Nobiletin Protects Against Diabetes-induced Testicular Injury via Hypophysis-gonadal Axis Up-regulation and Amelioration of Oxidative Stress

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Research Article

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Abstract

Background: Testicular injury is one of the most serious problems of Diabetes mellitus. The present study aims to compare the effect of two different doses of nobiletin and the probable mechanisms against diabetes-induced testicular impairment in rats.

Methods and Results: Streptozotocin injection was used to induce diabetes. Diabetic rats received nobiletin (10 mg/kg) or (25 mg/kg) daily and orally for 30 days. Diabetic rats displayed a significant elevation in glucose, glycosylated hemoglobin (HbA1c), homeostasis model of insulin resistance (HOMA-IR), and pro-inflammatory cytokines. Levels of serum insulin, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were significantly reduced. Histological changes with positive caspase-3 and decreased Androgen receptors (AR) immunoexpressions were observed in diabetic rats. Both doses of nobiletin improved hyperglycemia, reduced pro-inflammatory cytokines, and augmented insulin, testosterone, LH, and FSH levels. Gene and protein manifestation of LH and FSH receptors and cytochrome P450 17 α -hydroxylase (CYP17A1) was markedly down-regulated in testicular tissues of diabetic group, an effect which was markedly increased with both doses of nobiletin. In addition, both doses significantly reduced lipid peroxidation, and caspase-3 immuno-expression and improved the activity of the antioxidant enzymes and AR in testicular tissues of diabetic group.

Conclusion: Both doses showed protective effects against diabetes-induced testicular injury by diminution of oxidative stress, hyperglycemia, inflammation, caspase-3 and up-regulation of the hypophysis-gonadal axis and androgen receptors. The high dose of nobiletin was more effective than the lower dose.

Introduction

Diabetes mellitus, a common and serious metabolic syndrome, is one of the most important health problems affecting millions of people. The worldwide incidence of diabetes mellitus is expected to reach about 642 million by the year of 2040 [1]. Although several investigators chiefly give care to the most prevalent problems of diabetes, like diabetic retinopathy, cardiomyopathy, and nephropathy, investigators are becoming more and more conscious of impaired male reproductive function in diabetic animals and humans [2].

Increasing oxidative stress, in diabetic patients, leads to impaired testicular functions [3] probably by depletion of testosterone levels, spermatogenesis, seminal fluid volume and sperm count leading to male infertility [4]. Furthermore, diabetes-induced oxidative stress can cause apoptosis [5]. Insistent hyperglycemia during diabetes contributes to the creation of advanced glycation end-products (AGEs). Glucose auto-oxidation and AGEs might lead to sexual dysfunction by creating ROS [6]. Thus, variation of hyperglycemia-induced oxidative stress illustrates an important plan for the handling of diabetes and its related testicular dysfunction.

Several studies have shown that natural products cause pancreatic β -cells regeneration and improve the secretion and viability of insulin [7, 8]. Additionally, most of the natural products which contain flavonoids and phenolic compounds have antioxidant property to reduce the oxidative stress associated with diabetes mellitus [9].

Nobiletin, a polymethoxyflavone, is a flavonoid found in peels of citrus fruits [10]. It has many pharmacological properties and shows anti-inflammatory [11], anti-atherogenic [12], anti-diabetic [13], immunomodulatory [14], nephroprotective [15], neuroprotective and antioxidant properties [16]. It has been reported that nobiletin markedly reduces testicular tissue lipid peroxidation and enhances biochemical and sperm parameters, in addition to enhancement in the epididymal functions [17]. Nobiletin was also known to ameliorate the development of type 2 diabetes mellitus by protecting β -cells from apoptosis [18].

Little data has been published regarding the effect of nobiletin on the modulation of hypophysis–gonadal axis dysregulation induced by diabetes. Consequently, this study aimed at assessing the effect of two doses of nobiletin in increasing the gonadotropins production and improving the manifestation of their receptors in testicular tissues of diabetic rats. Furthermore, the effects of nobiletin on cytochrome P450 17 α -hydroxylase (CYP17A1) manifestation, hyperglycemia induced oxidative stress, histological changes, and immunohistochemical manifestation of androgen receptors (AR) and caspase-3 in testicular tissues of type 2 diabetic rats have been investigated.

Materials And Methods

Chemicals

Streptozotocin (STZ), nicotinamide, nobiletin, dimethyl sulfoxide (DMSO), pyrogallol, reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), and 1,1,3,3-tetramethoxypropane were obtained from Sigma–Aldrich (USA). The other chemicals were of analytical grade and were purchased from standard commercial stores.

Animals

Adult male albino rats (130–150 g) were purchased from the National Institute of Ophthalmology, El-Giza, Egypt. This study was approved by Institutional Animal Care and Use Committee, Beni-Suef University (BSU-IACUC (No. 020-116)). The rats were kept in standard aired cages at 12 /12 hour light/dark cycle and $25 \pm 2^\circ\text{C}$. Rats were fed with standard pellet diet and allowed drinking water *ad libitum*. The animals received human care according to the guidelines of the Research Ethics Committee of Beni-Suef University and those developed by applicable international rules [19].

