	5.41 ± 0.52	5.26 ± 0.48

Results were presented as mean ± SEM. *$P < 0.00001$, **$P < 0.05$ different from normal groups; #$P < 0.001$ different from rat with hepatosteatosis (no treatment) and rat with hepatosteatosis given ICV NaCl.



(a) (b) (c)

Figure 1: Stereotaxical infusion protocol was shown in rat.



(a) (b)

(c) (d)

Figure 2: Liver histopathology ((a)-(b)): normal group liver, H&E (×40 and ×100 magnification), and ((c)-(d)): hepatosteatosis group liver (macrovesicular steatosis in liver cytoplasm).

rats ($P < 0.00001$). However, mean level of latency time in rats with hepatosteatosis treated with ICV exenatide was statistically significantly increased than that of rats with hepatosteatosis treated with ICV NaCl ($P < 0.001$).

4. Discussion

Overconsumption of sugar-sweetened beverages promotes the development of overweight and is associated with metabolic disturbances, including intrahepatic fat accumulation and metabolic syndrome [17]. In the present study, macrovesicular hepatosteatosis was found in rat that received fructose overconsumption. And also it was found that cognitive performance (decreased latency time) has been impaired in rat with hepatosteatosis compared to normal rat. However, cognitive functions were improved with GLP-1 analog such as exenatide treatment.

Diet can affect brain plasticity and cognitive functions such as learning and memory [18]. A high fructose diet produces large increases in plasma triglyceride concentrations and liver mass [19]. In addition to increases in visceral adipose tissue [20–22] and liver mass [23, 24] with high fructose diet in both male and female rats, behavioral status can change. In previous studies [25–27], acute injections of fructose have affected the cognitive functions in animals. Likewise, Ross et al. [28] reported that a high fructose diet impaired spatial water maze memory in male rats. Likewise, in the present study, mean levels of latency time were statistically significantly decreased in rats with hepatosteatosis than those of normal rats. However, Bruggeman et al. [29] suggested that the metabolism of fructose and the effects of a high fructose diet on learning and memory might be sex dependent.

GLP-1, an endogenous 30-amino-acid peptide produced in enteroendocrine cells of intestine, stimulates glucose-dependent insulin secretion [30, 31]. And also GLP-1 and GLP-1R have been considered a therapeutic target in neurodegenerative and cognitive disorders throughout the central and peripheral nervous systems [14, 15, 32, 33]. In a previous study, Oka et al. [14] indicated that GLP-1 receptors existed in the hippocampus and are involved in modulating hippocampal activity through an increase in the release of excitatory amino acid transmitters. Likewise, Perry et al. [32] reported that GLP-1R has been considered a therapeutic target in neurodegenerative and cognitive disorders throughout the central and peripheral nervous systems. And also GLP-1 has been reported to cross the blood-brain barrier (BBB) and facilitate insulin signaling [34, 35]; exenatide, a GLP-1 receptor agonist, could enhance neuronal progenitor proliferation in the brain of diabetic mouse [35]. Recently, Chen et al. [36] suggested that GLP-1 receptor agonist can protect neurons from diabetes-associated glucose metabolic dysregulation insults in vitro and from ICV streptozotocin insult in vivo. In another study, Gault et al. [37] showed that GLP-1 receptor agonist therapy improved cognitive function and ameliorated impaired hippocampal synaptic plasticity in dietary-induced obesity. In the present study, we found that mean levels of latency time in rats treated with ICV exenatide were increased than those of rats treated with ICV NaCl. To our findings, exenatide treatment improved learning and memory performance in rats with hepatosteatosis and metabolic syndrome. So, it may be a candidate for alleviation of memory and cognitive dysfunctions in metabolic disorders.

In summary, memory performance falls off in rats with hepatosteatosis feeding with fructose (decreased latency time). However, GLP-1 ameliorates cognitive functions (increased latency time) in rats with hepatosteatosis and related metabolic syndrome.

Conflict of Interests

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

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Research Article

Evaluation of Lung and Bronchoalveolar Lavage Fluid Oxidative Stress Indices for Assessing the Preventing Effects of Safranal on Respiratory Distress in Diabetic Rats

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We investigated the effects of antioxidant activity of safranal, a constituent of *Crocus sativus* L., against lung oxidative damage in diabetic rats. The rats were divided into the following groups of 8 animals each: control, diabetic, and three diabetic + safranal-treated (0.25, 0.50, and 0.75 mg/kg/day) groups. Streptozotocin (STZ) was injected intraperitoneally (i.p.) at a single dose of 60 mg/kg for diabetes induction. Safranal was administered (i.p.) from 3 days after STZ administration to the end of the study. At the end of the 4-week period, malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) contents, activity of superoxide dismutase (SOD), and catalase (CAT) were measured in the bronchoalveolar lavage fluid (BALF) and lung tissue. Safranal in the diabetic groups inhibited the level of MDA and NO in BALF supernatant and lung homogenate. The median effective dose (ED_{50}) values were 0.42, 0.58, and 0.48, 0.71 mg/kg, respectively. Safranal in the diabetic groups increased the level of GSH and the activity of CAT and SOD in BALF supernatant and lung homogenate. The ED_{50} values were 0.25, 0.33, 0.26 in BALF and 0.33, 0.35, 0.46 mg/kg in lung, respectively. Thus, safranal may be effective to prevent lung distress by amelioration oxidative damage in STZ diabetic rats.

1. Introduction

Oxidative stress has been implicated in the major complications of diabetes mellitus (DM), including retinopathy, nephropathy, neuropathy, and accelerated coronary artery disease. Recently, several epidemiological and experimental studies have been reported that DM is an independent risk factor for occurrence respiratory disorders such as asthma [1]. Hyperglycemia due to DM leads to increasing oxidative stress and inflammatory responses [2]. Oxidative stress and inflammatory mediators are responsible mechanisms for induction of the pulmonary distress. The combination of these mechanisms alters the production of the oxidants, causing cellular stress and consequently the structural damage [3]. The complications of diabetes mellitus are the main causes of morbidities and mortalities [4]. However, antidiabetic drugs can not prevent diabetes complications significantly [5]. Therefore, this is necessary to provide drugs with lesser adverse effects and greater benefit to control diabetes and its complications [5]. Nowadays, with attention this issue, ethnobotanical studies that have focused on the protective effects of natural antioxidants have been originated from plants directly or indirectly [6]. Saffron (dried stigmas of *Crocus sativus* L.) is a food additive that used in the traditional medicine for the treatment of numerous diseases including depression, cognitive disorders, seizures, and cancer [7, 8]. Scientific findings have showed that saffron and the important ingredients (safranal and crocin) have antitumor, antigenotoxic, memory and learning enhancing, neuroprotective, analgesic, and anti-inflammatory, anticonvulsant, antianxiety, anti-depressant, antihypertensive and antihyperlipidemic effects [7–10]. Recently, it was reported

that the saffron extract, crocin, and safranal exhibited significant radical scavenging activity and thus antioxidant activity [11, 12].

Considering the antioxidant effects of safranal, this study was designed to evaluate the protective activity of safranal against pulmonary damage due to oxidative stress in streptozotocin- (STZ-) diabetic rats.

