Nobiletin protects against diabetes-induced testicular injury via hypophysis–gonadal axis upregulation and amelioration of oxidative stress

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Abstract

Background Testicular injury is one of the most serious problems associated with diabetes mellitus. The present study aimed to compare the effects of two different doses of nobiletin and analyze its mechanisms of action against diabetes-induced testicular impairment in rats.

Methods and results Streptozotocin injection was used to induce diabetes. Diabetic rats received nobiletin orally at 10 or 25 mg/kg daily for 30 days. Diabetic rats displayed significant elevations in glucose, glycosylated hemoglobin (HbA1c), Homeostatic Model of Insulin Resistance (HOMA-IR), and pro-inflammatory cytokines, while the serum levels of insulin, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were significantly reduced. Histological changes to positivity for caspase-3 and decreased androgen receptors (AR) immunoexpression were observed in diabetic rats. Both doses of nobiletin improved hyperglycemia, reduced pro-inflammatory cytokines, and augmented insulin, testosterone, LH, and FSH levels. LH and FSH receptors and cytochrome P450 17 α-hydroxylase (CYP17A1) were markedly downregulated in terms of both gene and protein expression in testicular tissues of the diabetic group, effects that were markedly ameliorated with both doses of nobiletin. In addition, both doses significantly reduced lipid peroxidation and caspase-3 immunoeexpression and improved the activity of the antioxidant enzymes and AR in testicular tissues of the diabetic group.

Conclusion Both nobiletin doses showed protective effects against diabetes-induced testicular injury by reducing oxidative stress, hyperglycemia, inflammation, and caspase-3 and upregulating the hypophysis–gonadal axis and AR. The high dose of nobiletin was more effective than the lower one.

Keywords Diabetes mellitus · Nobiletin · Testis · Hypophysis · Gonadal axis · Rat

Introduction

Diabetes mellitus, a serious metabolic syndrome, is one of the most important health problems affecting millions of people. Its global incidence is expected to reach about 642 million by the year 2040 [1]. Although research groups have primarily focused on the most prevalent problems associated with diabetes, such as diabetic retinopathy, cardiomyopathy, and nephropathy, investigators are becoming increasingly conscious of impaired male reproductive function in diabetic animals and humans [2].

Increasing oxidative stress in diabetic patients leads to impaired testicular functions, probably via the depletion of testosterone levels, spermatogenesis, seminal fluid volume, and sperm count, leading to male infertility [3]. Furthermore, diabetes-induced oxidative stress can cause apoptosis [4]. 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 reactive oxygen species (ROS) [5]. Thus, altering hyperglycemia-induced oxidative stress is a potentially important option for the management of diabetes and its related testicular dysfunction.

Several studies have shown that natural products can promote the regeneration of pancreatic β cells and improve the secretion and viability of insulin [6, 7]. Additionally, most
natural products that contain flavonoids and phenolic compounds have antioxidant effects that reduce the oxidative stress associated with diabetes mellitus [8].

Nobiletin, a polymethoxyflavone, is a flavonoid found in the peels of citrus fruits. It has many pharmacological properties and shows anti-inflammatory, anti-atherogenic, antidiabetic, immunomodulatory, nephroprotective, neuroprotective, and antioxidant properties [9]. It has been reported that nobiletin markedly reduces lipid peroxidation in testicular tissue and enhances biochemical and sperm parameters, in addition to enhancing epididymal functions [10]. Nobiletin is also known to ameliorate the development of type 2 diabetes mellitus by protecting β cells from apoptosis [11].

Limited data have been published regarding the effect of nobiletin on modulating the dysregulation of the hypophysis–gonadal axis induced by diabetes. Consequently, this study aimed to assess the effects of two doses of nobiletin in increasing gonadotropin production and improving the expression of its receptors in testicular tissues of diabetic rats. Furthermore, the effects of nobiletin on cytochrome P450 17α-hydroxylase (CYP17A1) expression, hyperglycemia-induced oxidative stress, histological changes, and immunohistochemical expression of androgen receptors (AR) and caspase-3 were investigated in the testicular tissues of type 2 diabetic rats.

Materials and methods

Chemicals

Streptozotocin (STZ), nicotinamide, nobiletin, dimethyl sulfoxide (DMSO), pyrogallol, reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid, thiobarbituric acid, 1-chloro-2,4-dinitrobenzene, 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 sources.