Diabetes induction

Experimental type 2 diabetes mellitus was promoted in overnight-fasted animals via intraperitoneal injection of nicotinamide (110 mg/kg body weight) liquified in a physiological saline 15 minutes before intraperitoneal injection of recently prepared STZ (50 mg/kg body weight) liquified in 0.1 mol/L sodium citrate buffer (pH 4.5) [20]. The control rats were administered equal volume of sodium citrate buffer. After six hours from intraperitoneal injection of STZ, animals were given 10% glucose solution for 24 hours to avoid lethal hypoglycemic influence of pancreatic insulin discharge. One week after STZ injection, hyperglycemia was tested according to blood glucose levels. Animals with blood glucose levels ≥ 250 mg/dl were chosen for the experiment.

Experimental plan

One week after STZ injection, normal and diabetic rats were randomly selected and divided into five equal groups (with 6 rats/each) as follows:

Group I (Control): rats were given only DMSO for 30 days.

Group II (25 mg/kg nobiletin): normal rats were orally supplied with 25 mg/kg nobiletin, dissolved in DMSO, for 30 days.

Group III (Diabetic rats).

Group IV (Diabetic+10 mg/kg nobiletin): diabetic rats treated with 10 mg/kg body weight nobiletin by oral intubation for 30 days.

Group V (Diabetic + 25 mg/ kg nobiletin): diabetic rats treated with 25 mg/kg body weight nobiletin by oral intubation for 30 days.

The doses of nobiletin were selected according to Parkar et al. [21].

Collection and preparation of samples

Twenty-four hours after the last treatment, two blood samples were taken from each rat. The first sample was taken into a tube with an anticoagulant for glycosylated hemoglobin (HbA1c) assessment. The other sample was left at room temperature to coagulate then centrifuged at 3000 rpm for 15 minutes to obtain serum. Obtained sera were kept at -20°C till use. After decapitation, testes were rapidly removed then washed using cold saline. One testis from each animal was homogenized in cold phosphate buffered saline (10% w/v) by a Teflon homogenizer (Glas-Col, Terre Haute, Indiana, USA). The homogenate was centrifuged at 3000 rpm for 10 minutes then the clear supernatant was kept at -20°C . The second testis

was divided into two parts one of them used for histological and immunohistochemical study and the other part reserved frozen at -80°C for RNA isolation and Western blotting.

Biochemical analyses

Estimation of fasting and postprandial glucose levels

One day before sacrifice, blood samples were taken from lateral caudal vein of overnight fasted control and diabetic rats. Other blood samples were then obtained at 120 minutes after the supplementation of 3 gm/kg body weight glucose solution. The concentration of serum glucose was estimated according to the technique of Trinder [22], via reagent kits obtained from bioMerieux chemicals (France).

Estimation of insulin, interleukin-1 β (IL-1 β), tumor necrosis factor alpha (TNF- α), and HbA1c%

Serum insulin, IL-1 β , and TNF- α were estimated by precise enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D systems (USA) following the producer's directives. HbA1c% was assessed according to the technique of Abraham et al. [23] via a reagent kit obtained from Stanbio Company (Texas, USA).

Assessment of insulin resistance

Since estimation of glucose or insulin levels can imprecisely determine abnormalities in insulin action [24]. Thus, insulin resistance was assessed by estimation of homeostasis model of insulin resistance (HOMA-IR) according to Haffner [25] and quantitative insulin-sensitivity check index (QUICKI) according to Perseghin et al. [26] as follows:

$$\text{HOMA-IR} = \text{Fasting insulin } (\mu\text{U/ mL}) \times \text{Fasting glucose (mmol /L)} / 22.5$$

$$\text{QUICKI} = 1 / \log \text{fasting insulin } (\mu\text{U/ mL}) + \log \text{fasting glucose (mmol /L)}$$

An elevation in HOMA-IR and a diminution in QUICKI values reveal insulin resistance.

Determination of Serum Testosterone, LH and FSH Levels

Serum levels of testosterone, LH and FSH were estimated with ELISA by precise commercial rat ELISA analytic kits (ALPCO Diagnostics) as stated in the company protocol.

Oxidative stress and antioxidant enzymes assays

Testicular antioxidant/oxidative stress indicators were measured using their own supernatants following well-known methods. Thiobarbituric acid reactive substance (TBARS) level was estimated using the method of Preuss et al. [27]. Glutathione (GSH) content was estimated as described by Beutler et al. [28]. Superoxide dismutase (SOD) activity was estimated according to the method described by Marklund and Marklund [29]. Glutathione-S-transferase (GST) was assessed as stated by Mannervik and Gutenber [30].

RNA isolation and quantitative reverse transcription – polymerase chain reaction (qRT–PCR)

Gene expression investigation of LH-receptor (LHR), FSH-receptor (FSHR), and CYP17A1 in the testicular samples was implemented. Whole RNA was isolated from frozen testes by TRIzol reagent (Invitrogen) and treated with RNase-free Dnase (Invitrogen). Purified RNA was assessed at 260 nm. RNA samples with A260/A280 ratios ≥ 1.7 were chosen for reverse transcription. Furthermore, RNA integrity was guaranteed by formaldehyde-containing agarose gel electrophoresis. Reverse transcription was done with 5 μ g RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas). cDNA was augmented by SYBR Green master mix (Fermentas) in a total volume of 25 μ L via the primer set itemized in Table 1. The Polymerase chain reaction (PCR) comprised initial denaturation at 95 °C for 3 minutes, 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, and a final step at 60 °C increased about 0.5 °C every 10 s up to 95 °C. Melting curve analysis was done to test the particularity of the used primers. Each test comprised a distilled water control. Finally, the augmentation data were evaluated following the $2^{-\Delta\Delta CT}$ technique [31] and the values were regularized to β -actin.