2. Materials and Methods

2.1. Chemicals and Reagents. Streptozotocin and safranal were from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade and were obtained from Merck.

2.2. Animals. 45 male rats (2 months; 200 ± 13 g) were bred at the university experimental animal care centre. Animals were maintained under standard environmental conditions and had free access to standard rodent feed and water.

2.3. Study Design. 45 male Wistar albino rats were randomly allotted into five experimental groups, as follows: group 1, control (C; $n = 8$); group 2, diabetic (D; $n = 8$); group 3, diabetic+safranal-treated (0.25 mg/kg/day) (D + S1; $n = 8$); group 4, diabetic + safranal-treated (0.5 mg/kg/day) (D + S2; $n = 8$); and group 5, diabetic+safranal-treated (0.75 mg/kg/day) (D + S3; $n = 8$). Rats were kept in their own cages at constant room temperature (21 ± 2°) under a normal 12 hr light: 12 hr dark cycle with free access to food and water. The animals were housed according to regulation of the Walfare of experimented animals. The study was conducted in Mashhad Medical University Experimental Animal Research Laboratory. Protocols were approved by the Ethical Committee. On the first day of the study, the all above diabetic groups were given STZ in a single intraperitoneal (i.p.) injection at a dose of 60 mg/kg for induction of diabetes. Blood was extracted from the tail vein for glucose analysis 72 hours after streptozotocin injection. The rats with blood glucose levels higher than 250 mg/dL were accepted as diabetic. In the control groups (C), safranal vehicle (i.p.) was administered to the treatment groups from 3 days after STZ administration; the injection continued to the end of the study (for 4 weeks). Blood glucose level and body weights were recorded at weekly intervals. The animals were sacrificed under light anesthesia (diethyl ether) 1 day after the end of the treatment, at which time blood was collected from retro-orbital sinus. Trachea and lungs were removed immediately for preparation lung lavage and lung homogenate.

2.4. Preparation Lung Lavage. Lung lavage was performed by cannulating the trachea and instilling 8.0 mL of cold normal saline with a syringe. The lavage fluid was rinsed in and out three times before collection [13]. The sample was centrifuged (2000 g, 5 min, 4°C) and the supernatant frozen at −70°C until being assayed.

2.5. Preparation Lung Homogenate. Lung homogenate was obtained from the right lung. The tissue was homogenized with KCl in 1 : 10 ratio. The homogenate was centrifuged (9000 ×g, 30 min) and the supernatant was used for measurement of oxidative stress indices.

2.6. Measurement of Reduced Glutathione (GSH). GSH was determined by the method of Ellman (1959). We added 10% of trichloroacetic acid (TCA) to the homogenate then, centrifuged. 1.0 mL of supernatant was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5,5-dithiobisnitrobenzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm [14].

2.7. Measurement of Malondialdehyde (MDA). MDA levels, as an index of lipid peroxidation, were measured in the homogenate. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm. Three mL phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 mL of liver homogenate in a centrifuge tube and the mixture was heated for 45 min in a boiling water bath. After cooling butanol was added the mixture and vortex-mixed for 1 min followed by centrifugation at 20000 rpm for 20 min. The organic layer was transferred to a fresh tube and its absorbance was measured at 532 nm and compared with values obtained from MDA standards. Results are expressed as nmol/mg tissue [15].

2.8. Catalase (CAT). CAT was assayed colorimetrically at 620 nm and expressed as moles of H_2O_2 consumed/min/mg protein as described by Sinha, (1972). The reaction mixture contained phosphate buffer (0.01 M, pH 7.0), tissue homogenate and 2 M H_2O_2. The reaction was stopped by the addition of dichromate acetic acid reagents (5% potassium dichromate and glacial acetic acid were mixed in a ratio of 1 : 3), [16, 17].

2.9. Measurement of Superoxide Dismutase (SOD) Activity. SOD was measured based on inhibition of the formation of amino blue tetrazolium formazan in nicotinamide adenine dinucleotide, phenazine methosulfate and nitroblue tetrazolium (NADH-PMS-NBT) system, according to method of Kakkar et al. (1984). One unit of enzyme activity was expressed as 50% inhibition of NBT reduction [18].

2.10. Measurement of Nitric Oxide (NO). NO level can be determined spectrophotometrically by measuring the accumulation of its stable degradation products, nitrite and nitrate. The serum nitrite level was determined by the Griess reagent according to Hortelano et al., (1995). The Griess reagent, a mixture (1 : 1) of 1% sulfanilamide in 5% phosphoric acid and 0.1% 1-naphtyl ethylenediamine gives a red-violent diazo color in the presence of nitrite. The color intensity was measured at 540 nm. Results were expressed as μmol/l using a NaNO2 calibration graph [19].

2.11. Measurement of Protein Content. Protein content was determined by the method of Lowry and coworkers 1951, using bovine serum albumin (BSA) as a standard [20].

Table 1: GSH, MDA, SOD, CAT, and NO in BALF of control (C), diabetic (D), diabetic + (0.25 mg/kg/day) safranal-treated (D + S1), diabetic + (0.5 mg/kg/day) safranal-treated (D + S2), and diabetic + (0.75 mg/kg/day) safranal-treated (D + S3) rats following 4 weeks of study.

BALF	C	D	D + S1	D + S2	D + S3
MDA (nmol/mg protein)	0.74 ± 0.10	$2.08 \pm 0.12^{***}$	$1.68 \pm 0.10^{***}$	$1.52 \pm 0.15^{**,+}$	$0.88 \pm 0.13^{+++,\#\#,\times}$
GSH (nmol/mg protein)	2.38 ± 0.21	$0.46 \pm 0.10^{***}$	$1.36 \pm 0.12^{**,++}$	$1.82 \pm 0.12^{+++}$	$2.32 \pm 0.17^{+++,\#\#}$
SOD (U/mg protein)	4.70 ± 0.51	$1.54 \pm 0.27^{***}$	$3.26 \pm 0.30^{**,+}$	$4.00 \pm 0.30^{*,+++}$	$4.80 \pm 0.25^{+++,\#}$
CAT (U/mg protein)	2.24 ± 0.20	$0.44 \pm 0.12^{***}$	$1.12 \pm 0.12^{***,+}$	$1.84 \pm 0.13^{+++,\#}$	$2.14 \pm 0.11^{+++,\#\#\#}$
NO (μmol/L)	1.98 ± 0.71	$13.08 \pm 1.08^{***}$	$10.02 \pm 0.71^{***,+}$	$6.80 \pm 0.64^{*,+++,\#}$	$3.00 \pm 0.57^{+++,\#\#\#,\times}$

Each measurement has been done at least in triplicate and the values are the means ± SEM for eight rats in each group.
Statistical significance for the difference between the data of control versus other groups: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
Statistical significance for the difference between the data of diabetes versus treated groups: $^{++}P < 0.01$, $^{++}P < 0.01$, $^{+++}P < 0.001$.
Statistical significance for the difference between the data D + S1 versus D + S2 and D + S3 ($^{\#}P < 0.05$,$^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$).
Statistical significance for the difference between the data between D + S2 versus D + S3 ($^{\times}P < 0.05$).

