ORIGINAL ARTICLE

Naringenin attenuates testicular damage, germ cell death and oxidative stress in streptozotocin induced diabetic rats: naringenin prevents diabetic rat testicular damage

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Summary

The aim of this study was to investigate the protective effect of naringenin on oxidative stress, on pro-inflammatory cytokines like TGF-β1, IL-1β and on programmed cell death in the testicular damage resulting from streptozotocin (STZ) induced diabetes in rats. Diabetes was induced by a single intraperitoneal injection of STZ (50 mg/kg), and the rats were treated with naringenin (5 mg/kg and 10 mg/kg) administered once a day orally for 10 weeks, starting 3 days after the STZ injection. At the end of the study, all animals were sacrificed. Testis tissue and blood samples were collected for the assessment of sperm parameters, and for biochemical and histopathological analysis. Naringenin treatment significantly decreased the levels of elevated tissue TBARS (thio-barbituric acid) and increased the superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) enzyme activities in the testis tissues. The naringenin-treated rats in the diabetic group showed an improved histological appearance, sperm parameters, and serum testosterone levels, along with a decrement of terminal dUTP nick end-labeling (TUNEL) detected program cell death and a reduced over expression of TGF-β1, IL-1β in Sertoli cells and Leydig cells. These results suggest that naringenin is a food supplement potentially beneficial in reducing testicular damage in diabetic rats by decreasing the oxidative stress related to programmed cell death.

Key words: diabetes; oxidative stress; naringenin; testis; TGF-β1; IL-1β; programmed cell death

INTRODUCTION

Diabetes mellitus (DM) is a degenerative disease with alteration in carbohydrate homeostasis, that affects male reproductive function at multiple levels particularly the endocrine control of spermatogenesis,

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spermatogenesis itself or by impairing penile erection and ejaculation (Sexton and Jarow 1997). About 90% of diabetic patients generally experience sexual abnormalities such as sexual dysfunction, impotence and infertility (Amaral et al. 2006). Male infertility is a common threat nowadays and it has increased rapidly because of hyperglycaemia (Andy et al. 2009). which results in the generation of reactive oxygen species (ROS) and attenuates antioxidant defense systems, leading to oxidative stress in a variety of tissues (Brownlee 2001). Furthermore, antioxidative enzyme activities like superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) might also be reduced due to glycation or increased lipid peroxidation products (Sozmen et al. 2001). The generation of reactive oxygen species (ROS) is mainly due to glucose autoxidation, activation of the

polyol pathway, increased glycolysis, and activation of PK C (protein kinase C) and the hexosamine pathway (Hakeem et al. 2008).

A previous report has suggested that the expression of TGF-β1 plays a crucial role in the testis during pubertal maturation, in the regulation of steroidogenesis, spermatogenesis and the functional development of male secondary reproductive organs (Ingman and Robertson 2002). However, ROS is considered a strong stimulus for the release of cytokines. The cytokine transforming growth factor-β (TGF-β) is up-regulated in the corpora cavernosum of rats with streptozotocin-induced diabetes (Ahn et al. 2005). There is evidence that hyperglycaemia causes oxidative stress, stimulating macrophages and the over expression of pro-inflammatory cytokines as IL-1β in rat testis (Abeer 2010).

Programmed cell death is a genetically encoded form of cell suicide central to the development and homeostasis of multicellular organisms. This event is critical for normal spermatogenesis in the adult testis (Sinha and Swerdloff 1999). A recent study has shown that increased programmed cell death in the seminiferous tubule of streptozotocin (STZ) induced diabetic testis of mice and rats (Cai et al. 2000). Furthermore, oxidative stress has been recognized as a strong mediator of programmed cell death (Leon et al. 2005) and the mechanisms related to this are still unclear. The balance between ROS and antioxidants is a major mechanism in preventing damage by oxidative stress. Therefore, dietary supplement with antioxidants such as vitamins and flavonoids has been used to prevent the occurrence of many chronic diseases (Peluso 2006). Antioxidants may also be useful in the treatment of male infertility (Park et al. 2003).

Bioflavonoids have been used in the treatment of diabetic complications like testicular damage (Khaki et al. 2010). Naringenin is a flavonone that is considered to have a bioactive effect on human health as an antioxidant, a radical scavenger, an anti-inflammatory, carbohydrate metabolism promoter, an immunity system modulator (Felgines et al. 2000) and a regulator of cytochrome P450 (CYP450) enzymes (Kidambi et al. 2009). Previous studies have shown that naringenin prevents dyslipidaemia, hyperinsulinaemia (Mulvihill et al. 2009), has a cholesterol lowering effect in rabbits and rats (Jeon et al. 2007) and exhibits antiviral activity (Nahmias et al. 2008). Goldwasser et al. (2010) found that naringenin could replace the actions of fibrates (PPARα agonists), thiazolidenediones (PPARγ agonists), and statins in the treatment of type-2 diabetes or hyperlipidaemia.

Streptozotocin is an antibiotic derived from *streptomyces achromogenes*, structurally a nitrosourea derivative. It is one of the most commonly used substances to induce diabetes in experimental animals (Szkudelski 2001). Streptozotocin causes alkylation or breakage of DNA strands and a consequent increase in the activity of poly-ADP-ribose synthetase, an enzyme depleting the NAD^+ in β cells, leading to energy deprivation and finally apoptosis of the β cells of the pancreas (Roy et al. 2011) and in the diabetic rat testis (Guneli et al. 2008).

This study was designed to investigate the effect of naringenin on changes in the body weight, glucose homeostasis, serum biochemical parameters, sperm parameters, antioxidant defense systems, histological architecture, cell damage biomarkers and correlations with programmed cell death in streptozotocin-induced diabetic rat testicular damage.

MATERIALS AND METHODS

Animals and experimental design
Inbred male Albino Wistar rats (140–160 gm) were obtained from the Indian Institute of Chemical Biology (IICB), Kolkata. They were housed in polypropylene cages ($47 \times 34 \times 20$ cm) lined with husk, renewed every 24 h under 12 h light/dark cycle at around 22 °C. Rats were fed with a semi purified basal diet and de-mineralized drinking water *ad libitum*. The rats were allowed to acclimatize to the laboratory environment for a week before the start of the experiment. All experimental procedures were conducted with the approval of the Institutional Animal Ethics committee CPCSEA (Reg. No. 1458/PO/a/11/CPCSEA) for the care and use of animals and their guidelines were strictly followed throughout the study.