Table 1

Primer pairs for quantitative RT-PCR

Gene	GenBank accession number	Sequence (5'-3')
LHR	NM_012978.1	F: GGCACACCATCACCTATGCT
		R: AAAAGAGCCATCCTCCGAGC
FSHR	NM_199237.1	F: ATTCTTGGGCACGGGATCTG
		R: CGGTCGGAATCTCTGTCACC
CYP17A1	NM_012753.2	F: AGACCTGTCCACGCCTATCT
		R: GGCATCCACGATACCCTCAG
β -actin	NM_031144.3	F: AGGAGTACGATGAGTCCGGC
		R: CGCAGCTCAGTAACAGTCCG

Western blot

Testes samples reserved at -80°C were used to examine the influence of nobiletin on the manifestation levels of LHR, FSHR, CYP17A1, and β -actin as a loading control [32]. Briefly, testes specimens were homogenized in RIPA lysis buffer containing proteinase inhibitors, centrifuged and the protein concentration was measured in the clear supernatant by Bradford reagent. 30 mg proteins were separated on SDS-PAGE, transmitted to nitrocellulose membranes and blocked in 5% skimmed milk liquified in TBS Tween20 (TBST). The membranes were incubated with primary antibodies against LHR, FSHR, CYP17A1, and β -actin. After washing with TBST, the membranes were probed with the matching secondary antibodies and developed by enhanced chemiluminescence reagent (BIORAD, USA). The intensity of the band was quantified using Image J and normalized to β -actin

Histological, immunohistochemical and morphometric studies

Haematoxylin and eosin staining

Testicular specimens were fixed for 24 hour in neutral buffered formalin (10%). The specimens were routinely handled, embedded in paraffin wax and sectioned by a microtome at thickness of 4–5 μm then stained with hematoxylin and eosin (H&E) [33] to investigate testicular morphology.

Immunohistochemical determination of androgen receptors and caspase-3 protein

Five μm sections were prepared from the paraffin embedded testis then mounted on positively charged glass slides for immunohistochemical detection of androgen receptors (AR) and caspase-3. The sections were dewaxed, rehydrated then autoclaved for 10 minutes at 120°C in citrate buffer (10 mM at pH 6). After washing with phosphate buffered saline (PBS), endogenous peroxidase was blocked by 0.3% H_2O_2 in methanol (15 minutes). Slides were washed by using PBS and blocking was done by blocking buffer then incubated at room temperature (30 minutes). Primary monoclonal and polyclonal antibodies for AR (Catalogue Number MA1-150, Thermo Fisher Scientific Co., USA) [34] and caspase-3 (mouse monoclonal antibody (Lab Vision, Fremont, CA USA) [35], were added after dilution by using PBS ($2\mu\text{g}/\text{ml}$ and 1:1000, respectively), then incubated for 30 minutes. The slides were washed with PBS three times (3 minutes each). Biotinylated polyvalent secondary antibody (Cat. No. 32230, Thermo Scientific Co., UK) was added to the sections and co-incubated (30 minutes). The slides were washed three times (3 minutes each) by washing buffer. The reaction was established by applying the working solution of Metal Enhanced DAB substrate to the tissue then incubated (10 minutes). The slides were washed two times (3 minutes each) with washing buffer. Hematoxylin was used as counterstain to cover the entire tissue surface [36]. The negative control for each section was prepared without using primary antibody.

Morphometric study

Morphometric measurements were done by using computer-based image analysis software

(Leica Qwin 500, Cambridge, UK). Measurements of ten non over-lapping fields for each animal at $\times 100$ were used to determine the area percentage of AR and caspase-3 immunoreactivity and $\times 400$ to determine the number of Leydig cells/ $100\mu\text{m}^2$.

Statistical Analysis

The Statistical analysis was done using SPSS (version 16). Values were represented as mean $\pm$ standard deviations (SD). Comparisons between means were made by one-way ANOVA test followed by Tukey's test post hoc analysis. Values of ($P \leq 0.05$) were considered significant.

Results

Nobiletin suppresses hyperglycemia in diabetic rats

Diabetic rats showed a significant ($p \leq 0.001$) rise in the fasting and postprandial blood glucose levels comparing to control rats. Rats treated with both nobiletin doses showed a significant decrease ($p \leq 0.001$) in the blood glucose levels as compared to the diabetic group (Fig. 1a). Moreover, diabetic rats displayed a significant elevation ($P \leq 0.001$) in HbA1c% in comparison to that of control rats. Treatment of diabetic rats with both doses of nobiletin markedly ($P \leq 0.05$) improved blood HbA1c% (Fig. 1b).