Table 2: GSH, MDA, SOD, CAT, and NO in lung homogenate of control (C), diabetic (D), diabetic + (0.25 mg/kg/day) safranal-treated (D + S1), diabetic + (0.5 mg/kg/day) safranal-treated (D + S2), and diabetic + (0.75 mg/kg/day) safranal-treated (D + S3) rats following 4 weeks of study.

Lung	C	D	D + S1	D + S2	D + S3
MDA (nmol/mg protein)	4.12 ± 0.86	$14.61 \pm 0.88^{***}$	$11.56 \pm 0.54^{***,+}$	$9.50 \pm 0.48^{*,+++}$	$7.92 \pm 0.36^{+++,\#\#}$
GSH (nmol/mg protein)	21.60 ± 0.92	$13.88 \pm 0.28^{***}$	$17.24 \pm 0.40^{***,+++}$	$18.74 \pm 0.25^{**,+++}$	$21.16 \pm 0.48^{+++,\#\#\#,\times}$
SOD (U/mg protein)	8.40 ± 0.43	$2.00 \pm 0.35^{***}$	$4.00 \pm 0.31^{***,++}$	$5.40 \pm 0.43^{***,+++}$	$7.88 \pm 0.26^{+++,\#\#\#,\times\times}$
CAT (U/mg protein)	6.16 ± 0.35	$2.84 \pm 0.28^{***}$	$4.12 \pm 0.22^{***,+}$	$5.11 \pm 0.26^{+++}$	$5.68 \pm 0.28^{+++,\#\#}$
NO (μmol/L)	19.41 ± 3.88	$86.00 \pm 1.70^{***}$	$71.00 \pm 2.91^{***,+}$	$49.61 \pm 3.32^{***,+++,\#\#\#}$	$30.41 \pm 3.23^{+++,\#\#\#,\times\times}$

Each measurement has been done at least in triplicate and the values are the means ± SEM for eight rats in each group.
Statistical significance for the difference between the data of control versus other groups: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
Statistical significance for the difference between the data of diabetes versus treated groups: $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$.
Statistical significance for the difference between the data D + S1 versus D + S2 and D + S3 ($^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$).
Statistical significance for the difference between the data between D + S2 versus D + S3 ($^{\times}P < 0.05$, $^{\times\times}P < 0.01$).

2.12. Statistical Analysis. The data were expressed as means ± SEM. Statistical analysis was performed by SPSS/16 statistical software for Microsoft Windows, (Professional Statistic). Data were analyzed using one-way analysis of variance (ANOVA). The homogeneity of variance was tested by use of the Levene test. The Tukey honestly significant difference test was used for post hoc wise analysis of the data with homogenous variances, whereas Tamhane's post hoc pair wise analysis of data was used for data sets with nonhomogenous variances. Statistically significant differences in results of morphometric quantifications were determined by the Student's t-test. A 2-tailed P value less than 0.05 was considered statistically significant. Statistical analysis of the results concerning SOD, NO, GSH, and MDA levels were performed by one-way ANOVA followed by the Bonferroni post hoc test for multiple comparisons.

3. Result

STZ injection produced significant oxidative stress in the BALF and lung homogenate of diabetic rats 4 weeks after DM induction which was manifested by increased lipid peroxidation products (MDA) and with decreased GSH compared to control group ($P < 0.001$), (Tables 1 and 2).

Safranal treatment significantly decreased the MDA in the BALF and lung homogenate and also increased in the glutathione in diabetic safranal (0.25, 0.5 and 0.75 mg/kg/day)—treated groups versus the nontreated diabetic group ($P < 0.001$). However, the effect of the lowest concentration of safranal on MDA level in BALF was with similar value as in nontreated diabetic rats. The activity of GSH in BALF of animals has received the high safranal concentration were significantly greater than the low concentration ($P < 0.01$) (Table 1). Our results also showed that GSH activity in lung homogenate of diabetic rats treated with the high concentration was also significantly increased compared with the low ($P < 0.001$) and middle concentrations ($P < 0.05$), (Table 2). Safranal in the diabetic groups increased the level of GSH in BALF supernatant and lung homogenate. The ED_{50} values were 0.25 and 0.33 mg/kg, respectively. In addition, the levels of MDA in BALF of animals that have been administrated the high safranal concentration were significantly lower than the low ($P < 0.05$) and middle concentrations ($P < 0.01$) (Table 1); however, our data indicated that MDA levels in lung homogenate of diabetic rats treated with the high concentration were significantly lower than the low concentration ($P < 0.01$) (Table 2). Safranal in the diabetic groups inhibited the level of MDA in BALF supernatant and lung homogenate. The median effective dose (ED_{50}) values were 0.42 and 0.48 mg/kg, respectively.

There was a decrease in SOD and CAT in the STZ-diabetic group compared with respective control group ($P < 0.001$). The safranal concentrations (0.25, 0.5, and 0.75 mg/kg/day) significantly increased in SOD activity among the

diabetic-treated groups compared with the diabetic group ($P < 0.001$). The CAT activity in diabetic safranal (0.25, 0.5, and 0.75 mg/kg/day)—treated groups, was significantly higher than nondiabetic group ($P < 0.05$, $P < 0.001$). So that, there was not a significant difference between diabetic safranal (0.75 mg/kg/day)—treated groups and control group (Tables 1 and 2).

CAT activity in BALF of animals that have received the high and middle safranal concentrations was significantly greater than that with the low concentration ($P < 0.05$, $P < 0.001$), (Table 1) and that of high concentration inlung homogenate was significantly greater than the low concentration ($P < 0.01$) (Table 2). In addition, the SOD activity of BALF and lung homogenate of animals that have been administrated the high safranal concentration was significantly greater than the low concentration ($P < 0.05$, $P < 0.001$) (Table 1) as well as in lung homogenate was higher than the middle concentration ($P < 0.01$), (Table 2). Safranal in the diabetic groups increased the activity of CAT and SOD in BALF supernatant and lung homogenate. The ED_{50} values were 0.33, 0.26 in BALF and 0.35, 0.46 mg/kg in lung, respectively.

STZ injection produced significant increase of NO compared to control group ($P < 0.001$). The safranal concentrations (0.25. 0.5, and 0.75 mg/kg/day) significantly decrease NO level in BALF and lung homogenate in diabetic-treated groups compared with the nontraeted diabetic group ($P < 0.001$) (Tables 1 and 2). NO levels in BALF and lung homogenate of animals that have received the high safranal concentration were significantly lower than the low and medium concentrations (0.25 and 0.5 mg/kg/day), and those of medium were lower than the low concentration ($P < 0.001$) (Tables 1 and 2). Safranal in the diabetic groups inhibited the level of NO in BALF supernatant and lung homogenate. The ED_{50} values were 0.58 and 0.71 mg/kg, respectively.