Animals

Adult male albino rats (130–150 g) were purchased from the National Institute of Ophthalmology, El-Giza, Egypt. This study was approved by the Institutional Animal Care and Use Committee, Beni-Suef University (BSU-IACUC, approval No. 020-116). The rats were kept in standard aired cages under a 12/12 h light/dark cycle and 25 ± 2 °C. They were fed a standard pellet diet and given access to drinking water ad libitum. The animals received humane care in accordance with the guidelines of the Research Ethics Committee of Beni-Suef University and related international guidance [12].

Diabetes induction

Experimental type 2 diabetes mellitus was promoted in animals fasted overnight via the intraperitoneal injection of nicotinamide (110 mg/kg body weight) liquified in physiological saline 15 min before the intraperitoneal injection of recently prepared STZ (50 mg/kg body weight) liquified in 0.1 mol/L sodium citrate buffer (pH 4.5) [13]. The control rats were administered an equal volume of sodium citrate buffer. Six hours after the intraperitoneal injection of STZ, the animals were given 10% glucose solution for 24 h to avoid a lethal hypoglycemic influence of pancreatic insulin discharge. One week after STZ injection, hyperglycemia was tested by measuring the 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 administered 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; and.
- 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 in accordance with the work of Parkar et al. [14].

Collection and preparation of samples

Twenty-four hours after the last treatment, two blood samples were taken from the retro-orbital venous plexus for each rat. The first sample was taken into a tube with an anticoagulant to assess glycosylated hemoglobin (HbA1c). The other sample was left at room temperature to coagulate, followed by centrifugation at 3000 rpm for 15 min to obtain serum. Obtained sera were kept at − 20 °C until use. After decapitation, the testes were rapidly removed and 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 min and...
then the clear supernatant was kept at − 20 °C. The second testis was divided into two parts, one of which was used for histological and immunohistochemical study, while the other was kept 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 the lateral caudal vein of overnight fasted control and diabetic rats. Other blood samples were then obtained at 120 min after the supplementation of 3 g/kg body weight glucose solution. The concentration of serum glucose was estimated following the method reported by Trinder [15], via reagent kits obtained from bioMerieux Chemicals (France).

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

Serum insulin, IL-1β, and TNF-α were estimated using precise enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems (USA), following the manufacturer’s instructions. HbA1c% was assessed following the method reported by Abraham et al. [16] using a reagent kit obtained from Stanbio Company (Texas, USA).

**Assessment of insulin resistance**

The estimation of glucose or insulin levels can lead to imprecise determination of abnormalities in insulin activity [17]. Thus, insulin resistance was assessed using Homeostatic Model of Insulin Resistance (HOMA-IR), in accordance with the work of Haffner [18], and quantitative insulin sensitivity check index (QUICKI), in line with the procedure of Perseghin et al. [19], as follows:

\[
\text{HOMA-IR} = \frac{\text{Fasting insulin} \times \text{Fasting glucose}}{22.5}
\]

\[
\text{QUICKI} = \frac{1}{\log \text{fasting insulin}} + \log \text{fasting glucose}
\]

Elevation in HOMA-IR and reduction in QUICKI reflect insulin resistance.

**Determination of serum testosterone, LH, and FSH levels**

Serum levels of testosterone, LH, and FSH were estimated by ELISA using precise commercial rat ELISA analytical kits (ALPCO Diagnostics), as per the manufacturer’s instructions.

**Oxidative stress and antioxidant enzyme assays**

Testicular antioxidant/oxidative stress indicators were measured using their own supernatants following well-known methods. The level of thiobarbituric acid reactive substances was estimated using the method of Preuss et al. [20]. Glutathione (GSH) content was estimated as described by Beutler et al. [21]. Superoxide dismutase (SOD) activity was estimated following the method described by Marklund and Marklund [22]. Glutathione-S transferase (GST) was assessed as reported by Mannervik and Guthenberg [23].

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

The expression of LH-receptor (LHR), FSH-receptor (FSHR), and CYP17A1 genes in testicular samples was investigated. Whole RNA was isolated from frozen testes using 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 confirmed by formaldehyde-containing agarose gel electrophoresis. Reverse transcription was performed with 5 µg of RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas). cDNA was augmented by SYBR Green Master Mix (Fermentas) in a total volume of 25 µL via the primer set indicated in Table 1. The polymerase chain reaction (PCR) comprised initial denaturation at 95 °C for 3 min; 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 starting at 60 °C and ramping up by about 0.5 °C every 10 s to 95 °C. Melting curve analysis was performed to test the specificity of the used primers. Each test included a sample of distilled water as a control. Finally, the augmentation data were evaluated using the $2^{-\Delta\Delta CT}$ technique [24] and the values were normalized to β-actin.