Nobiletin ameliorates serum insulin release and insulin sensitivity in diabetic rats

Data represented in Table 2 display the impact of both doses of nobiletin on insulin release and insulin sensitivity indices (HOMA-IR and QUICKI) in normal and diabetic animals. Serum insulin level was markedly ($P \leq 0.001$) declined in the diabetic group compared to the control rats. Treatment of diabetic rats with the low and high dose of nobiletin markedly ($P \leq 0.05$) and ($P \leq 0.001$) increased insulin level, respectively.

Diabetic rats displayed a significant elevation in HOMA-IR ($P \leq 0.01$) and reduction in QUICKI ($P \leq 0.05$). High dose of nobiletin administration showed a marked amelioration in insulin sensitivity as manifested by decreasing ($P \leq 0.01$) HOMA-IR and increasing ($P \leq 0.05$) QUICKI.

Table 2

Effect of both doses of nobiletin on insulin levels, HOMA-IR, and QUICKI in normal and diabetic rats.

	Insulin ($\mu\text{U/ml}$)	HOMA-IR	QUICKI
Control	15.56 $\pm$ 0.75	4.38 $\pm$ 0.57	0.50 $\pm$ 0.015
N25	15.23 $\pm$ 0.89	4.39 $\pm$ 0.74	0.50 $\pm$ 0.019
Diabetic	8.26 $\pm$ 0.60 ^c	6.84 $\pm$ 1.11 ^b	0.45 $\pm$ 0.014 ^a
Diabetic+N10	10.71 $\pm$ 0.77 ^{cd}	4.76 $\pm$ 0.31 ^d	0.49 $\pm$ 0.006
Diabetic+N25	12.93 $\pm$ 0.61 ^{bfg}	4.42 $\pm$ 0.36 ^e	0.50 $\pm$ 0.008 ^d
Homeostasis model of insulin resistance (HOMA-IR) Quantitative insulin-sensitivity check index (QUICKI) a ($P \leq 0.05$), b ($P \leq 0.01$), c ($P \leq 0.001$) vs Control d ($P \leq 0.05$), e ($P \leq 0.01$), f ($P \leq 0.001$) vs Diabetic g ($P \leq 0.05$) vs Diabetic+N10			

Nobiletin decreases pro-inflammatory cytokine production in diabetic rats

To test the impact of nobiletin on hyperglycemia induced-inflammation, serum levels of the pro-inflammatory cytokines, TNF- α and IL-1 β were determined (Figs. 2a,b). Serum TNF- α level of diabetic rats revealed a significant increase ($P \leq 0.001$) in comparison to the control group. Supplementation of either 10 mg or 25 mg nobiletin showed a significant decrease ($P \leq 0.001$) in serum TNF- α level of diabetic rats. Likewise, IL-1 β was significantly increased ($P \leq 0.001$) in the serum of diabetic rats in comparison to the control group and significantly decreased ($P \leq 0.001$) after treatment with both doses in comparison to the diabetic group.

Nobiletin up-regulates the hypophysis–gonadal axis in the diabetic rats

To assess if the hypophysis–testes axis is modified by nobiletin in the diabetic animals, serum hormonal levels of LH, FSH, and testosterone were figured out using the ELISA technique (Figs. 3a,b,c). Additionally, testicular LHR, FSHR, and CYP17A1 mRNA, in addition to their protein manifestation levels were quantified by RT–PCR and Western blot technique (Figs. 4 a-f). Serum levels of LH, FSH and testosterone in diabetic rats were significantly decreased ($P \leq 0.001$) in comparison to the control group. Administration of the low dose of nobiletin for 30 days significantly increased serum levels of LH ($P \leq 0.01$) and FSH ($P \leq 0.05$) in diabetic rats but non significantly increased testosterone serum level . High nobiletin dose produced a significant elevation of LH, FSH ($P \leq 0.001$), and testosterone ($P \leq 0.05$) in diabetic animals. The abundance of LHR and FSHR mRNA in the testes of diabetic rats revealed a significant down-regulation ($P \leq 0.001$) in comparison to the control group. Oral administration of either low or high dose of nobiletin revealed a significant ($P \leq 0.001$) up-regulation of LHR and FSHR mRNA manifestation in the testes of diabetic rats (Figs. 4a,b). CYP17A1 showed a similar manifestation pattern. It was significantly down-regulated ($P \leq 0.001$) in the testes of diabetic rats (Fig. 4c). Low and high doses of nobiletin significantly ($P \leq 0.01$) and ($P \leq 0.001$) increased CYP17A1 mRNA manifestation in diabetic rats, respectively.

Data from Western blotting (Figs. 4d,e,f) revealed a significant ($P \leq 0.001$) reduction in protein expression levels of LHR, FSHR, and CYP17A1 in testes of diabetic rats. Administration of either 10 mg/kg nobiletin or 25 mg/kg nobiletin showed a significant ($P \leq 0.001$) elevation in protein manifestation levels of LHR and FSHR in testicular tissues of the diabetic rats. Similarly, administration of low ($P \leq 0.01$) and high ($P \leq 0.001$) doses of nobiletin significantly up-regulated CYP17A1 protein expression.