4. Discussion

The results of the present study indicate that intraperitoneal injection of safranal significantly ameliorated increased biomarkers of oxidative stress in rats' lung after STZ administration. We observed the significant elevation in GSH, CAT, and SOD with reduction in the MDA and NO, in both BALF and lung of safranal treated diabetic rats compared with non-treated diabetic group. These results are compatible with the findings reported by other investigations using saffron and its active constituents, crocin, and safranal to improve oxidative damage due to STZ and alloxan diabetic rats [21–24]. In the present study, significant decline in GSH level and antioxidant enzymes activity including SOD, CAT in BALF supernatant and in lung homogenates of STZ diabetic rats in relation to the controls group as well as increased MDA and NO indicates that the increase of oxidative stress and possible damages to the lung structure caused by DM. These data are in accordance with the findings of other authors, [25] who demonstrated the increase of the oxidative stress and the decrease of the antioxidant enzyme SOD in the lungs of diabetic rats. An increase in the expression of inducible nitric oxide synthase in the lung tissue of the diabetic animals has been also indicated by these authors. The same finding was demonstrated by another group of authors [26]. However, they used, as an experimental model, alloxan-induced DM in rabbits. One of the factors responsible for pulmonary alterations can be oxidative stress. The mechanism responsible for this development is hyperglycemia, which activates the polyol pathway, increasing the production of sorbitol. This increase results in cellular stress that leads to a decrease in the intracellular antioxidant defenses. It can also result in the concentration of the products of advanced glycosylation, thus altering cell function. However, hyperglycemia can also activate nuclear transcription factors, triggering an increase in the expression of the inflammatory mediators. The combination of these mechanisms alters the production of oxidants, causing cellular stress and consequently the structural damage [27]. Several studies showed that STZ produces imbalance between plasma oxidant and antioxidant content results in the development of DM and its complications. STZ enters the ß cell via the low affinity glucose protein-2 transporter, inducing the selective destruction of the insulin producing islets' ß cells and, in turn, a drastic reduction in insulin production. The cytotoxic effect of STZ could result from the combined action of DNA alkylation [27] and the cytotoxic effects of ROS [28] or the intracellular liberation of NO directly or indirectly through the formation of peroxynitrite [29, 30].

The improvement of variable measurements in the BALF and lung homogenate of STZ-diabetic rats after safranal treatment might suggest a protective influence of safranal against STZ action on lung tissue damage that might be mediated through suppression of oxygen free radicals induced by STZ. Safranal, monoterpene aldehyde, which is the major constituent of the essential oil of saffron showed good antioxidant activity.

Treatment with safranal reversed diabetic effects on lung GSH level and SOD and CAT activity. Treatment with safranal also decreased MDA and NO in lung of diabetic rats. These results indicate that safranal therapy may reverse diabetic oxidative stress in an overall sense.

Safranal induced an increase in cellular GSH content which might enhance the GSH/GSSG ratio and decrease lipid peroxidation, therefore, improve glucose regulation. In addition, SOD is responsible for removal of superoxide radicals and catalase decomposes hydrogen peroxide to water and oxygen; thus, these enzymes may contribute to the modulation of redox state of lung [31]. This observation perfectly agrees with those of Rahbani et al. 2011 who demonstrated hypoglycemic and antioxidant activity of ethanolic saffron in streptozotocin induced diabetic rats [24]. Similarly, Kianbakht and Hajiaghaee 2011 observed that saffron, crocin, and safranal may effectively control glycemia in the alloxan induced diabetes model of the rat [22]. Furthermore, Kianbakht and Mozaffari, in 2009 indicated that saffron, crocin, and safranal may prevent the gastric mucosa damage due to their antioxidant properties by increasing the glutathione levels and diminishing the lipid peroxidation in the rat gastric mucosa. These studies indicated that safranal was a potent

antioxidant and able to protect body organs against certain toxic materials [21].

In conclusion, findings of the present study show that safranal treatment may be effective to prevent lung damage in diabetic rat by modulation oxidative stress. These finding supports the efficacy of safranal as natural antioxidant for diabetes and its complication management.

Conflict of Interests

The authors report no conflict of interest.

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Research Article

A Novel Role of Globular Adiponectin in Treatment with HFD/STZ Induced T2DM Combined with NAFLD Rats

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Aims. To evaluate the effects of globular adiponectin (gAd) on treatment of type 2 diabetic rats combined with NAFLD. *Materials and Methods*. Twenty-one male wistar rats were fed with normal diet (7 rats) or high fat diet (HFD) (14 rats) for 4 weeks, and then HFD-fed rats were injected with streptozotocin (STZ) to induce type 2 diabetes mellitus (T2DM). Half of T2DM rats were randomly injected with gAd intraperitoneally for 7 days. The expressions of adiponectin receptors (adipoR1/R2) in liver and skeletal muscle tissues were detected through western blotting or RT-qPCR, respectively. *Results*. Globular adiponectin alleviated the hepatic steatosis and increased insulin secretion. In liver, both the protein and mRNA expressions of adipoR2 in T2DM group decreased ($P < 0.05$, resp.) in contrast to NC group and increased ($P < 0.05$ and $P < 0.001$, resp.) after gAd treatment. But the protein and mRNA expressions of adipoR1 increased ($P < 0.05$, resp.) in T2DM group and no change was found in the gAd-treated group. In skeletal muscle, the protein and mRNA expressions of adipoR1 and adipoR2 were upregulated in T2DM group and were downregulated after gAd treatment. *Conclusions*. Globular adiponectin could ameliorate the hepatic steatosis and vary the expressions of adiponectin receptors in liver and skeletal muscle by stimulating insulin secretion.

1. Introduction

The occurrence of NAFLD in type 2 diabetes mellitus (T2DM) patients is reported to range from 50% to 75% [1, 2]. NAFLD not only increases the risk of T2DM, but also worsens glycemic control and contributes to the pathogenesis of major chronic complications of diabetes [3]. Presently, there is no consensus on treatment of NAFLD. Most interventions evaluated for the treatment of NAFLD are those commonly used for the treatment of T2DM and they exerted an indirect effect through improvement in insulin resistance (IR).

Adiponectin is a 30 KDa protein, secreted specifically and abundantly in adipose tissue, which has anti-inflammatory, antidiabetic, and antiatherogenic properties [4]. It was confirmed that the level of adiponectin in serum was decreased under conditions of obesity, insulin resistance, NAFLD, and T2DM [5–7]. Accordingly, adiponectin-deficient mice exhibited insulin resistance and diabetes [8]. Conversely, administration of adiponectin included transgenic overexpression of adiponectin or injected recombinant adiponectin prevented development of diabetes and hyperlipidemia [9–11]. Hence, it had been revealed that adiponectin played an important role in the progression of diabetes.

Biological functions of adiponectin depend on not only the serum circulating concentration of hormone but also the expression level and function of its specific receptors (including adipoR1 and adipoR2). AdipoR1 and adipoR2 are ubiquitous; adipoR1 is most preferentially expressed in skeletal muscle and adipoR2 is most preferentially expressed in liver [12]. Some evidences supported that deficiency of adiponectin receptors caused hyperglycemia and hyperinsulinemia [13, 14]. Knockout mice lacking adipoR1 and adipoR2 [15] exhibited loss of the metabolic actions and effects of adiponectin and showed an increased tendency of tissue triglyceride content, inflammation, oxidative stress, insulin resistance, and glucose intolerance. The activation of adipoR1 and adipoR2 resulted in increased hepatic and skeletal

muscle fatty acid oxidation, increased skeletal muscle lactate production, reduced hepatic gluconeogenesis, increased cellular glucose uptake, and inhibition of inflammation and oxidative stress [12]. It indicated that adipoR1 and adipoR2 were the predominant mediators of the metabolic effects of adiponectin and they played an important role in transmitting the signal of adiponectin. Nevertheless, the changes of adiponectin receptors stimulated by various factors in adiponectin signal path were controversial. It seemed that both adpoR1 and adipoR2 were with similar patterns to transmit the signal of adiponectin. But Bjursell et al. [16] showed that adipoR1 and adipoR2 were clearly involved in energy metabolism but had opposing effects. The expressions of adipoR1 and adipoR2 in liver, skeletal muscle, and fat were differentially displayed in genetically modified animal models of obesity and diabetes, such as ob/ob and db/db mice [13, 17].