The animals were randomly divided into 6 groups with 10 animals each.
Group I: Control group.
Group II: STZ treated group (50 mg/kg body weight).
Group III: STZ + Naringenin (5 mg/kg body weight).
Group IV: STZ + Naringenin (10 mg/kg body weight).
Group V: Naringenin (5 mg/kg body weight) treated control rats.
Group VI: Naringenin (10 mg/kg body weight) treated control rats.

After 10 weeks of the experiment, blood samples were collected for the biochemical estimations, then the rats were sacrificed by ether anaesthesia I.P (Indian Pharmacopoeia) and the testis were collected for sperm parameters, antioxidant, histopathological, immunohistochemical, and apoptotic studies.

Induction of diabetes

Diabetes was induced in groups II, III, IV rats following overnight fasting, by an i.p. injection of a single dose of streptozotocin (50 mg/kg). Streptozotocin was dissolved in a freshly prepared 0.01 M citrate buffer at pH 4.5 while the control rats were injected with the buffer alone. The streptozotocin-injected animals were given 5% glucose for 24 h to prevent initial streptozotocin-induced hypoglycaemic mortality. The experiment was conducted for 10 weeks. Rats with blood glucose levels of more than 200 mg/dl were considered diabetic and included in the experiment.

Naringenin dose selection and treatment

The doses of naringenin 5 mg and 10 mg/kg bodyweight/day were selected based on previously reported protective and antioxidant properties of this compound in rats. These doses have been found to suppress STZ induced type-I diabetes and improve the sperm quality in rodent model (Akondi et al. 2011). In the present study, rats were treated with naringenin (5 mg/kg and 10 mg/kg) given once a day orally for 10 weeks, starting 3 days after STZ injection.

Drugs and chemicals

Naringenin, streptozotocin, 3, 3'-Diamino benzidine tetra hydrochloride (DAB), bovine serum albumin (BSA) were purchased from Sigma chemicals Ltd (Sigma USA). The rat anti -mouse TGF-β1 and IL-1β antibodies were purchased from Biolegend Pvt, Ltd (Biolegend USA). TUNEL detected programmed cell death kit was purchased from Takara INC, Ltd (Japan). Proteinase K was purchased from Chromous Biotech Pvt. Ltd (Mumbai). HRP conjugated secondary goat anti rabbit IgG, purchased from (Bio-Genei), RIA kit (Immunotech Beckman Coulter Co., CA, USA).

All other chemicals and reagents used were of analytical grade and purchased in purest form, available from local firms.

Biochemical assays

Blood glucose level, serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, LDL (low density of lipoprotein), VLDL (very low density of lipoprotein) creatinine, urea, and albumin were estimated by using commercially available biochemical estimation kits (Autospan, India).

Serum testosterone analysis

Blood samples were collected, and the sera were separated and kept at –20 °C until testosterone concentrations were measured by means of the radioimmunoassay technique. Serum concentrations of total testosterone were measured by using a double antibody RIA kit (Immunotech Beckman Coulter Co.,

CA, USA). The assay sensitivity per tube was 0.025 ng/ml (Huang et al. 1995).

Evaluation of sperm count, sperm motility and sperm viability

Epididymal spermatozoa were collected by cutting the cauda region of the epididymis into small pieces in 2 ml of normal saline pre-warmed to 37 °C. Sperm was forced out of the cauda epididymis with fine forceps by putting pressure on the lower region of the cauda epididymis, not forcing out excess material i.e. immature cells. In this study, sperm motility, count, and viability were evaluated by using conventional methods (Raji et al. 2003). Progressive sperm motility was measured immediately after the collection of the sperm. The number of motile spermatozoa was calculated per unit area and expressed as percentage of sperm motility. Sperm counts were done using a haemocytometer and the results were expressed as millions/ml of suspension. Sperm viability was measured using Eosin and Nigrosin stain. The dead sperm took up the stain. Hundreds of sperm cells were counted in order to obtain the percentage of live/dead ratio.

Measurement of in vivo antioxidant system

The lipid peroxidation in the control and all treated groups of animals was measured by the quantification of thiobarbituric acid reactive substances (TBARS) determined by the method of Buege and Aust (Buege and Aust 1984). The activity of superoxide dismutase in the testis of control and treated rats were measured by the xanthine-xanthine oxidase cytochrome C method (Reddi and Bollineni 1997). The activities of glutathione peroxidase and catalase were measured by the modified method of Pagila and Valentine (Pagila and Valentine 1967) and Johansson and Hakan Borg (Johansson and Hakan 1988) method.

Histological examination

Ten weeks after the experiment, animals from each group were randomly selected, and a portion of the testis was excised from the ether anaesthetized rat, fixed in 10% formalin and processed for histological studies. Tissues are dehydrated through 70%, 90%, and 100% alcohol and embedded in low melting point paraffin wax. Sections of 5 μm thickness were cut and placed serially on glass slide. The sections were deparaffinized in xylene and rehydrated through 100%, 90%, and 70% alcohol. Three continuous sections were made from each testis tissue and stained with hematoxylin and eosin for histological evaluation using light microscopy. Spermatogenesis was assessed histopathologically using Johnsen's mean testicular biopsy score criteria (Johnsen 1970).

Moreover, the testes sections were observed and evaluated for the average number of spermatogonia, spermatocytes, spermatids and Sertoli cells per tubule.

Immunohistochemical analysis
Immunostaining of TGF-β1 and IL-1β: immuno-histochemical detection of TGF-β1 and IL-1β protein in cold acetone- fixed, paraffin-embedded testis section was performed by the avidin-biotin-peroxidase complex method (Jin et al. 2002). 5-μm thin sections of lysin-coated slides were deparaffinized and rehydrated through 100%, 90%, and 70% alcohol. For immunolabelling of TGF-β1 and IL-1β antigen retrievals were facilitated by heating the sections in citrate buffer pH 6.0, for 20 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 in 0.1 M Tris-NaCl, (pH 7.6) for 30 min. After incubation in 5% normal goat serum, sections were then separately incubated overnight at 4 °C with the primary rat anti-mouse TGF-β1 and IL-1β antibody. Sections were then incubated with a secondary goat anti rabbit IgG for 30 min at 37 °C with 1:100 dilution. This was followed by incubation with H_2O_2 and methanol (1:4) for 1 h. After that, 100–400 μl of DAB reagent was added to each section; as soon as sections turned brown, the slides were immersed in double-distilled water (ddH$_2$O). Then the slides were counter-stained with harris hematoxylin for 2 min. The tissue sections washed in ddH$_2$O for 5 min each. The sections were dehydrated and mounted with DPX (distyrene plasticizer xylene) and served as a positive control. The percentage of immunopositive cells were counted under a light microscope.