Normal animals treated with nobiletin revealed nonsignificant changes ($P \geq 0.05$) in serum hormones, testicular gene and protein manifestation levels of LHR, FSHR, and CYP17A1.

Nobiletin suppresses oxidative stress in diabetic rats

The effect of nobiletin on testicular lipid peroxidation and antioxidant enzymes activity under normal and diabetic conditions is shown in Fig.5. Diabetic rats showed a significant ($P \leq 0.001$) increase in testicular

MDA levels as compared to the control group. Treatment of diabetic animals with either low or high dose of nobiletin significantly ($P \leq 0.001$) decreased MDA content (Fig. 5a). In contrast, testicular GSH content (Fig. 5b) of diabetic rats was significantly decreased ($P \leq 0.001$). Supplementation of the high dose of nobiletin significantly ($P \leq 0.05$) increased testicular GSH content in diabetic rats. Supportive of the GSH data, the antioxidant activity of SOD (Fig. 5c) and GST (Fig. 5d) enzymes were significantly ($P \leq 0.001$) decreased in testes of diabetic animals. After administration of both doses of nobiletin, the activity of SOD was significantly ($P \leq 0.01$) increased. GST activity increased significantly with low ($P \leq 0.05$) and high ($P \leq 0.001$) nobiletin doses.

Nobiletin ameliorates the histological changes in the testes of diabetic rats

Light microscopic investigation of testicular sections of the control group showed closely arranged seminiferous tubules with normal histological architecture lined by spermatogenic epithelium at various phases of development and Sertoli cells between them (Fig. 6a). Spermatogenic cells comprised spermatogonia, primary spermatocytes, spermatids and spermatozoa. Spermatogonia are small rounded cells that rest on intact and regular basement membrane (Fig. 6b). The largest cells are the primary spermatocytes that have large rounded nuclei (Fig. 6b). Spermatids look like small round cells with rounded nuclei and their site towards the lumen (Fig. 6b). Spermatozoa appeared with intensely stained heads and detected near the lumen of the seminiferous tubules (Fig. 6b). Sertoli cells seemed between the spermatogenic cells as tall cells with triangular nuclei (Fig. 6b). External to the basement membrane of the seminiferous tubules; smooth muscle-like cells were found, which called myoid cells (Fig. 6b). The interstitial tissue, in between the seminiferous tubules, contained blood vessels and Leydig cells that arranged into small clumps with spherical or polyhedral shape and have vesicular nuclei (Figs. 6a,b). Histological investigation of testicular sections from nobiletin group showed the same histological structures. The negative control testicular sections are represented for them.

Histological investigation of testicular sections from the diabetic group showed greatly affected tissues (Figs. 6c,d,e,f). Most seminiferous tubules appeared with a lumen containing few or no spermatozoa (Fig. 6c). The seminiferous tubules were separated by wide interstitial spaces containing congested blood vessels, edema and few numbers of Leydig cells (Fig. 6c). In few seminiferous tubules, the spermatogenic cells increased in number and seemed as a solid mass occupying the tubular lumen (Fig. 6d). Most seminiferous tubules appeared with irregular outlines and separated from their basement membranes (Figs. 6e, f). Many seminiferous tubules had few layers of spermatogenic cells with vacuolated cytoplasm (Figs. 6e,f). The spermatogenic cells appeared with pyknotic exfoliated nuclei (Fig. 6f).

Histological investigation of testicular sections from low nobiletin dose-treated diabetic group showed that many seminiferous tubules looked regular, lined by layers of spermatogenic cells and contain many sperms in their lumina whereas, few tubules contained degenerated spermatogenic cells (Figs. 7a,b).

The testicular sections of diabetic rats treated with the high dose of nobiletin displayed a great enhancement (Figs. 7c,d). The seminiferous tubules and their germinal epithelium seemed to be similar to those of the control rats. They seemed with a regular and intact basement membrane, spermatogenic cells and more sperm flagella are present in their lumina.

There was a significant reduction ($P \leq 0.001$) in the mean number of Leydig cells of the diabetic group (Fig. 7e) as compared to that of the control group. The mean number of Leydig cells was markedly increased ($P \leq 0.001$) in the diabetic group supplemented with either the low or high dose of nobiletin (Fig. 7e) as compared with that of the diabetic group.

Nobiletin upregulates androgen receptors and downregulates caspase-3 immunoexpression in diabetic testes

Testicular sections from control and control nobiletin groups, stained with anti-androgen, displayed a positive immunoreaction in the Leydig cells (Figs. 8a,b). Weak immunoreaction was found in the Leydig cells of diabetic rats (Fig. 8c). Moderate androgen receptor immunoreaction was observed in Leydig cells of diabetic rats treated with the low dose of nobiletin (Fig. 8d), while strong positive androgen receptor immunoreaction in Leydig cells of diabetic rats treated with the high dose of nobiletin (Fig. 8e) was observed.

There was a significant reduction ($P \leq 0.001$) in the AR immunopositive reaction in the diabetic group (Fig. 8f) compared with that of the control group. The AR immunoreaction was significantly ($P \leq 0.001$) increased in the diabetic group supplemented with either the low or high dose of nobiletin (Fig. 8f) as compared with that of the diabetic group.