**Western blot**

Testis samples preserved at − 80 °C were used to examine the influence of nobiletin on the expression levels of LHR, FSHR, CYP17A1, and β-actin as a loading control.
Briefly, these specimens were homogenized in RIPA lysis buffer containing protease inhibitors, centrifuged, and the protein concentration was measured in clear supernatant by Bradford reagent. A total of 30 mg of proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 5% skimmed milk liquified in TBS Tween20 (TBST). These membranes were then incubated with primary antibodies against LHR, FSHR, CYP17A1, and β-actin. After washing with TBST, the membranes were probed with matching secondary antibodies and developed with an enhanced chemiluminescence reagent (Bio-Rad, USA). The intensity of each band was quantified using Image J and normalized to β-actin.

Histological, immunohistochemical, and morphometric studies

Hematoxylin and eosin staining

Testicular specimens were fixed for 24 h in neutral buffered formalin (10%). The specimens were routinely handled, embedded in paraffin wax, and sectioned by a microtome at a thickness of 4–5 μm, followed by staining with hematoxylin and eosin (H&E) [26] to investigate testicular morphology.

Immunohistochemical determination of AR and caspase-3 protein

Five micrometer sections were prepared from the paraffin-embedded testis and then mounted on positively charged glass slides for the immunohistochemical detection of AR and caspase-3. The sections were dewaxed, rehydrated, and then autoclaved for 10 min at 120 °C in citrate buffer (10 mM at pH 6). After washing with phosphate-buffered saline (PBS), endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol (15 min). Slides were washed using PBS and blocked with blocking buffer, followed by incubation at room temperature (30 min). Primary monoclonal and polyclonal antibodies for AR (Catalog Number MA1-150; Thermo Fisher Scientific Co., USA) [27] and caspase-3 (mouse monoclonal antibody; Lab Vision, Fremont, CA USA) [28], were added after dilution using PBS (2 μg/ml and 1:1000, respectively), and then incubated for 30 min. The slides were washed with PBS three times for 3 min each. Biotinylated polyclonal secondary antibody (Cat. No. 32230; Thermo Scientific Co., UK) was added to the sections and co-incubated for 30 min. The slides were washed three times for 3 min each with washing buffer. The reaction was established by applying working solution of Metal Enhanced DAB substrate to the tissue, followed by incubation for 10 min. The slides were washed two times for 3 min each with washing buffer. Hematoxylin was used as a counterstain to cover the entire tissue surface [29]. A negative control for each section was prepared without using primary antibody.

Statistical analysis

Statistical analysis was performed using SPSS (version 16). Values are presented as mean ± standard deviation (SD). Comparisons between means were made by one-way ANOVA, followed by Tukey’s post hoc analysis. Values of P ≤ 0.05 were considered significant.

Results

Nobiletin suppresses hyperglycemia in diabetic rats

Diabetic rats showed significant (p ≤ 0.001) increases in fasting and postprandial blood glucose levels compared with control rats. Rats treated with both nobiletin doses showed a significant decrease (p ≤ 0.001) in blood glucose level compared with the diabetic group (Table 2). Moreover, diabetic rats displayed a significant elevation (P ≤ 0.001) in HbA1c% in comparison to control rats. The treatment of diabetic rats with both doses of nobiletin markedly (P ≤ 0.05) improved blood HbA1c% (Table 2).

Table 1 Primer pairs for quantitative RT-PCR

| Gene   | Gene Bank accession number | Sequence (5′–3′)               |
|--------|----------------------------|--------------------------------|
| LHR    | NM_012978.1                | F: GGCACACCATACACTATGCT         |
| FSHR   | NM_199237.1                | R: AAAAGAGCCATCCTCGAGC          |
| CYP17A1| NM_012753.2                | F: AGACCTGTCCACGCCTATCT         |
| β-actin| NM_031144.3                | R: GGATCCAGATGATCGGC            |
Nobiletin ameliorates serum insulin release and insulin sensitivity in diabetic rats

The data presented in Table 2 indicate 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 ≤ 0.001) decreased in the diabetic group compared with that in the control rats. The treatment of diabetic rats with low and high doses of nobiletin markedly (P ≤ 0.05 and P ≤ 0.001, respectively) increased the insulin level.

Diabetic rats displayed a significant elevation in HOMA-IR (P ≤ 0.01) and reduction in QUICKI (P ≤ 0.05). Administration of the high dose of nobiletin markedly ameliorated insulin sensitivity, as manifested by decreasing (P ≤ 0.01) HOMA-IR and increasing (P ≤ 0.05) QUICKI.