TUNEL assay detect programmed cell death
Programmed cell death in testis tissue sections were determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. The sections were digested with proteinase K (20 μg/ml) in PBS, for 15 min at room temperature, and rinsed with double-distilled water. Slides were then quenched by 2% H_2O_2 for 5 min at room temperature, equilibrated with buffer. Slides were then incubated with TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), followed by TdT reaction solution containing TdT and dUTP for 90 min at 37 °C, then washed with 2% standard saline citrate for 10 min at room temperature to stop the reaction. The slides were then washed with PBS for 5 min each and incubated with H_2O_2 and methanol for 30 min (1:4) at room temperature. Color was developed using 0.05% 3,3′-dimethyl aminobenzene in 0.01% H_2O_2 diluted with Tris-HCl (pH 7.5) then lightly counterstained with methyl green. The sections were then washed, dehydrated, and mounted. The cells undergoing programmed cell death were identified by a brown stain over the nuclei.

Statistical analysis
The results were expressed as mean value ± the standard error of the mean (SEM). Analysis of the variance (ANOVA) was used to test for the differences among all the groups at the significance level 2α=0.05. To find out where the significant difference occurred among the groups, the Duncan's multiple comparison test was used to compare the means. All statistical analyses were carried out using the appropriate Graph Pad Prism (Version 5).
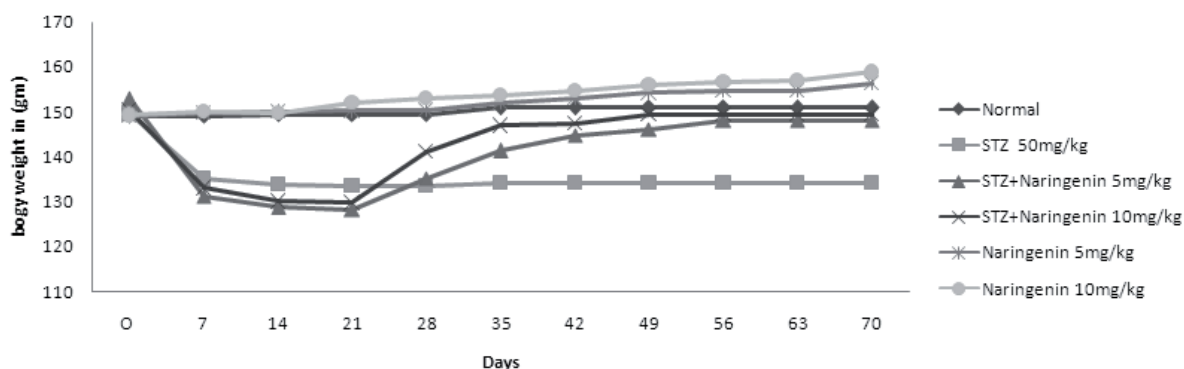

Fig. 1. **The effects of naringenin on body weight within groups.** Group I – control rats; group II – STZ control rats; group III – STZ + naringenin (5 mg/kg) treated rats; group IV – STZ + naringenin (10 mg/kg); group V – naringenin (5 mg/kg) and group VI – naringenin (10 mg/kg) treated rats.

RESULTS

Body weight changes

Fig. 1 depicts the body weight changes of different groups of rats and a decrease of body weight was observed in the STZ treated group during the experimental tenure. After 21 days of streptozotocin challenge, the naringenin treated groups (group III and IV) increased their body weight as compared to STZ treated group. The result also suggested that naringenin treated group (group IV) showing better efficacy on maintaining body weights in comparison with group III. Furthermore, gradual increases in body weights of the naringenin alone treated groups (groups V and VI) were similar to control rats.

Effect of naringenin on blood glucose level

The effect of naringenin on blood glucose levels in diabetic and non diabetic rats is shown in (Fig. 2). After 3 days of STZ injection a modest increase in the blood glucose levels was observed in all the groups (group II, III, IV) reaching an optimum level on the 7th day. From that day onwards naringenin treated diabetic groups maintained the decrement of their blood glucose levels for the remainder of the experiment. However, naringenin treated rats (group IV) showed better efficacy in comparison with group III. The naringenin treated alone groups (groups V and VI) did not show any affect on blood glucose levels.

Effect of naringenin on serum biochemical parameters

After the STZ challenge the serum total cholesterol, triglycerides, LDL, VLDL, albumin, creatinine and urea were significantly increased in the STZ treated group (group II) represented in Table 1. The diabetic groups treated by oral administration of naringenin showed a significantly decrease in above serum levels towards normal. STZ insult resulted in a significant reduction in HDL cholesterol in diabetic rats. The level of HDL cholesterol was significantly increased in naringenin treated diabetic groups (III and IV) as compared to STZ treated group. Significant differences were observed between the naringenin treated diabetic groups. Naringenin control rats (groups V and VI) did not show any effect of serum biochemical parameters and it was similar to that of the control (group I) (data not shown).

Serum testosterone levels

STZ-induced diabetic rats showed significantly lower serum testosterone levels (1.85±0.04) compared with all the experimental groups; however, these levels were reversed by administration of 5 mg and 10 mg of naringenin treated groups (2.17±0.07), (3.89±0.12) respectively, represented in Table 1. Naringenin control rats (groups V and VI) did not show any effect on serum testosterone levels, as with the control rats (data not shown).