Negative nuclear caspase-3 immunoreaction was observed in cells lining the seminiferous tubules of control and control nobiletin testicular sections (Figs. 9a,b). Strong positive caspase-3 immunoreaction was observed in cells lining the testicular tubules of diabetic rats (Fig. 9c). Moderate positive caspase-3 immunoreaction was observed in the cells lining the testicular tubules of diabetic rats treated with low nobiletin dose (Fig. 9d), while few caspase-3 immunoreactive cells lining the testicular tubules of diabetic rats treated with high nobiletin dose (Fig. 9e) were observed.

There was a significant ($P \leq 0.001$) increase in the caspase-3 immunopositive reaction in the diabetic group (Fig. 9f) as compared to the control group. The caspase-3 immunoreaction was significantly decreased ($P \leq 0.001$) in the diabetic group supplemented with either the low or high dose of nobiletin (Fig. 9f) as compared to that of the diabetic group.

Discussion

Diabetes mellitus has been reported to affect the male reproductive system negatively. Also, there are obvious effects of oxidative stress on testicular tissues of diabetic rats [37]. Type 2 diabetes mellitus is characterized by a continually high blood glucose level due to insulin resistance and insulin deficiency [38]. Therefore, the treatment that can enhance glycemic control and reduce testicular oxidative stress is a hopeful plan for the inhibition of reproductive disorders related to diabetes. The current study aimed to examine the effect of two doses of nobiletin on testicular tissue damage, oxidative stress and hypophysis–gonadal axis dysregulation induced by hyperglycemia in Type 2 diabetes mellitus rat model.

Insulin deficiency and hyperglycemia increase testicular impairment through ROS-induced DNA damage, reduction of sperm count, and delay of the process of spermatogenesis [39], leading to male sterility. In the present study, diabetic animals revealed hyperglycemia joined with reduced serum insulin concentration. The diabetic state was established from the augmented HbA1c%. Measuring HbA1c concentration is a good indicator for the diagnosis and prediction of diabetic problems [40]. Moreover, the insulin sensitivity indices (HOMA-IR and QUICKI) proposed that insulin resistance has been established, as formerly stated [41].

Decreased levels of FSH, LH, and testosterone hormones in diabetic rats of the current study indicate testicular damage as observed by the histological studies. Diabetes has been stated to deteriorate the FSH and LH levels which seriously affect androgen biosynthesis [42]. Hyperglycemia augments resistance of the testis to these hormones, causing low testosterone levels [43]. In the current study, FSH and LH resistance-induced by diabetes was established by FSHR and LHR down regulation in testicular cells of the diabetic rats. Consequently, CYP17A, a main steroidogenic enzyme in testosterone formation, was similarly down regulated, elucidating the decreased testosterone levels. Former studies established a negative link among serum testosterone and HbA1c levels [44]. Moreover, decreased testosterone level is related to an increased hazard of insulin resistance and type 2 diabetes mellitus in healthy males [45]. So, insulin sensitizing agents could increase testosterone secretion.

Numerous studies have related diabetes mellitus to the interruption of the hypothalamic–pituitary–gonadal (HPG) axis, so changing the testosterone, LH, and FSH concentrations in males. Schoeller et al. [42] have revealed that diabetes mellitus markedly represses the HPG axis and decreases FSH and LH responses to gonadotropin releasing hormone (GnRH) in Type 1 diabetes mellitus states. Repressed testosterone secretion due to reduced Leydig cell function has been recorded in type 2 diabetes mellitus [44]. Reduced serum levels of reproductive hormones (FSH, LH, and testosterone) have also been stated in several diabetic animal models [46, 47].

In the present study, diabetic animals revealed a significant increase in TNF- α and IL-1 β cytokines. IL-1 β has been informed to promote β -cell failure [48]. Also, β -cells themselves produce IL-1 β upon glucose stimulation [49]. Additionally, IL-1 β stimulates its secretion in β -cells [50] and attracts macrophages [48] which represent an additional source of IL-1 β and different cytokines.

In diabetes, ROS can induce transcription factors as nuclear factor kappa B (NF- κ B) resulting in the manifestation of the pro-inflammatory cytokines TNF- α and IL-6 [51]. These moderators diminish insulin

receptor substrate (IRS)-1 tyrosine phosphorylation. Also, TNF- α rises the circulating free fatty acids level [52] and accordingly contributes to the pathogenesis of insulin resistance.

Oxidative injury in type 2 diabetic rats was determined in the present study. Diabetic rats revealed a significant increase in the lipid peroxidation marker (MDA) with adjuvant declined GSH content, SOD and GST activity in the testes. Raised lipid peroxidation in the testicular tissues was revealed to harm testicular function [53]. Moreover, hyperglycemia-induced ROS creation causes DNA damage [54] and is identified to be connected with male infertility [39]. The effect of oxidative stress in the progress of diabetes and its reproductive problems has been proposed in many experimental studies [55,37].