The globular and full length forms of adiponectin exhibit different affinities for two adiponectin receptor (AdipoR1/R2) isoforms [18] and have been shown to mediate distinct effects [9, 19]. The globular adiponectin had been reported to exert more potent effects [20]. Hence, it is suggesting that the recombination globular adiponectin may have a potential effect in the treatment of T2DM, especially combined with NAFLD.

Therefore, we aim to evaluate the effects of globular adiponectin in treatment of type 2 diabetic combined with NAFLD rats induced by high-fat/STZ and further explore the interaction between gAd and adipoR1/R2 in liver and skeletal muscle.

2. Materials and Methods

2.1. Experimental Model. Twenty-one male adult wistar rats (190–210 grams) supplied by Hayes Lake experimental animals company (Shanghai, China) were acclimatized in communal cages at 25 ± 2°C with a 12 h light and 12 h dark cycle for 1 week with normal diet. Then the rats were randomly divided into three groups, NC group: normal control rats ($n = 7$), T2DM group: type 2 diabetic control rats ($n = 7$), and gAd-treatment group: type 2 diabetes rats treated with gAd ($n = 7$). At the beginning, NC group rats were fed with normal diet, while those of T2DM and gAd-treated group were fed with a high-fat diet (10% fat, 10% carbohydrate, 5% cholesterol, and 75% basis diet) provided by the Animal Experimental Center of Xiamen University.

After 4 weeks, rats of T2DM group and gAd-treated group were intraperitoneally injected with a freshly prepared solution of streptozotocin (STZ; 28 mg/kg, Sigma, St. Louis, MO, USA) in 0.1 M citrate buffer (PH 4.21) to induce type 2 diabetic model compared to those of NC group which were administered with an equal volume of 0.1 M citrate buffer. After STZ injection for 72 h, fourteen rats with random blood glucose level above 16.7 mmol/L were considered as diabetic. Then, seven type 2 diabetic rats were selected randomly into gAd-treated group and were injected intraperitoneally with gAd (BioVision, CA, USA) at a dose of 3.5 ug daily at 9 a.m. for one week, while NC and T2DM group rats received an equal volume of 0.9% saline, respectively. All rats were euthanized at the end of globular adiponectin or 0.9% saline intervention.

All experiments were approved by the Laboratory Animal Care and Use Committee of Xiamen University.

2.2. Biochemical Sampling and Analysis. Blood samples were collected from rat hearts under anesthesia after euthanizing. Plasma insulin concentrations were determined by ELISA using commercial kits (Millipore Corporation, USA). Fasting plasma glucose and triglyceride (TG) were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's directions.

2.3. Liver Histological Evaluation. The liver tissues in formalin solution were fixed overnight in 10% buffered formalin and embedded in paraffin. The severity of hepatic histologic changes was assessed in hematoxylin-eosin (HE) stained samples and blindly scored by two pathologists who were unaware of the treatments for rats. Steatosis, inflammation, and fibrosis were semiquantitatively evaluated according to Guidelines for Managements of Nonalcoholic Fatty Liver Disease [21]. In NAFLD activity score (NAS), (1) steatosis was scored from 0 to 3 based on a four grades scoring system from S0 to S3, S0: no steatosis or less than 5%, S1: 5–33%, S2: 33–66%, and S3: >66%; (2) lobular inflammation was graded as follows, stage 0: no foci, stage 1: <2 foci per 200x field, stage 2: 2–4 foci per 200x field, and stage 3: >4 foci per 200x field; (3) ballooning degeneration of liver cells was evaluated as follows: grade 0: absent, grade 1: few cells and grade 2: many cells. The histological NAS score was defined as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2), thus ranging from 0 to 8. Diagnostic criteria of simple steatosis, borderline NASH, and NASH were based on scores of 0–2, 3-4, and 5 or greater, respectively.

2.4. Western Blotting Analysis. Total protein was extracted from liver and skeletal muscle using Protein Extraction Kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's protocol, respectively. Total protein levels were determined by the bicinchoninic acid (BCA) method (Applygen Technologies Inc., Beijing, China). Equal amounts of protein samples were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to 0.45 um PVDF membranes. Blotted membranes were blocked with 5% skim milk in TBS with 0.1% Tween 20 and incubated at 4°C overnight, respectively, with one of the following primary antibodies: goat anti-rat adipoR1 polyclonal antibody (diluted to 1 : 1000 with TBS with 0.1% Tween 20; Novus Biologicals, Littleton, CO, USA) or goat anti-rat adipoR2 polyclonal antibody (diluted to 1 : 1000 with TBS with 0.1% Tween 20; Novus Biologicals, Littleton, CO, USA). After three washes in TBS with 0.1% Tween 20, the membranes were incubated with 1 : 5,000 secondary HRP-conjugated anti-goat antibody (MultiSciences Biotech Co., Hangzhou, China) at room temperature for 1 h. Membranes were exposed to the ECL system (Applygen Technologies Inc.,