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-1β were determined (Table 3). The serum TNF-α level of diabetic rats was shown to significantly increase (P ≤ 0.001) compared with that in the control group. Supplementation with either 10 mg or 25 mg of nobiletin significantly decreased (P ≤ 0.001) the serum TNF-α level of diabetic rats. Likewise, IL-1β was significantly increased (P ≤ 0.001) in the serum of diabetic rats in comparison with that in the control rats and was significantly decreased (P ≤ 0.001) after treatment with both nobiletin doses in comparison to that in the diabetic group.

Nobiletin upregulates the hypophysis–gonadal axis in diabetic rats

To assess whether the hypophysis–gonadal axis is modified by nobiletin in diabetic animals, serum levels of LH, FSH, and testosterone were measured by ELISA (Table 3). Additionally, testicular LHR, FSHR, and CYP17A1 mRNA and protein expression levels were quantified by RT-PCR and western blotting (Fig. 1a–f). Serum levels of LH, FSH and testosterone in diabetic rats were significantly decreased (P ≤ 0.001) in comparison with those in the control group. The administration of a low dose of nobiletin for 30 days significantly increased the serum levels of LH (P ≤ 0.01) and FSH (P ≤ 0.05) in diabetic rats; it also increased the serum testosterone level, albeit not significantly. The high nobiletin dose produced significant elevations of LH, FSH (P ≤ 0.001), and testosterone (P ≤ 0.05) in diabetic animals.

LHR and FSHR mRNA in the testes of diabetic rats underwent significant downregulation (P ≤ 0.001) in comparison to the levels in the control group. The oral administration of either the low or the high dose of nobiletin significantly (P ≤ 0.001) upregulated LHR and FSHR mRNA in the testes of diabetic rats (Fig. 1a,b). CYP17A1 showed a similar pattern. It was significantly downregulated (P ≤ 0.001) in the testes of diabetic rats, while the low and high doses of nobiletin significantly (P ≤ 0.01 and P ≤ 0.001, respectively) increased CYP17A1 mRNA in diabetic rats (Fig. 1c).

Data from western blotting (Fig. 1d–f) revealed significant (P ≤ 0.001) reductions in the protein expression levels of LHR, FSHR, and CYP17A1 in the testes of diabetic rats. The administration of either 10 or 25 mg/kg nobiletin significantly (P ≤ 0.001) elevated the protein levels of LHR and FSHR in the testicular tissues of diabetic rats.

### Table 2

Effect of low (N10) and high (N25) nobiletin doses on fasting and postprandial glucose levels, HbA1c (%), insulin levels, HOMA-IR, and QUICKI in control and diabetic rats

|                    | Fasting glucose level (mg/dL) | Postprandial glucose level (mg/dL) | HbA1c (%) | Insulin (µU/ml) | HOMA-IR | QUICKI |
|--------------------|------------------------------|-----------------------------------|-----------|-----------------|---------|--------|
| **Control**        | 112.2 ± 8.87                 | 155.8 ± 10.35                     | 3.57 ± 0.66 | 15.56 ± 0.75 | 4.38 ± 0.57 | 0.50 ± 0.015 |
| **N25**            | 119.2 ± 11.07                 | 138.4 ± 20.80                     | 4.06 ± 0.72 | 15.23 ± 0.89 | 4.39 ± 0.74 | 0.50 ± 0.019 |
| **Diabetic**       | 316.8 ± 33.23<sup>c</sup>     | 397.8 ± 69.38<sup>e</sup>         | 7.8 ± 1.4<sup>d</sup> | 8.26 ± 0.60<sup>c</sup> | 6.84 ± 1.11<sup>b</sup> | 0.45 ± 0.014<sup>a</sup> |
| Diabetic + N10     | 174.0 ± 12.14<sup>e</sup>     | 221.4 ± 51.24<sup>d</sup>         | 5.166 ± 0.40<sup>d</sup> | 10.71 ± 0.77<sup>ed</sup> | 4.76 ± 0.31<sup>d</sup> | 0.49 ± 0.006<sup>d</sup> |
| Diabetic + N25     | 137.4 ± 10.99<sup>ef</sup>    | 163.0 ± 25.25<sup>d</sup>         | 4.63 ± 1.02<sup>d</sup> | 12.93 ± 0.61<sup>be</sup> | 4.42 ± 0.36<sup>e</sup> | 0.50 ± 0.008<sup>d</sup> |

HbA1c % glycosylated hemoglobin concentration, HOMA-IR homeostasis model of insulin resistance, QUICKI quantitative insulin-sensitivity check index

<sup>a</sup>(P ≤ 0.05)
<sup>b</sup>(P ≤ 0.01)
<sup>c</sup>(P ≤ 0.001) vs control
<sup>d</sup>(P ≤ 0.05)
<sup>e</sup>(P ≤ 0.01)
<sup>f</sup>(P ≤ 0.001) vs diabetic
<sup>g</sup>(P ≤ 0.05) vs diabetic + N10
Similarly, the administration of low ($P \leq 0.01$) and high ($P \leq 0.001$) doses of nobiletin significantly upregulated CYP17A1 protein expression. Normal animals treated with nobiletin revealed nonsignificant changes ($P \geq 0.05$) in serum hormones (Table 3), and testicular gene and protein levels of LHR, FSHR, and CYP17A1 (Fig. 1).