The testes of the diabetic untreated group, in the current study, showed irregular seminiferous tubules and reduction in germ cell layers. Also, apoptosis was seen in the testicular tissues of diabetic rats stained for caspase-3. The present morphometric study confirmed this result. Degenerated germ cells and atrophy of the seminiferous tubules were reported by Guneli et al. [56] and Kanter et al. [2]. Oxidative stress has been concerned in diabetes-induced altered testicular structure in diabetic animals [57,58]. Testicular tissue is susceptible to oxidative stress due to the profusion of highly unsaturated fatty acids [59]. ROS has been stated to induce testicular apoptosis in diabetic rats through stimulation of DNA injury-mediated p53 signaling pathway and up-regulation of proapoptotic proteins [60].

Some authors stated that spermatogenic cells were the goal of diabetes mellitus as it causes atrophy of the seminiferous tubules and apoptosis in spermatogenic cells [61]. Decreased spermatogenic cells is a histopathological pointer of spermatogenesis failure in diabetic rats [62]. Reduction of insulin levels has been stated to suppress the process of spermiogenesis by decreasing FSH rate [62]. Some authors have recorded an augmented activity of caspase-3 in testicular tissues of diabetic rats [63, 64]. Increasing the pro-apoptotic protein (Bax) and decreasing the antiapoptotic protein (Bcl-2) have been recorded in Type2 diabetic animals [57], with an elevation in caspases 8 and 3 expression [65] in Type 1 diabetic rats.

Androgen hormone and AR are essential for preservation of spermatogenesis and maturation of sperms [66]. The present findings showed a significant decrease in the area percentage of AR and the number of Leydig cells in testicular tissues of diabetic rats. Diabetes-induced alterations in Leydig cells can be explained through two parallel mechanisms (1) the effect of reduced insulin level on serum LH level, and (2) the combined effects of the reduced LH and insulin levels on Leydig cells [67].

Treatment of the diabetic animals with nobiletin markedly improved serum LH, FSH, and testosterone levels, and upregulated LHR, FSHR, and CYP17A1 expression in the testicular tissue. Also, nobiletin reduced TNF- α and IL-1 β . These results could be clarified as a result of enhanced glycemic control in diabetic animals treated with nobiletin. Nobiletin suppressed hyperglycemia, and HbA1c%, and augmented insulin levels and sensitivity.

Nobiletin has revealed a capability of modifying insulin signal transduction and elevating insulin sensitivity [68]. It prevents the activity of protein tyrosine phosphatase that negatively controls insulin signaling [12], and controls the manifestation of genes essential for glucose-stimulated insulin secretion

[68]. The hypoglycemic effect of nobiletin was additionally assumed to be mediated by the protection of β -cells [69,18], reduction of intestinal glucose absorption [70], and improving glucose uptake and utilization [71]. The elevated insulin levels in the present study were also in line with the results of Jung [72] who reported the same in type 2 diabetic mice treated with the citrus flavonoid, hesperidin.

The useful effects of nobiletin on the hypophysis–gonadal axis and its consequent elevated testosterone levels might have a good effect on insulin sensitivity. Therapy with testosterone replacement has shown an enhancement on insulin resistance and glycemic control in type 2 diabetic males with hypogonadism [73]. Moreover, testosterone might repress the proinflammatory cytokines which are believed to improve insulin resistance [74].

The current study also showed that both doses of nobiletin enhanced the histological structures of the testicular tissues, reduced caspase-3 and increased androgen positive cells. These results were confirmed by the morphometric study. The beneficial effects of nobiletin could be also clarified by their capability to reduce oxidative stress in the testes of diabetic animals as indicated in the present study. Administration of either low or high dose of nobiletin reduced lipid peroxidation and returned changed levels of the antioxidant defenses. Many studies have revealed that nobiletin could improve the antioxidant defense and decreases lipid peroxidation [75,76,17]. This antioxidant effect may be gifted by modifying the antioxidants gene manifestation [77], free radical reducing action [17], or by decreasing hyperglycemia [78]. The antioxidant effect of nobiletin is in agreement with the results of Bi et al. [75]. Moreover, the useful effect of nobiletin could be elucidated, at least in part, by their capability to diminish the pro-inflammatory cytokines production [68].

Conclusion

the current study reveals that nobiletin supplementation have an anti-hyperglycemic role, elevate insulin sensitivity and secretion, reestablish FSH and LH levels and the manifestation of their receptors, enhance testosterone levels by up-regulation of CYP17A1 manifestation and AR, reduce testicular oxidative stress, tissue damage, TNF- α , IL-1 β and caspase-3 manifestation. Given the main role of oxidative stress in diabetes related testicular dysfunction, the observed antioxidant and antidiabetic effects of nobiletin make it an applicant as a co-therapy to diminish testicular injury.

Declarations

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Conflicts of interest:

The authors declared no conflict of interest.