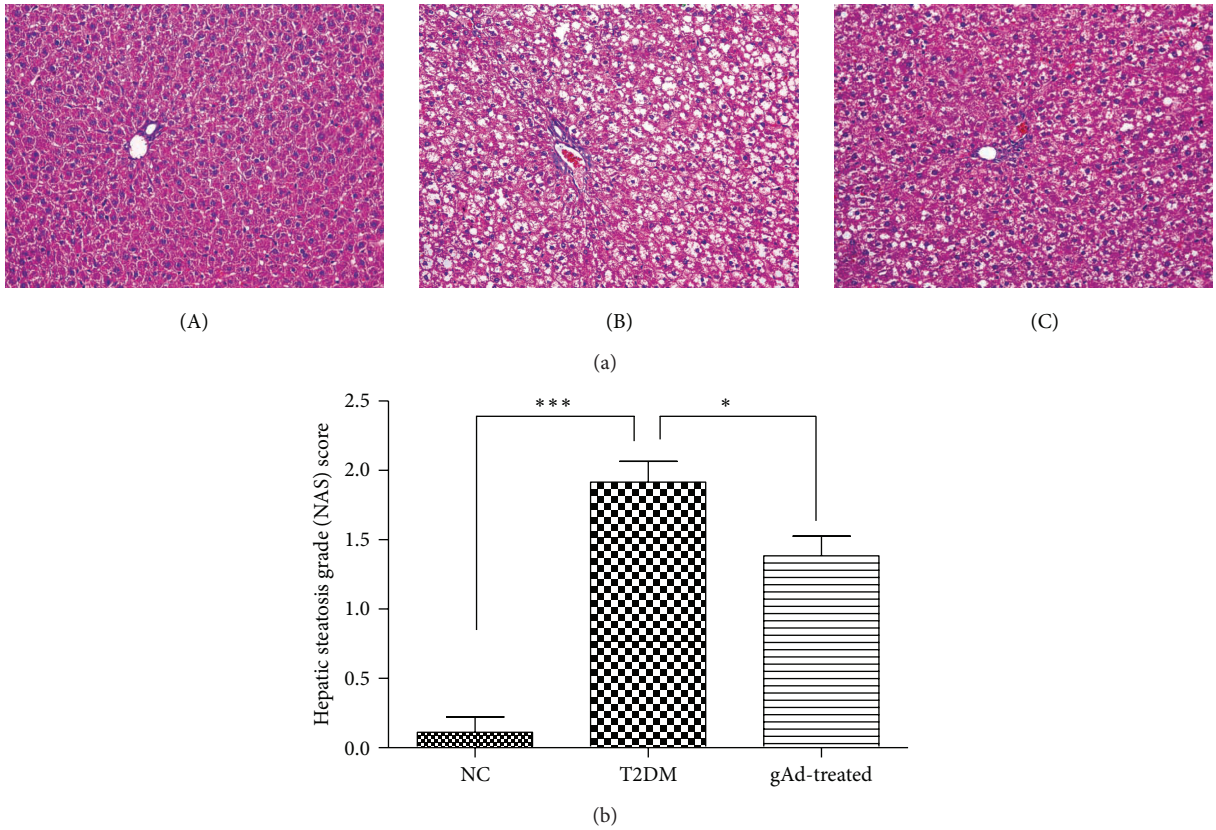

Figure 1: HE staining of liver specimens. (a) HE staining of liver specimens for NC group (A), T2DM group (B), and gAd-treated group (C) (×200). (b) NAFLD activity scores of liver specimens of three groups. $^{*}P < 0.05$, $^{***}P < 0.001$ as compared to T2DM group.

Beijing, China) and the bands were quantified with the use of Adobe Photoshop CS5.0 software (Adobe Company, USA).

2.5. RT-qPCR Analysis. Total RNA was extracted using Trizol reagent (Invitrogen, SanDiego, CA, USA) from liver and skeletal muscle, respectively, according to manufacturer's instructions. After determination of RNA concentrations by measuring the absorbance at 260 nm and 280 nm, 4 μL RNA as template was reversely transcribed to cDNA by using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA). Real-time PCR was performed on 7500 real-time PCR system (ABI Applied Biosystems) using power SYBR Green PCR master Mix. The primer sequences were listed as follows: AdipoR1, forward 5′-GCTGGCCTTTATGCTGCTCG-3′ and reverse 5′-TCTAGGCCGTAACGGAATTC-3′; AdipoR2, forward 5′-CCACAACCTTGCTTCATCTA-3′ and reverse 5′-GATACTGAGGGGTGGCAAAC-3′; β-actin, forward 5′-GTAGCCATCCAGGCTGTGTT-3′ and reverse 5′-AACACAGCCTGGATGGCTAC-3′. The cycling conditions were listed as follows: 10 minutes at 95°C as an initial step, 15 seconds at 95°C, and 1 minute at 60°C for 40 cycles for adipoR1/R2. A melting curve analysis was used to confirm specificity of the PCR product, which was demonstrated as a single peak (data not shown). The expression of β-actin served as the internal control. Every sample was analyzed in triplicate. A comparative Ct method reported preciously was used in data analysis of real-time PCR.

2.6. Statistical Analysis. The variability of results was expressed as mean ± standard deviation. The significance of differences was determined by one-way ANOVA. The difference between two groups was used Student's t-test. A two-tailed P value of <0.05 was considered statistically significant. SPSS 13.0 for Windows (SPSS Software, Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. Liver Pathology. HE staining of liver specimens from T2DM group exhibited steatosis which suggested that the rat model of T2DM was combined with NAFLD. The NAFLD activity score (NAS) of T2DM group was 1.92 ± 0.51, higher than that of NC group (0.11 ± 0.33) ($P < 0.001$). Globular adiponectin treatment made an improvement in the steatosis as compared to T2DM group (1.39 ± 0.51 versus 1.92 ± 0.51, $P < 0.05$) (Figure 1).

3.2. Plasma Insulin Level and Glycolipid Metabolism Analysis. The results of fasting plasma insulin, glucose, and TG were summarized in Table 1. Compared to NC group, fasting plasma insulin level decreased significantly in T2DM group ($P < 0.01$) and it increased in gAd-treated group compared with T2DM group ($P < 0.05$) (Figure 2). The glucose level of T2DM group was higher than NC group ($P < 0.01$), and it was lower in gAd-treated group compared to T2DM group

Table 1: The fasting plasma insulin, glucose, and TG levels of rats ($\overline{x} \pm s$).

	NC group ($n = 7$)	T2DM group ($n = 7$)	gAd-treated group ($n = 7$)
Insulin (mIU/L)	26.2 ± 9.5	12.8 ± 1.8**	18.0 ± 3.2#
Glucose (mmol/L)	7.1 ± 0.8	24.8 ± 1.3**	20.9 ± 1.9##
TG (mmol/L)	1.3 ± 0.1	1.8 ± 0.2**	1.4 ± 0.2#

**$P < 0.01$ compared with NC group, #$P < 0.05$, ##$P < 0.01$ compared with T2DM group.

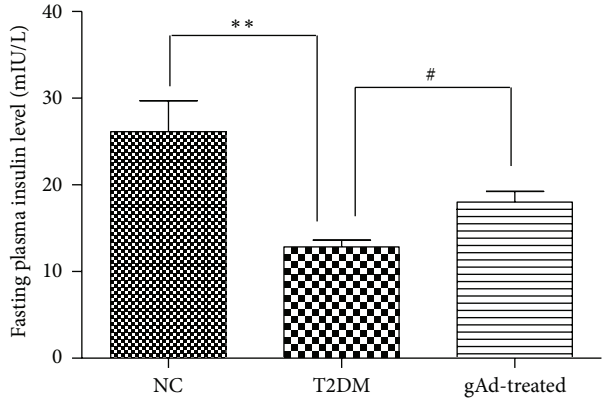

Figure 2: Comparison of plasma insulin levels of all groups.** $P < 0.01$ compared with NC group, # $P < 0.05$ compared with T2DM group.

($P < 0.01$). In addition, the T2DM group had increased level of TG compared with NC group ($P < 0.01$). The concentration of TG in gAd-treated group was lower than T2DM group ($P < 0.05$), which decreased to nearly normal level.

3.3. Effects of gAd on AdipoR1/R2 Protein and mRNA Expressions in Liver. Western blotting analysis in rat liver tissues showed that the expression of adipoR1 increased in T2DM group ($P < 0.05$) and no change was found in gAd-treated group as compared to T2DM group. While the protein expression of adipoR2 in T2DM group decreased significantly in contrast to those of NC group ($P < 0.05$) and increased significantly in gAd-treated group as compared to T2DM group ($P < 0.05$). In addition, the alterations of adipoR1/R2 mRNA expressions were in accord with the alterations of the protein expressions (Figure 3).