### Nobiletin suppresses oxidative stress in diabetic rats

The effects of nobiletin on testicular lipid peroxidation and antioxidant enzyme activity under normal and diabetic conditions are shown in Table 4. Diabetic rats showed a significant ($P \leq 0.001$) increase in testicular MDA levels compared with the control group. The treatment of diabetic animals with either the low or the high dose of nobiletin significantly ($P \leq 0.001$) decreased MDA content. In contrast, the testicular GSH level of diabetic rats was significantly decreased ($P \leq 0.001$). Supplementation with the high dose of nobiletin significantly ($P \leq 0.001$) increased the testicular GSH content in diabetic rats. Supportive of the GSH data, the antioxidant activities of SOD and GST enzymes were significantly ($P \leq 0.001$) decreased in the testes of diabetic animals. After the administration of both

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**Table 3** Effect of low (N10) and high (N25) nobiletin doses on TNF-α, IL-1β, LH, FSH and testosterone levels in serum of control and diabetic rats

|                  | TNF-α (pg/ml) | IL-1β (pg/ml) | LH (mg/ml) | FSH (ng/ml) | Testosterone (ng/ml) |
|------------------|---------------|---------------|------------|-------------|----------------------|
| Control          | 36.13 ± 4.31  | 35.20 ± 2.85  | 7.3 ± 0.43 | 11.36 ± 1.15 | 1.73 ± 0.21          |
| N25              | 42.13 ± 7.35  | 36.66 ± 4.63  | 6.55 ± 0.52| 10.59 ± 1.04 | 1.63 ± 0.3           |
| Diabetic         | 143.03 ± 3.25c| 136.9 ± 7.26c | 2.41 ± 0.41| 3.76 ± 0.65c | 0.58 ± 0.03c         |
| Diabetic + N10   | 93.53 ± 3.27cf| 72.36 ± 2.40cf| 4.39 ± 0.40c| 6.16 ± 0.21cd| 0.92 ± 0.06c         |
| Diabetic + N25   | 74.73 ± 5.41cfh| 54.6 ± 4.50gh | 5.41 ± 0.36bd| 8.03 ± 0.35fr | 1.08 ± 0.02bd        |

*TNF-α tumor necrosis factor-alpha, IL-1β interleukin-1β, LH luteinizing hormone, FSH follicle-stimulating hormone*

$b (P \leq 0.01)$

c $(P \leq 0.001)$ vs control

d $(P \leq 0.05)$

e $(P \leq 0.01)$

f $(P \leq 0.001)$ vs diabetic

h $(P \leq 0.01)$ vs diabetic + N10
doses of nobiletin, the activity of SOD was significantly (P ≤ 0.01) increased. GST activity increased significantly with low (P ≤ 0.05) and high (P ≤ 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. 2a). Spermatogenic cells comprised spermatogonia, primary spermatocytes, spermatids, and spermatzoa. The spermatogonia were small rounded cells that rested on intact and regular basement membrane (Fig. 2b). The largest cells were the primary spermatocytes, exhibiting large rounded nuclei (Fig. 2b). The spermatids appeared as small round cells with rounded nuclei and localized toward the lumen (Fig. 2b). The spermatzoa appeared with intensely stained heads and were detected near the lumen of the seminiferous tubules (Fig. 2b). Sertoli cells appeared between the spermatogenic cells as tall cells with triangular nuclei (Fig. 2b). External to the basement membrane of the seminiferous tubules, smooth muscle-like cells were found, called myoid cells (Fig. 2b). The interstitial tissue, between the seminiferous tubules, contained blood vessels and Leydig cells arranged in small clumps with a spherical or polyhedral shape and exhibiting vesicular nuclei (Fig. 2a,b). Histological analysis of the testicular sections from the nobiletin group showed the same histological structures. The negative control testicular sections are presented for them.