Sperm parameters

The percentage of sperm count, sperm motility and sperm vitality were significantly decreased ($2\alpha=0.05$) in diabetic control group in comparison with other experimental groups. Values of sperm count, motility percentage and sperm vitality percentage were given in Table 2. All the above parameters were significantly increased in the case of all the treated groups but naringenin (10 mg/kg) treatment significantly
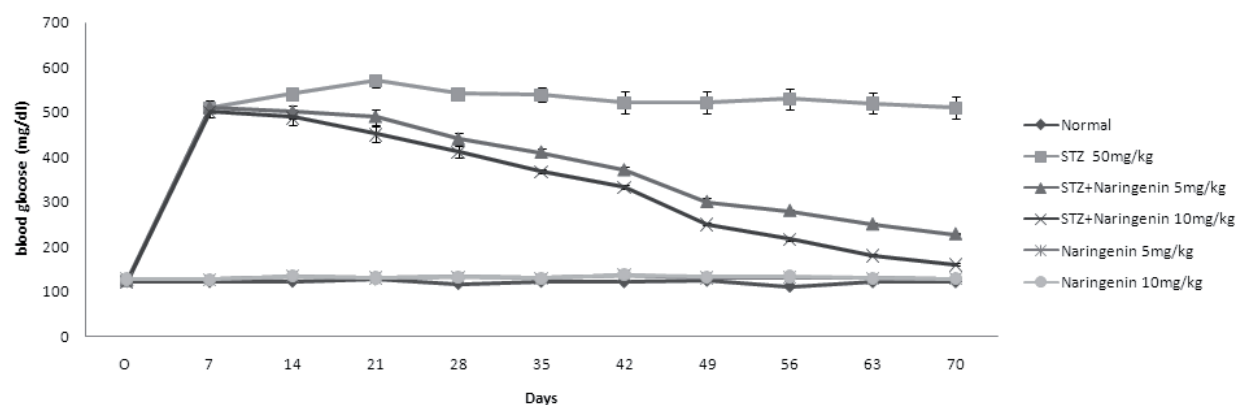

Fig. 2. **Blood glucose levels within groups after 10 weeks.** Group I – control rats; group II – STZ control rats; group III – STZ + naringenin (5 mg/kg) treated rats; group IV – STZ + naringenin (10 mg/kg); group V naringenin (5 mg/kg) and group VI naringenin (10 mg/kg) treated rats. Values are given as mean ± SEM for groups of ten animals each.

increased (2α= 0.05) the sperm count, motility and viability as compared to naringenin (5 mg/kg) treated alone. Naringenin control rats (groups V and VI) did not show any effect on sperm parameters levels as with the control rats (data not shown).

Table 1. **Effect of naringenin on serum biochemical parameters in rats.**

Biochemical parameters (unit)	Control (I)	STZ 50 mg/kg (II)	Naringenin 5 mg/kg (III)	Naringenin 10 mg/kg (IV)
Cholesterol (mg/dl)	52.4±0.60*	236.4±2.90	61.04±1.38*	55.59±0.82*#
Triglycerides (mg/dl)	86.32±4.90*	292.3±16.91	93.3±1.42*	88.43±1.12*#
HDL cholesterol (mg/dl)	39.6±0.7*	23.3±1.04	32.58±0.94*	37.87±0.69*#
LDL cholesterol (mg/dl)	11.30±0.26*	62.12±2.15	35.85±1.38*	12.51±0.64*#
VLDL cholesterol (mg/dl)	5.28±0.27*	51.0±2.27	21.10±0.80*	7.41±0.47*#
Albumin (gm %)	3.045±0.10*	6.7±0.38	4.35±0.22*	3.37±0.10*#
Creatinine (mg %)	0.571±0.033*	1.69±0.15	1.00±0.09*	0.65±0.04*#
Urea (mg %)	16.26±0.72*	167.6±7.12	67.89±2.79*	18.6±1.03*#
Testosterone (ng/ml)	4.11±0.05*	1.85±0.04	2.17±0.07*	3.89±0.12*#

Values are given as mean ± SEM for groups of 10 animals each. * Statistically significant as compared with streptozotocin group. # Statistically significant as compared with naringenin (5 mg/kg) treated diabetic rat.

Table 2. **Effect of naringenin on sperm parameters (Sperm motility, viability and Count) and counting of spermatogenic cells for each group.**

Parameters	Normal control (I)	STZ control 50 mg/kg (II)	Naringenin 5 mg/kg (III)	Naringenin 10 mg/kg (IV)
Sperm motility (%)	62.90±1.13*	21.25±0.98	32.60±0.93*	57.66±0.96*#
Sperm viability (%)	71.40±1.15*	23.20±0.82	33.30±1.31*	68.80±0.77*#
Sperm count (10^6/ml)	27.19±0.67*	7.78±0.57	10.14±0.60*	24.60±0.84 *#
Spermatogonia	16.22±0.81*	7.22±0.65	13.01±0.77*	16.44±0.81*#
Spermatocytes	125.01±1.6*	35.28±2.76	89.32±1.7*	95.56±0.88*#
Spermatids	124.0.4±2.44*	38.55±2.66	91.12±2.1*	97.65±1.2*#
Sertoli cells	15.65±0.87*	6.28±0.98	10.66±0.68*	13.77±0.88*#

Symbols as in Table 1.

Measurement of in-vivo antioxidant status
Fig. 3 indicates the effect of naringenin on the activities of testicular enzymatic antioxidants such as SOD, catalase, GSH and TBARS levels in the control and experimental groups of rats. SOD (0.52±0.03), catalase (7.91±0.1) and glutathione peroxidase (4.3±0.68) were significantly decreased in STZ-induced diabetic rats testis, while both the naringenin treated diabetic rats significantly increased the activities of SOD, catalase, and glutathione peroxidase. Naringenin treated control rats (groups V and VI) exhibited SOD, catalase, and glutathione peroxidase activities as with the control rats (group I; data not shown). The lipid peroxidation (TBARS) in the diabetic rats was significantly increased (1.99±0.06). Administration of 5 mg/kg naringenin (1.55±0.04) and 10 mg/kg naringenin (1.03±0.32) for 10 weeks significantly decreased the TBARS levels. The naringenin control rats (groups V and VI) did not show any marked changes in the TBARS levels (data not shown).
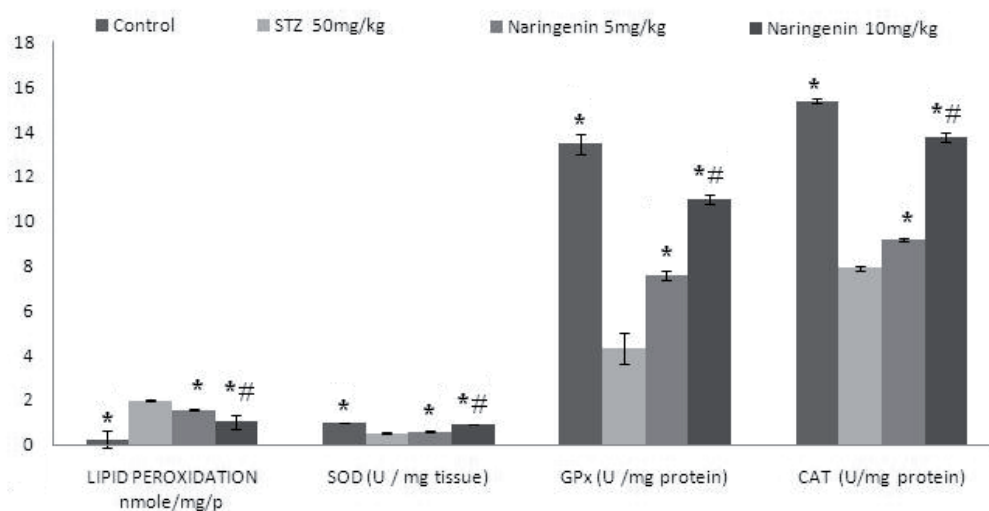
Fig. 3. **Effect of naringenin on the activity of lipid peroxidation, SOD, glutathione peroxidase (GPx) and catalase (CAT) in rat testis tissue.** * Statistically significant as compared with streptozotocin control. # Statistically significant as compared with naringenin (5 mg/kg) treated diabetic rat.