3.4. Effects of gAd on AdipoR1/R2 Protein and mRNA Expressions in Skeletal Muscle. Western blotting results in skeletal muscle showed that the protein expression of adipoR1 in T2DM group was higher than NC group ($P < 0.01$). Treatment with globular adiponectin significantly downregulated the expressions of adipoR1 protein as compared to the T2DM group rats ($P < 0.05$). The protein expression of adipoR2 also increased in T2DM group compared with NC group ($P < 0.001$) and decreased in gAd-treated group as compared to T2DM group ($P < 0.001$). The alterations of adipoR1/R2 mRNA expressions were in accordance with the alterations of the protein expressions in skeletal muscle (Figure 4).

4. Discussion

In our study, high-fat diet and low-dose STZ were used to induce the T2DM rat model. It was close to mimicing the natural history and the metabolic characteristics of type 2 diabetes in humans [22]. The rats exhibited hyperglycemia, together with insulin resistance and deficiency which were in accord with the characteristic of T2DM. In addition, progression of hypoinsulinemia can be detected in severe type 2 diabetic patients clinically at the later stage [23]. The occurrence of NAFLD in T2DM patients is reported to range from 50% to 75% [1, 2]. In our study, we found that the NAS of T2DM group was 1.92 ± 0.51, which was diagnosed as simple steatosis (0–2). Therefore, we induced the T2DM rat model characterized with hypoinsulinemia and hyperglycemia, which mimics T2DM at a later stage closely and is combined with NAFLD characterized with simple steatosis.

Adiponectin is secreted specifically and abundantly in adipose tissue and has anti-inflammatory, antidiabetic, and antiatherogenic properties [4]. It exists as full-length or globular fragment, named full-length adiponectin or globular adiponectin, respectively [9]. The globular adiponectin which had been reported exerted more potent effects [20]. As we know, the specific therapy for T2DM combined with NAFLD was so far lacking. In our study, the recombination globular adiponectin was used for the treatment which had been reported to play an important role in T2DM and NAFLD. It showed that treatment with globular adiponectin reduced hyperglycemia and hypertriglyceridemia of the rats induced by HFD/STZ. Globular adiponectin also stimulated insulin secretion in our experiment. The analysis of liver pathology indicated that globular adiponectin alleviated the steatosis even though it was injected for only one week. The NAS of gAd-treated group decreased significantly as compared to T2DM group (1.39 ± 0.51 versus 1.92 ± 0.51, $P < 0.05$). It indicated that gAd had potential effects on the treatment of T2DM combined with NAFLD.

Liver and skeletal muscles are two vital organs maintaining energy homeostasis with respective mechanism. Skeletal muscle has a fundamentally important role in the maintenance of normal glycolipid homeostasis and in regulating whole-body glycolipid metabolism [24, 25]. Hepatic steatosis may also be associated with the changes of glycolipid metabolism in skeletal muscle.

An evidence supported that globular adiponectin had a higher binding affinity to the membrane fractions of skeletal muscles than full-length adiponectin and had more effects on downstream signaling pathways, whereas only full-length adiponectin did so in the liver [26]. Kadowaki and Yamauchi [12] reported that in skeletal muscle, both globular and full-length adiponectin activated AMPK and then stimulated phosphorylation of ACC, fatty-acid oxidation, and glucose uptake. They also activated PPAR-α, thereby stimulating fatty-acid oxidation and decreasing TG content. In the liver, only full-length adiponectin activated AMPK and reduced PEPCK (phosphoenolpyruvate carboxykinase) or

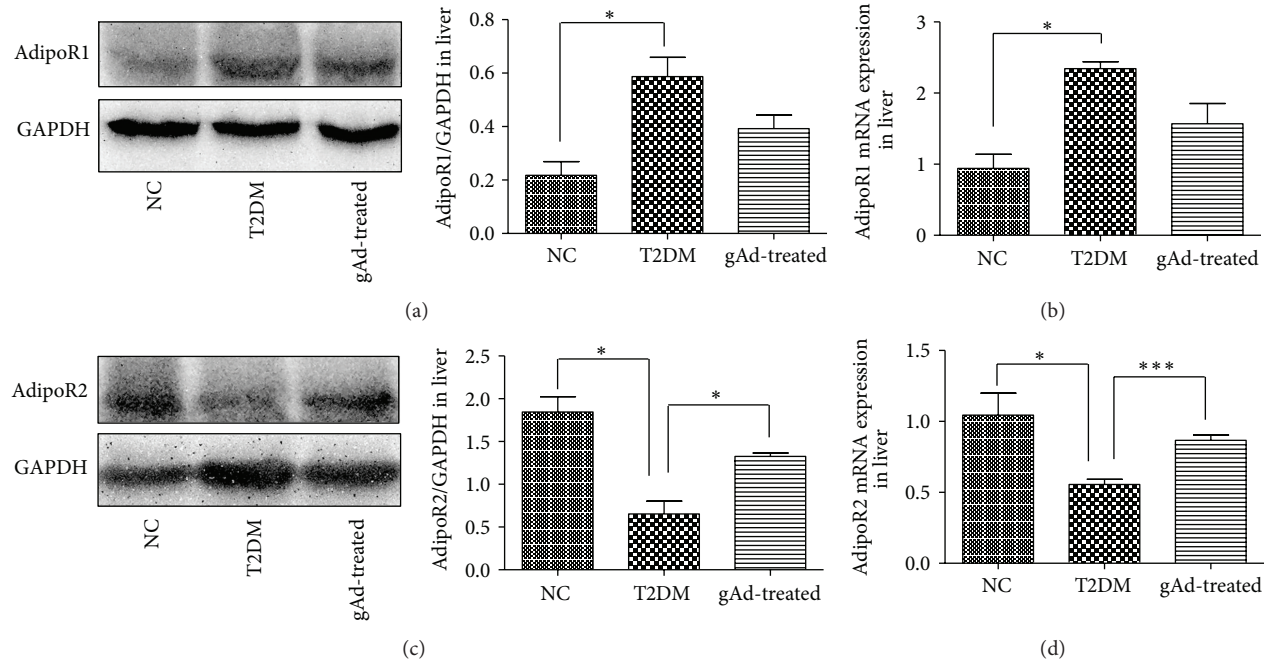

Figure 3: (a) Relative adipoR1 protein expression in liver was determined through western blotting using GAPDH as a reference protein. (b) Relative adipoR1 mRNA expression in rat liver. (c) Relative adipoR2 protein expression in liver was determined through western blotting using GAPDH as a reference protein. (d) Relative adipoR2 mRNA expression in rat liver. $^{*}P < 0.05$, $^{***}P < 0.001$ as compared to T2DM group.