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Data will be made available on reasonable request

Code availability:

Not applicable

Authors' contributions:

Conceptualization, methodology, data curation, and investigation: M.S., S.M.K., and K.A.I. Original draft preparation: M.S. and S.M.K. Review and editing, validation and formal analysis: M.S. and S.M.K. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Effect of low (N10) and high (N25) nobiletin doses on (a) Glucose level (b) glycosylated hemoglobin (HbA1c) concentration in serum of control and diabetic rats (DM). c ($P \leq 0.001$) versus Control d ($P \leq 0.05$), f ($P \leq 0.001$) versus DM g ($P \leq 0.05$) versus DM+N10

Figure 2

Effect of low (N10) and high (N25) nobiletin doses on (a) TNF- α and (b) IL-1 β levels in serum of control and diabetic rats (DM). b ($P \leq 0.01$), c ($P \leq 0.001$) versus Control f ($P \leq 0.001$) versus DM h ($P \leq 0.01$) versus DM+N10

Figure 3

Effect of low (N10) and high (N25) nobiletin doses on serum levels of (a) LH, (b) FSH and (c) testosterone in control and diabetic rats (DM). b ($P \leq 0.01$), c ($P \leq 0.001$) versus Control d ($P \leq 0.05$), e ($P \leq 0.01$), f ($P \leq 0.001$) versus DM h ($P \leq 0.01$) versus DM+N10

≤ 0.001) versus DM

Figure 4

Effect of low (N10) and high (N25) nobiletin doses on testicular tissue mRNA and protein expression levels of LHR (a, d), FSHR (b, e), and CYP17A1 (c, f) in control and diabetic rats (DM). a ($P \leq 0.05$), b ($P \leq 0.01$), c ($P \leq 0.001$) versus Control e ($P \leq 0.01$), f ($P \leq 0.001$) versus DM g ($P \leq 0.05$), i ($P \leq 0.001$) versus DM+N10

Figure 5

Effect of low (N10) and high (N25) nobiletin doses on (a) lipid peroxidation (MDA) (b) reduced glutathione (GSH) and the activity of (c) superoxide dismutase (SOD) and (d) glutathione-S-transferase (GST) in testicular tissue of control and diabetic rats (DM). a ($P \leq 0.05$), b ($P \leq 0.01$), c ($P \leq 0.001$) versus Control d ($P \leq 0.05$), e ($P \leq 0.01$), f ($P \leq 0.001$) versus DM

Figure 6

a, b) Photomicrographs of testicular tissue from control rats showing (a) closely arranged seminiferous tubules (S) with normal histological architecture and interstitial tissue (I) in between them. (b) Small rounded spermatogonia (Sg) that rest on intact and regular basement membrane, primary spermatocytes (Ps) that have large rounded nuclei, Spermatids (Sd) with rounded nuclei, Spermatozoa (Sp) with intensely stained heads, and Sertoli cells (Sc) between the spermatogenic cells with triangular vesicular

nuclei. Note the myoid cells (M) external to the basement membrane of the seminiferous tubules and the interstitial tissue containing Leydig cells (arrows). (c-f) Photomicrographs of testicular tissue from diabetic rats showing (c) widely separated seminiferous tubules (S) with depletion of most spermatogenic cells, few or no sperm flagella were observed in the lumens, oedema (arrow) and congested blood vessels (V) in the interstitial tissues. (d) Increase in the number of spermatogenic cells that filled the lumen of a seminiferous tubule (S). (e) Irregular seminiferous tubules (S) which separated from their basement membranes (arrow) and wide interstitial spaces between the seminiferous tubules. (f) Seminiferous tubule lined by few layers of vacuolated spermatogenic cells (V), wide lumina, exfoliated cells with dark pyknotic nuclei (thin arrow) and separated basement membrane (thick arrow).

Figure 7

(a, b) Photomicrographs of testicular tissue from diabetic rats treated with low dose of nobiletin showing (a) apparent normal seminiferous tubules (S). (b) A seminiferous tubule lined by several layers of spermatogenic cells and mature sperms (Sp) and nearly normal basement membrane. (c, d) Photomicrographs of testicular tissue from diabetic rats treated with high dose of nobiletin showing (c) several regular and closely packed seminiferous tubules (S) lined by several layers of spermatogenic

cells. (d) Mature sperms (Sp) and intact basement membrane (arrows). (e) The mean number of Leydig cells among different studied groups. c ($P \leq 0.001$) versus control and f ($P \leq 0.001$) versus DM.

Figure 8

Photomicrographs of testicular sections stained with anti-androgen. (a) Control group (b) Control nobiletin showing strong positive immunoreaction in Leydig cells (arrows). (c) Diabetic group showing weak immunoreaction in Leydig cells. (d) Diabetic rats treated with low nobiletin dose showing moderate

immunoreaction in Leydig cells (arrow). (e) Diabetic rats treated with high dose of nobiletin showing strong positive immunoreaction in Leydig cells (arrow). (f) The mean area percentage of AR among different studied groups. c ($P \leq 0.001$) versus control and f ($P \leq 0.001$) versus DM.

Figure 9

Photomicrographs of testicular sections stained with anti-caspase-3. (a) Control group (b) Control nobiletin showing negative caspase-3 immunoreaction in spermatogenic and Leydig cells. (c) Diabetic

group showing strong caspase-3 positive immunoreaction in spermatogenic and Leydig cells (arrows). (d) Diabetic rats treated with low nobiletin dose showing moderate positive caspase-3 immunoreaction in the spermatogenic and Leydig cells (arrows). (e) Diabetic rats treated with high nobiletin dose showing weak caspase-3 immunoreaction in the spermatogenic and Leydig cells (arrows). (f) The mean area percentage of caspase-3 protein among different studied groups. c ($P \leq 0.001$) versus control and f ($P \leq 0.001$) versus DM.