Histopathological changes

The photograph of the testicular histopathological section of control, STZ treated and as well as naringenin treated rats is shown in (Fig. 4). Fig. 4a depicts the histological testicular tissue sections of control rat's demonstrating a well preserved normal cellular architecture of the testis comprised of spermatogonia, Sertoli cells, Leydig cells, primary spermatocytes, spermatids, late spermatids, spermatozoa with an albugineous layer and the complete seminiferous tubule cell series. Fig. 4b represent the architectural changes of the diabetic rat testis with cluster of the interstitial Leydig cells and seminiferous tubules were observed prominently. Fig. 4c and d shows that the size of the seminiferous tubules was strongly reduced when the morphology of the epithelium was severely impaired with vacuolated cytoplasm. The seminiferous tubule structure in the diabetic rats was found to be disrupted, and there was a considerable decrease in the number of spermatogenic cell series like spermatogonia, Sertoli cells, primary spermatocytes, spermatids (shown in Table 2) . Also in diabetic rats, atrophy of the tubules with varying degrees of spermatogenetic arrest or reduced formation of spermatozoa was detected. Some degenerated spermatozoa were profound in the lumen of the tubules. It was observed from histological examinations that naringenin (5 mg/kg) treatment to diabetic rats somehow corrects the architectural changes and there was an improvement in the seminiferous tubule structure compared with the diabetic group which faintly appeared in the Leydig cells and a few spermatogenic cell series (Fig. 4e, f). The naringenin (10 mg/kg) treated diabetic group regained of the seminiferous tubules lined by several layers of spermatogenic cell series, similar to that of control, is shown in (Fig. 4g, h). The naringenin control rats (groups V and VI) showed no sign of architectural changes in testis tissue sections (figure not shown).

Effect of naringenin on expression of TGF-β1

Fig. 5 depicts the expression of TGF-β1 in testis tissue examined immunohistochemically. The testicular sections of control group showed a complete absence of immunostaining of TGF-β1 (Fig. 5a) and the percentage of immunostaining was expressed in Fig. 7. Positive staining of this protein was observed in the Sertoli and Leydig cells lined by several spermatogenic epithelium of STZ-treated diabetic rat testis (26.5±0.21) (Fig. 5b). In contrast, a decrement of TGF-β1 immunopositivity was observed in naringenin-(5 mg/kg) and naringenin (10 mg/kg) treated diabetic rats (17.87±0.29), (10.27±0.28) respectively compared to the STZ treated group (Fig. 5c, d). Moreover a significant reduction of TGF-β1 immunopositivity was observed in 10 mg/kg of the naringenin treated diabetic group as compared to 5 mg/kg of naringenin treated group.
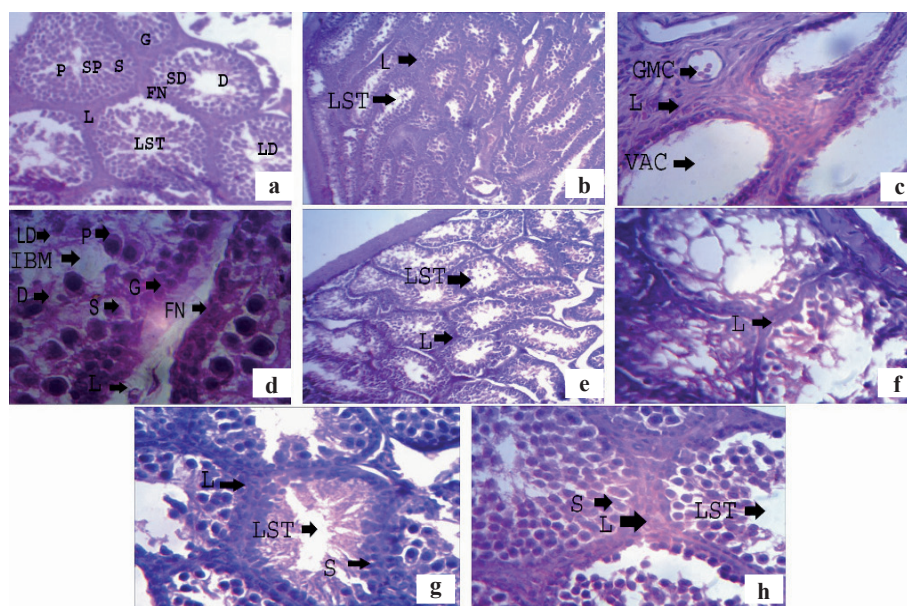
Fig. 4. **Histological profile of representative testis tissue section in experimental animals.** (*a*) Represents normal control rat testis (group I) showing spermatogonia (G), cytoplasm of sertoli cell (S), spermatozoon (SP), leydig cell (L); varying stages of sperm development (SD); Lumen of seminiferous tubule (LST), spermatogonia (G) and sertoli cells (S) resting on the basement membrane which was surrounded by myoid cells with their flat nuclei (FN), Primary spermatocytes (P), spermatids (D) and late spermatids (LD) were also observed (H & E ×100); (*b*) STZ control group showing irregular seminiferous tubules and derangement of the testicular tissue section (H & E ×100); (*c*) showing giant multinucleated cells (GMC), clustered of interstitial leydig cells (L), thickened interstitial tissue and vacuolated cytoplasm (VAC) (H & E ×200); (*d*) showing irregular basement membrane (IBM), few seminiferous tubules lined with spermatogenic cell series (H & E ×200); (*e*) represented naringenin (5 mg/kg) treated diabetic groups, showing faintly appearance of seminiferous tubules (LST), leydig cells and rearrangement of the testis tissues (H & E ×100); (*f*) represented naringenin (5 mg/kg) treated diabetic group showing slightly appearance of spermatogenic cell series leydig cells (H & E ×200); (*g, h*) represented naringenin 10 mg/kg treated diabetic group, showing completely recovered seminiferous tubules (LST) lined with spermatogenic cell series, spermatogonia (G), sertoli cells(s), leydig cells (L), primary spermatocytes (P), (H & E ×200, scale bar, 50 μm).