Histological investigation of testicular sections from the diabetic group showed pronounced tissue disruption (Fig. 2c–f). Most seminiferous tubules appeared with a lumen containing few or no spermatzoa (Fig. 2c). The seminiferous tubules were separated by wide interstitial spaces containing congested blood vessels, edema, and a few Leydig cells (Fig. 2c). In the few seminiferous tubules, the spermatogenic cells increased in number and appeared as a solid mass occupying the tubular lumen (Fig. 2d). Most seminiferous tubules appeared with irregular outlines and were separated from their basement membranes (Fig. 2e,f). Many seminiferous tubules had few layers of spermatogenic cells with vacuolated cytoplasm (Fig. 2e,f). The spermatogenic cells appeared with pyknotic exfoliated nuclei (Fig. 2f).

Histological investigation of the testicular sections from the diabetic group treated with a low nobiletin dose showed that many seminiferous tubules had a regular appearance, being lined by layers of spermatogenic cells and containing many sperm in their lumina, whereas few tubules contained degenerated spermatogenic cells (Fig. 3a,b).

The testicular sections of diabetic rats treated with a high dose of nobiletin displayed a great enhancement (Fig. 3c,d). The seminiferous tubules and their germinal epithelium appeared to be similar to those of the control rats. They had a regular and intact basement membrane, spermatogenic cells, and more sperm flagella present in their lumina.

There was a significant reduction (P ≤ 0.001) in the mean number of Leydig cells of the diabetic group (Fig. 3e) compared with that in the control group. The mean number of Leydig cells was markedly increased (P ≤ 0.001) in the diabetic group supplemented with either the low or the high dose of nobiletin (Fig. 3e), compared with that of the diabetic group.

Nobiletin upregulates AR 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 (Fig. 4a,b). A weak

| Table 4 Effect of low (N10) and high (N25) nobiletin doses on MDA, GSH and the activity of SOD and GST in testicular tissue of control and diabetic rats |
|-----------------|-----------------|-----------------|-----------------|
|                 | MDA (nmol/100 mg) | GSH (nmol/100 mg) | SOD (U/g)       | GST (U/g)       |
| Control         | 29.45 ± 3.74     | 22.20 ± 2.68     | 25.46 ± 3.32    | 81.4 ± 7.19     |
| N25             | 34.29 ± 5.56     | 18.28 ± 3.11     | 23.81 ± 0.22    | 77.73 ± 3.82    |
| Diabetic        | 79.99 ± 7.86c    | 7.51 ± 0.83c     | 12.75 ± 1.42c   | 49.25 ± 1.52c   |
| Diabetic + N10  | 51.06 ± 1.77bd   | 10.93 ± 1.33c    | 19.93 ± 1.67ae  | 62.77 ± 3.08bd  |
| Diabetic + N25  | 39.27 ± 5.45f    | 15.78 ± 3.13d    | 20.14 ± 2.15c   | 73.55 ± 6.13l   |

MDA lipid peroxidation, GSH reduced glutathione, SOD superoxide dismutase, GST glutathione-S-transferase

| aP ≤ 0.05       | bP ≤ 0.01       | cP ≤ 0.001 vs control | dP ≤ 0.05       | eP ≤ 0.01       | fP ≤ 0.001 vs Diabetic |

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immunoreaction was found in the Leydig cells of diabetic rats (Fig. 4c). A moderate AR immunoreaction was observed in the Leydig cells of diabetic rats treated with the low dose of nobiletin (Fig. 4d), while a strongly positive AR immunoreaction was found in the Leydig cells of diabetic rats treated with the high dose of nobiletin (Fig. 4e). There was a significant reduction (P ≤ 0.001) in the AR-immunopositive reaction in the diabetic group (Fig. 4f), compared with that of the diabetic group supplemented with either the low or the high dose of nobiletin (Fig. 4f), compared with that of the diabetic group.

A negative nuclear caspase-3 immunoreaction was observed in cells lining the seminiferous tubules of control and control nobiletin testicular sections (Fig. 5a, b). A strongly positive caspase-3 immunoreaction was observed in cells lining the testicular tubules of diabetic rats (Fig. 5c). A moderately positive caspase-3 immunoreaction was observed in the cells lining the testicular tubules of diabetic rats treated with a low nobiletin dose (Fig. 5d), while there...
were few caspase-3 immunoreactive cells lining the testicular tubules of diabetic rats treated with a high nobiletin dose (Fig. 5e).

There was a significant ($P \leq 0.001$) increase in the caspase-3 immunopositive reaction in the diabetic group (Fig. 5f) compared with that in the control group. The caspase-3 immunoreaction was significantly decreased ($P \leq 0.001$) in the diabetic group supplemented with either the low or the high dose of nobiletin (Fig. 5f), compared with that of the diabetic group.