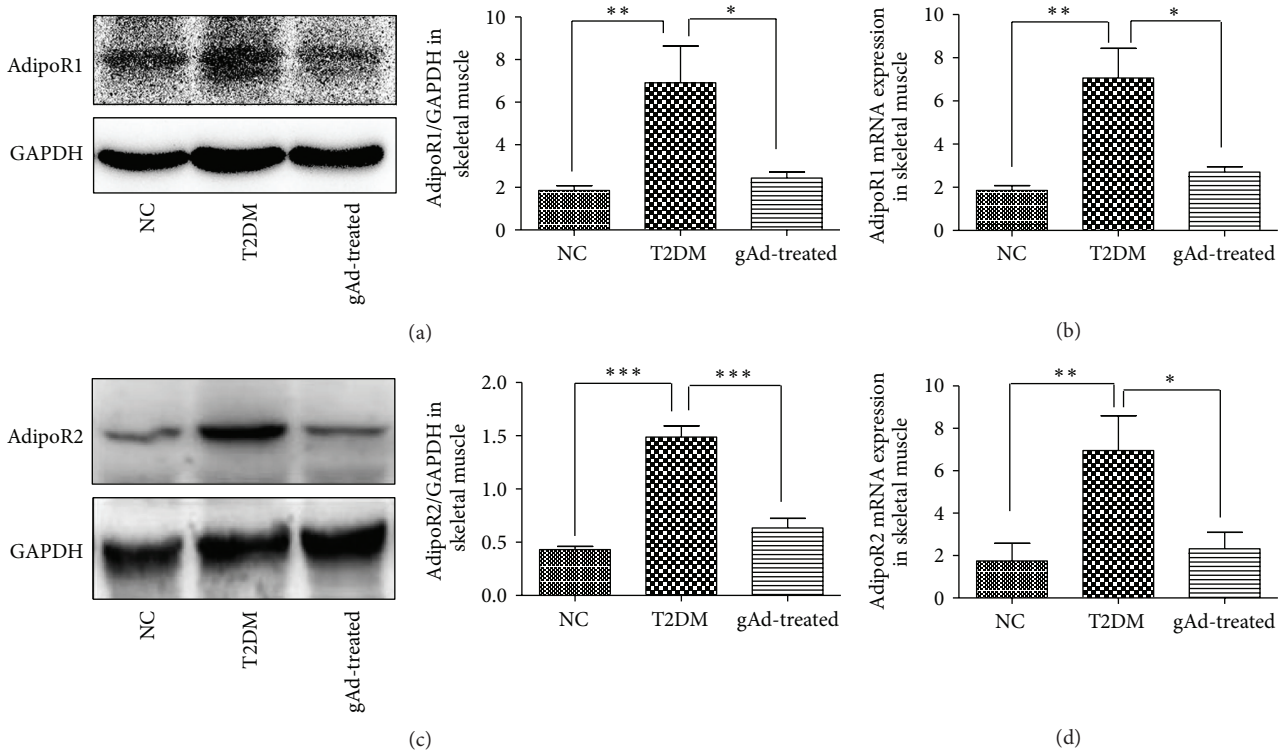

Figure 4: (a) Relative adipoR1 protein expression in skeletal muscle was determined through western blotting using GAPDH as a reference protein. (b) Relative adipoR1 mRNA expression in rat skeletal muscle. (c) Relative adipoR2 protein expression in skeletal muscle was determined through western blotting using GAPDH as a reference protein. (d) Relative adipoR2 mRNA expression in rat skeletal muscle. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to T2DM group.

G6Pase (glucose-6-phosphatase G6Pase) involved in gluconeogenesis and increased fatty-acid oxidation and decreased tissue TG content just as in skeletal muscle [12]. Our results exhibited that treatment with globular adiponectin reduced hyperglycemia and hypertriglyceridemia induced by HFD/STZ and alleviated the hepatic steatosis. Therefore globular adiponectin may alleviate the hepatic steatosis by improving glycolipid metabolism in skeletal muscle.

The biological functions of adiponectin depend not only on the serum circulating concentration but also on the expression levels and functions of its specific receptors (adipoR1/R2). Some evidences indicated that adipoR1 and adipoR2 were the predominant mediators of the metabolic effects of adiponectin and played an important role in transmitting the signal of adiponectin [26, 27]. AdipoR1 is a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin, while adipoR2 is an intermediate-affinity receptor for full-length and globular adiponectin [18]. But the interaction between adiponectin and adipoR1/R2 still remained controversial. Some evidences supported that adpoR1/R2 were with a similar pattern to transmit the signal of adiponectin [13, 17], while Bjursell et al. [16] observed that adipoR1 and adipoR2 were clearly involved in energy metabolism but had opposing effects. We detected the alterations of adipoR1/R2 in liver and skeletal muscle after globular adiponectin intervention. The results showed that the protein and mRNA expressions of adipoR2 in liver decreased in T2DM group which were in accordance with the report of Beylot et al. [28] and adipoR2 was upregulated after gAd treatment. We also found that the protein and mRNA expressions of adipoR1 were increased in T2DM group and no change was found after gAd treatment as compared to T2DM group. It was postulated that adipoR2 expression decreased in liver and there might be a complementary mechanism which caused the overexpression of adipoR1 in T2DM group. But the fact that the expression of adipoR1 did not change in liver after treatment with gAd might be due to the fact that the dominating tissue which globular adiponectin affected was muscle tissue. Yamauchi et al. [18] found that adipoR1 or adipoR2 enhanced both globular and full-length adiponectin binding, which were associated with increase in PPAR-α ligand activity and fatty-acid oxidation. In skeletal muscle, we found that the alterations of adipoR1/R2 differed from the alterations in liver. Both the protein and mRNA expressions of adipoR1 or adipoR2 in T2DM group were higher than NC group and downregulated significantly after globular adiponectin treatment in skeletal muscle. The protein and mRNA expressions of adipoR1/R2 were downregulated after globular adiponectin treatment in skeletal muscle suggesting that it might have a complementary mechanism between liver and skeletal muscle. Some other evidences also supported our results. Inukai et al. [17] reported that adipoR1 mRNA increased in STZ induced diabetic mice, whereas it was reversed by administration of insulin (values for muscle adipoR2 mRNA were not reported). In the vitro experiments of murine C2C12 myotubes cells, Staiger et al. found a trend toward lower adipoR1 mRNA levels with higher insulin concentrations. Staiger et al. [29] also observed that insulin deficiency induced by STZ increased and insulin replenishment reduced the expression of adipoR1/R2 in vivo. Moreover, they found the expressions of adipoR1/R2 in ob/ob mice were significantly decreased in skeletal muscle, which exhibited hyperglycemia and hyperinsulinemia, as compared to control mice [13]. These observations suggested that insulin may negatively regulate the protein and mRNA expressions of adipoR1/R2 in skeletal muscle. In our study, plasma insulin concentration was enhanced after globular adiponectin treatment which was associated with the decreased expressions of adipoR1/R2 in gAd-treated group as compared to T2DM group, suggesting that globular adiponectin may affect the expressions of adipoR1/R2 in skeletal muscle by stimulating insulin secretion.

In conclusion, globular adiponectin varied the expressions of adiponectin receptors in liver and skeletal muscle. the fact that it decreased the expressions of adipoR1/R2 in skeletal muscle might be due to the stimulation of insulin secretion. Globular adiponectin may ameliorate the hepatic steatosis by stimulating insulin secretion and improving glycolipid metabolism in skeletal muscle. Globular adiponectin might be considered as a potential therapy for T2DM combined with NAFLD.

Conflict of Interests

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

Authors' Contribution

Hong Ma and Guo-Ping You contributed equally to this work.

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