Effect of naringenin on expression of IL-1β

Fig. 6 depicts the representative photomicrographs of the immunohistochemical staining of IL-1β in testis tissues. Fig. 7 shows the percentage of expression of IL-1β immunopositive cells of various experimental groups. The testis sections of the control group shows a complete absence of immunostaining of IL-1β (Fig. 6a). Positive staining of the pro-inflammatory cytokines was observed in the Sertoli cell and Leydig cells lined by several spermatogenic epithelium of STZ-treated diabetic rat testis (152.5±0.69) (Fig. 6b). In contrast, a decrement of IL-1β immunopositivity was observed in group III (110.8±1.3) and in group IV (84.9±1.93) compared to the STZ group (Fig. 6c, d). Furthermore a significant difference was observed between the naringenin treated diabetic groups.

Effect of naringenin on programmed cell death

Programmed cell death was performed by TUNEL methods (Fig. 8) and the percentage of TUNEL positive cells is shown in Fig. 9. The chromogen generated dark brown strain was an indication of TUNEL positive cells. It should be noted that in almost every case the brown stain overlaps the condensed chromatin of apoptotic bodies, conforming that TUNEL assay correlates to the morphological appearance of programmed cell death. The rate of TUNEL positive cells was generally very low in the control group (Fig. 8a) and more positive staining was expressed in STZ-induced testis sections (Fig. 8b), whereas fewer TUNEL positive cells were observed in naringenin-treated groups (group III and IV) (Fig. 8c, d). The percentage of TUNEL positive cells

was detected and the value of the streptozotocin treated group was found as (16.10±0.64). A reduction of TUNEL positive cells by 5 mg/kg of naringenin (6.95±0.3) and 10 mg/kg of naringenin (1.54±0.15) treated diabetic rat

was statistically significant compared to STZ treated group. The percentage of TUNEL positive cells of 5 mg/kg of naringenin treated diabetic rat was significantly less compared to the 10 mg/kg of naringenin group.
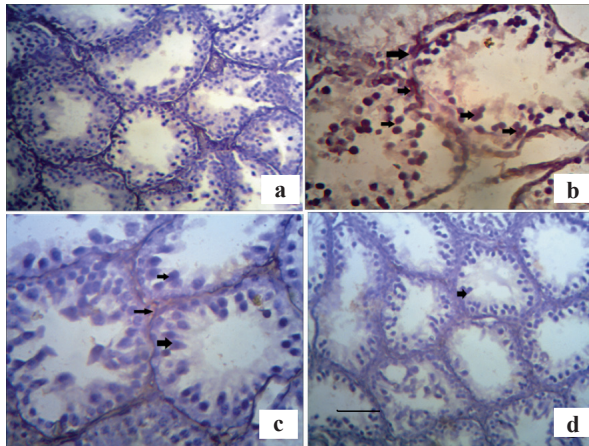

Fig. 5. **Immunohistochemical localization of TGF-β1 in the rat testis tissue.** The figure shows representative DAB staining for TGF-β1 against hematoxylin counter stain. (*a*) Represents normal testis (group I) (×100); (*b*) represents STZ control testis (group II) (×200); (*c*) represents naringenin (5 mg/kg) + STZ (group III) (×200); (*d*) represents section from naringenin (10 mg/kg) + STZ (group IV) (×100). Testis sections of STZ control group rats show more intensive staining (scale bar, 50 µm). Arrow represents the expression of TGF-β1.
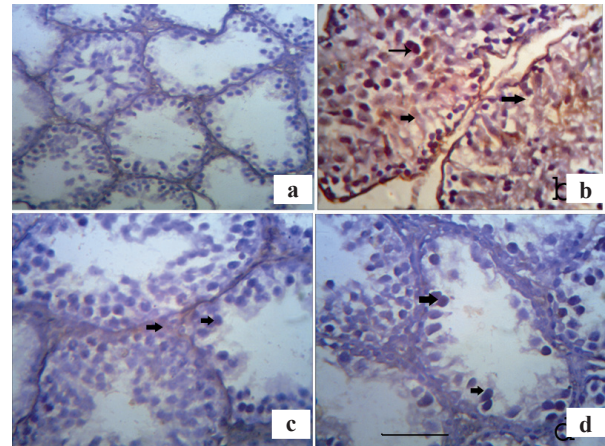
Fig. 6. **Immunohistochemical localization of IL-1β in the rat testis tissue.** The figure shows representative DAB staining for IL-1β against hematoxylin counter stain. (*a*) Represents normal testis (group I). (×100); (*b*) represents STZ control testis (group II) (×200); (*c*) represents naringenin (5 mg/kg) + STZ (group III) (×200); (*d*) represents section from naringenin (10 mg/kg) + STZ (group IV) (×200). Testis sections of STZ control group show more intensive staining (scale bar, 50 µm). Arrow represents the expression of IL-1β.
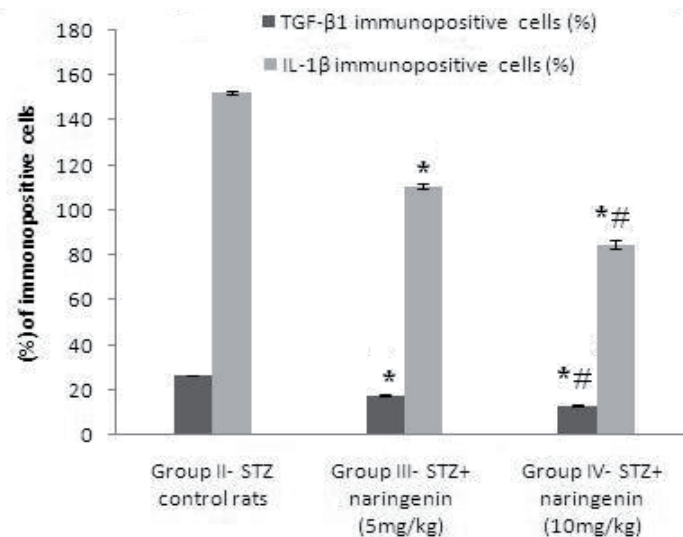