**Fig. 3**  
**a, b** Photomicrographs of testicular tissue from diabetic rats treated with low dose of nobiletin showing 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 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

**Discussion**

Diabetes mellitus has been reported to adversely affect the male reproductive system. Pronounced effects of oxidative stress have also been revealed in the testicular tissues of diabetic rats [30]. Type 2 diabetes mellitus is characterized by a continually high blood glucose level due to insulin resistance and insulin deficiency [31]. Therefore, treatment that can enhance glycemic control and reduce testicular oxidative stress should help to inhibit the reproductive disorders related to diabetes. The current study
examined the effects of two doses of nobiletin on testicular tissue damage, oxidative stress, and dysregulation of the hypophysis–gonadal axis induced by hyperglycemia in a rat model of type 2 diabetes mellitus.

In the present study, the diabetic animals exhibited hyperglycemia combined with a reduced serum insulin concentration. The diabetic state was established from the augmented HbA1c%. The measurement of HbA1c concentration is a good indicator for the diagnosis and prediction of diabetic problems [32]. Moreover, it was previously asserted that insulin sensitivity indices (HOMA-IR and QUICKI) can reflect the establishment of insulin resistance [33].

In our work, nobiletin suppressed hyperglycemia and HbA1c%, and augmented insulin levels and sensitivity. Nobiletin has also been previously reported to be able to modify insulin signal transduction and elevate insulin sensitivity [9]. It prevents the activity of protein tyrosine phosphatase, which negatively controls insulin signaling [34], and controls the expression of genes essential for glucose-stimulated insulin secretion [9]. In addition, the hypoglycemic effect of nobiletin was assumed to be
mediated by the protection of β cells [11], reduction of intestinal glucose absorption [35], and improvements of glucose uptake and utilization [36]. The elevated insulin levels as identified in the present study are also in line with the results of Jung [37], who reported the same in type 2 diabetic mice treated with the citrus flavonoid hesperidin.

In the present study, the type 2 diabetic rats were demonstrated to exhibit oxidative injury. They also revealed a significant increase in the lipid peroxidation marker MDA with associated declines in GSH content, and SOD and GST activity in the testes. The elevated oxidative stress in type 2 diabetes mellitus is a result of various abnormalities, including hyperglycemia and insulin resistance, each of which stimulates excessive mitochondrial superoxide overproduction [38]. Hyperglycemia causes mitochondrial dysfunction and endoplasmic reticulum stress, which promotes the accumulation of ROS that can in turn immediately harm DNA, proteins, or lipids and alter intracellular signaling pathways. Examples of this include mitogen-triggered protein kinases and redox-sensitive transcription elements causing alterations in protein expression and, consequently, irreversible oxidative changes [39].
Testicular tissue is susceptible to oxidative stress due to the profusion of highly polyunsaturated fatty acids, leading to damage to sperm vitality, motility, and structure [10].

In line with previous results, in the current study the testes of the untreated diabetic group showed irregular seminiferous tubules and reduction in germ cell layers. In addition, apoptosis was seen in the testicular tissues of diabetic rats stained for caspase-3. This was also confirmed by the results of morphometric study. Degenerated germ cells and atrophy of the seminiferous tubules were reported by Kanter et al. [2]. Oxidative stress has been reported to be a concern in the altered testicular structure induced by diabetes in diabetic animals [40]. It has been stated that ROS induce testicular apoptosis in diabetic rats through stimulating the DNA injury-mediated p53 signaling pathway and the upregulation of proapoptotic proteins [41].

Some researchers stated that spermatogenic cells were a target of diabetes mellitus as this disease causes atrophy of the seminiferous tubules and apoptosis in spermatogenic cells [42]. A decrease in spermatogenic cells is a histopathological marker of failure of spermatogenesis in diabetic rats [43]. Some researchers also recorded augmented activity of caspase-3 in the testicular tissues of diabetic rats [44]. An increase in a pro-apoptotic protein (Bax) and a decrease in an antiapoptotic one (Bcl-2) have also been recorded in type 2 diabetic animals [40], along with elevations in caspase 8 and 3 expression [45] in type 1 diabetic rats.

These damaging changes were shown to be effectively mitigated by nobiletin. The current study showed that both doses of nobiletin enhanced the histological structures of the testicular tissues and reduced the frequency of caspase-3-positive cells. The beneficial effects of nobiletin could be explained by its ability to reduce oxidative stress in the testes of diabetic animals. The administration of either the low or the high dose of nobiletin reduced lipid peroxidation and allowed recovery of the changed levels of components of the antioxidant defense system. Many studies have revealed that nobiletin could improve antioxidant defense and decrease lipid peroxidation [10, 46]. This antioxidant effect may be conferred via modifications of antioxidant gene expression [47], activity to reduce free radicals [10], or a decrease in hyperglycemia [48].

In the present study, diabetic animals revealed significant increases in TNF-α and IL-1β cytokines. IL-1β has been reported to promote β-cell failure [49]. In addition, β cells themselves produce IL-1β upon glucose stimulation [50]. Additionally, IL-1β stimulates its own secretion in β cells [51] and attracts macrophages [49], which represent an additional source of IL-1β and other cytokines. In diabetes, ROS can induce transcription factors such as nuclear factor kappa B (NF-κB), resulting in expression of the pro-inflammatory cytokines TNF-α and IL-6 [52]. These moderators reduce insulin receptor substrate (IRS)-1 tyrosine phosphorylation. Moreover, TNF-α increases the level of circulating free fatty acids [53] and accordingly contributes to the pathogenesis of insulin resistance. Treatment with nobiletin was reported to reduce TNF-α and IL-1β at least in part by the ability to lower the production of pro-inflammatory cytokines [9, 48]. Lin et al. [54] suggested that the anti-inflammatory action of nobiletin is very similar to those of anti-inflammatory steroids and considered nobiletin as a novel immunomodulatory and anti-inflammatory drug.

In the current study, there were decreased levels of FSH, LH, and testosterone in diabetic rats. Reduced serum levels of reproductive hormones have also been reported in a previously presented diabetic rat model [55]. It has also been asserted that a reduction of insulin level suppressed the process of spermiogenesis by decreasing FSH [43]. Hyperglycemia augments resistance of the testis to these hormones, causing low testosterone levels [56]. It has also been reported that diabetes’ effects of lowering the FSH and LH levels seriously impact on androgen biosynthesis [57]. Moreover, it has been presented that androgen and AR are essential for preserving spermatogenesis and the maturation of sperm [58]. The findings of the present study 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 reduced LH and insulin levels on Leydig cells [59]. Repressed testosterone secretion due to reduced Leydig cell function has been recorded in type 2 diabetes mellitus [60].

Similarly, diabetes-induced FSH and LH resistance was shown to be established by the downregulation of FSHR and LHR in the testicular cells of diabetic rats. Consequently, CYP17A, a main steroidogenic enzyme in testosterone formation, was similarly downregulated, explaining the decreased testosterone levels. Previous studies established a negative correlation between serum testosterone and HbA1c levels [60]. Moreover, decreased testosterone level has been reported to be related to an increased risk of insulin resistance and type 2 diabetes mellitus in healthy males [61]. Therefore, insulin-sensitizing agents could increase testosterone secretion.

Numerous studies have described the relationship between diabetes mellitus and disruption of the hypothalamic–pituitary–gonadal (HPG) axis, so changing the concentrations of testosterone, LH, and FSH in males could be effective for treating this disease. Schoeller et al. [57] revealed that diabetes mellitus markedly suppressed the HPG axis and decreased FSH and LH responses to gonadotropin releasing hormone (GnRH) in type 1 diabetes mellitus.

The treatment of diabetic animals with nobiletin markedly improved serum LH, FSH, and testosterone levels, and
upregulated LHR, FSHR, and CYP17A1 expression in testicular tissue. It was proven that nobiletin could increase the level of cyclic adenosine monophosphate (cAMP) [62], a molecule that potentially upregulates the expression of genes encoding steroidogenic enzymes [63], thus enhancing testosterone formation.

Moreover, the advantageous effects of nobiletin on the hypophysis–gonadal axis and the consequent elevated testosterone levels might have beneficial effects on insulin sensitivity. Therapy with testosterone replacement has been shown to enhance insulin resistance and glycemic control in males with type 2 diabetic mellitus and hypogonadism [64]. Moreover, testosterone might suppress pro-inflammatory cytokines, which is believed to improve insulin resistance [65].

In conclusion, the current study reveals that nobiletin supplementation has an anti-hyperglycemic effect; elevates insulin sensitivity and secretion; reestablishes FSH and LH levels and the expression of their receptors; enhances testosterone levels by upregulating CYP17A1 and AR; and reduces testicular oxidative stress, tissue damage, TNF-α, IL-1β, and caspase-3 expression. Given the main role of testosterone in male sexual function, fertility, and bone health, it is suggested that nobiletin may be a promising candidate as one component of a therapeutic strategy to diminish testicular injury in diabetes mellitus.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The Institutional Animal Care and Use Committee of Beni-Suef University, Egypt, approved the entire conducted procedures (BSU-IACUC, Approval No. 020-116).

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