Fig. 7. **Effects of naringenin on TGF-β1 and IL-1β immunopositivity in rat testis.** Approximately 200 cells were counted per field, five fields were examined per slide and five slides were examined per group. Results were analyzed by one-way ANOVA, confirmed by post hoc Duncan's multiple comparison test. * Statistically significant as compared with streptozotocin control. # Statistically significant as compared with naringenin (5 mg/kg) treated diabetic rat.
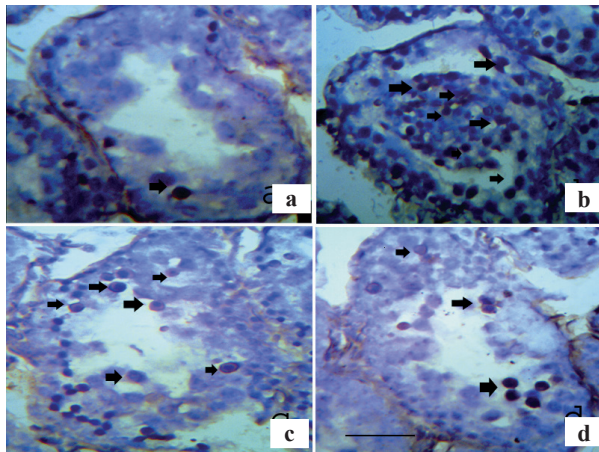
Fig. 8. **Representative immunohistochemistry pictures of TUNEL positive cells in testis sections (×200).** Arrow represented the expression of TUNEL-detected programmed cell death. (*a*) Immunostaining of rat testis from normal control (group I) showed absence of brown staining that represented the normal programmed cell death of testis sections; (*b*) STZ group (group II) showing more staining indicative cells undergoing intensive programmed cell death; (*c*) naringenin (5 mg/kg) + STZ (group III) exhibited less cells undergoing programmed cell death; (*d*) naringenin (10 mg/kg) + STZ (group IV) exhibited very few number of stained nuclei represented minimal appearance of programmed cell death (scale bar, 50 µm).

DISCUSSION

The present investigation demonstrated the protective potential of naringenin against hyperglycaemia-mediated oxidative stress in streptozotocin induced diabetic testicular damage. Diabetes mellitus is a chronic disease affecting many tissues and systems of the body. Some of these manifestations were spermatogenic and steroidogenic alterations (Shrilatha and Muralidhara 2007). The STZ induced diabetic rodents exhibited frequent abnormal architectural changes in the seminiferous tubule lined by spermatogenic cell series, Sertoli cells and Leydig cells, which leads to a decrease in plasma testosterone levels (Ricci et al. 2009). Our results suggested that diabetes mellitus in male rats caused testicular dysfunctions, and that naringenin treatment improved these functional deficits by providing protection against the impairment of seminiferous tubules and the loss of spermatogenic cell series. The Sertoli cells formed the basal and apical surface of the seminiferous tubule and provided the cyto-architectural framework for the developing germinal cells (Petersen and Soder 2006). Thus, the interactions of all three somatic cells, Sertoli, peritubular, and Leydig, are important for regulation of normal spermatogenic function in the testis (Skinner 1991).

In this study, the decrease in body weight observed in uncontrolled diabetes might be the result of protein
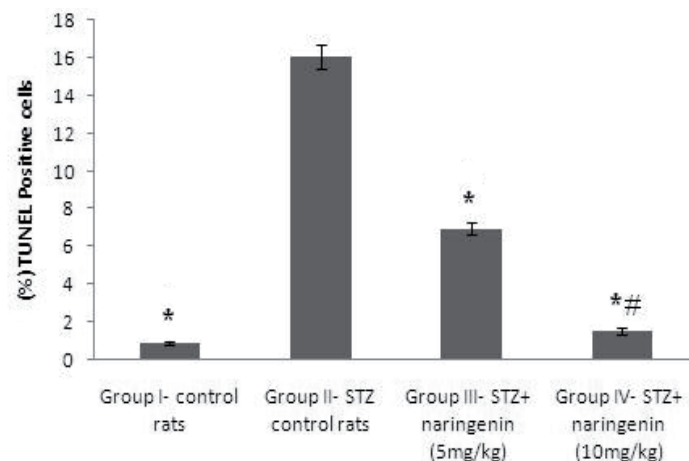

Fig. 9. **Percentage (%) of TUNEL positive cells of rat testis.** Approximately 200 cells were counted per field, five fields were examined per slide and five slides were examined per group. Results were analyzed by one-way ANOVA, confirmed by post hoc Duncan's multiple comparison test. * Statistically significant as compared with streptozotocin control. # Statistically significant as compared with naringenin (5 mg/kg) treated diabetic rat.

wasting due to the unavailability of carbohydrate for utilization as an energy source. Body weights were increased extensively by the administration of naringenin compared with the diabetic group. Likewise in our study, reduced body weights were improved by the administration of naringenin in the diabetic rats (Faramarz and Mehrdad 2012).

STZ causes diabetes by the rapid depletion of the beta cells mass which leads to a reduction in insulin release and hyperglycaemia (Gupta et al. 2004). In this study, the diabetic group had a drastically higher blood glucose level relative to the control group and naringenin treated rats showed a modest decrease in blood glucose levels. The naringenin might be producing its hypoglycaemic effect by an extra-pancreatic action, e.g., possibly by stimulating glucose utilization in the extra-hepatic tissues or increases in the expression of insulin receptors in the liver plasma membranes (Pinent et al. 2004).

The present study was intended to investigate the actions of naringenin on serum biochemical parameters in diabetic rats. The degree of hypercholesterolaemia is directly proportional to the severity of the diabetes and the diabetic rats had a noticeable decrease in HDL cholesterol (Al-Shamaony et al. 1994). Administration of naringenin to the diabetic rats decreased the levels of triglycerides, total cholesterol, LDL, VLDL and increased HDL cholesterol levels. Several reports have demonstrated that naringenin is a potent agent for the inhibition of HMG-CoA reductase and also beneficial for lowering serum cholesterol levels (Ana et al. 2009). Profound changes in protein metabolism occur in diabetes, and the enhanced catabolism of muscle proteins in diabetes elevates the serum creatinine levels (Almdal and Vilstrup 1988). Our findings concluded that naringenin has a positive effect in diabetic rats by decreasing the creatinine levels. The diabetic hyperglycaemia induces the elevation of serum levels of urea and albumin. In our study, STZ-induced diabetic rats increased serum urea, albumin concentrations. Administration of naringenin decreased the levels of serum urea and albumin.

Previous studies have indicated that STZ induced diabetes caused a significant decrease in the total serum testosterone level in the diabetic (Khaki et al. 2010). In this experiment, the serum testosterone levels were distinctly decreased in the diabetic group. These levels were significantly inverted by administration of naringenin to the treated diabetic group.

Type I diabetes mellitus may affect endocrine function and spermatogenesis (Sexton and Jarow 1997). In diabetes mellitus, hyperglycaemia increases oxidative stress (ROS) and causes DNA damage in testis: a significant reduction in sperm parameters like sperm motility, sperm count and sperm viability (Amaral et al. 2006). Naringenin significantly altered the sperm parameters by modulating the antioxidant status of the testis. Furthermore, naringenin (10 mg/kg) had shown better efficacy in maintaining the sperm parameters.

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems (Fang et al. 2002). When the balance is disrupted towards an over abundance of ROS, oxidative stress (OS) occurs in STZ induced diabetic rats (Roy et al. 2011). Oxidative stress plays a role in the development of diabetic complications (Sexton and Jarow 1997). Certain enzymes play an important role in antioxidant defense, to maintain ability for viable reproduction; a protective mechanism against oxidative stress is of importance. These enzymes include SOD, glutathione peroxidase and catalase, which convert free radicals or reactive oxygen intermediates to non-radical products. Previous reports have suggested that SOD, catalase, and GSH are major enzymes that scavenge harmful ROS in male reproductive organs (Fujii et al. 2003). In this study, we observed in diabetic rat that the decreased activities of SOD, catalase, and glutathione peroxidase in testis, play an important role in scavenging free radicals. In line with this, our results also showed that oral administration of naringenin to diabetic rats for 10 weeks increased the activities of testis SOD, catalase, and glutathione peroxidase. In the diabetic state, lipid peroxidation can be induced by protein glycation and glucose auto-oxidation that can further lead to the formation of free radicals (Mullarkey et al. 1990). The main free radicals that occur in this diseased state were superoxide, hydroxyl and peroxyl radicals. These free radicals all might play a role in DNA damage, glycation and protein modification reactions, and in lipid oxidative modification in diabetes (Hunt et al. 1990). The concentration of testis TBARS (thiobarbituric acid) was high in STZ-induced diabetic rats, whereas these levels in naringenin treated rats significantly decreased the concentrations of TBARS which indicate the decreased rate of lipid peroxidation. Several reports have demonstrated that naringenin possesses a lot of activities including antioxidative activity (Felgines et al. 2000).

Under an elevated oxidative stress status, ROS cause cellular injury via several mechanisms including lipid peroxidation and oxidative damage of proteins and DNA (Buttke and Sandstrom 1994). It has previously been shown that diabetes mellitus increases oxidative stress in the diabetic male testis (Baynes and Thorpe 1999). The seminiferous tubules are the site of spermatogenesis where germ cells develop into spermatozoa in close interaction with Sertoli cells. The Sertoli cell is an important testicular somatic cell which controls the

germ cell environment by the secretion and transport of nutrients and regulatory factors (Skinner 1991). Mitochondria play an important role in the apoptotic process. The mitochondrial dysfunction induced by oxidative stress can lead to the release of cytochrome c and then caspase activation, which results in apoptotic cell death (Leon et al. 2005). It is well known that diabetes mellitus induces testicular dysfunction by causing apoptotic cell death (Cai et al. 2000). In this revision, the number of TUNEL-positive germ cells significantly increased in the diabetic group. Naringenin treatment reduced the reactivity and the number of TUNEL-positive germ cells.

The present study addresses the application of an animal model to evaluate the effects of diabetes on the histological structure of the testis. STZ induced diabetes provides a relevant model to study the effect of diabetes. The changes in sexual and reproductive tract functions were similar to those encountered in man and rats (Hassan et al. 1993). Histological findings clearly showed that the normal architecture of the testis tissue was altered due to administration of STZ and it released reactive oxygen species causing degeneration in seminiferous tubules lined by several spermatogenic cell series, Sertoli cells and Leydig cells. In contrast, rats treated with naringenin showed noticeable improvement in histopathological parameters. There are no studies available on the protective nature of naringenin on the testis tissue during experimental diabetes. Hence, this investigation should be considered an innovative assessment for the testicular tissue protective nature of naringenin in rats insulted by streptozotocin.

The Sertoli cell is responsible for the overall control of testis development. The interstitial space around the seminiferous tubules contains another somatic cell type, the Leydig cell, which is responsible for testosterone production. Although the Leydig cell has numerous secretory products (Skinner 1991) testosterone is the most significant secretory product of these cells. TGF-β1 is expressed by the Sertoli cell (Esposito et al. 1991) and may be important for spermatogenesis (Nargolwalla et al. 1990). Sertoli cell production of TGF- β1 may be targeted to the germinal cell population due in part to the blood-testis barrier (Mullaney and Skinner 1993). Interleukins-1β (IL-1β) is produced in the testis by Sertoli cells and Leydig cells. These interleukins can regulate Sertoli, Leydig, and germ cell growth and differentiation functions (Meroni et al. 2000). Previous report have suggested that STZ induced diabetes enhances the expression of TGF-β1 and IL-1β in testis and changes in testicular function (Salama et al. 2001). In the present investigation we demonstrated that injection of STZ induces rapid and transient increases in the levels of proteins, and pro-inflammatory cytokines in the rat testis. In our recent findings, over expression of TGF-β1 and IL-1β in STZ induced rats was markedly reduced by naringenin-treated groups.

In summary, our findings provide substantial evidence that naringenin improves testicular damage. To the best of our knowledge, this is the first report indicating that naringenin improves diabetes induced testicular dysfunction in diabetic rats. Naringenin can contribute to a balanced oxidant-antioxidant status and modulate the levels of ROS, the number of TUNEL-positive cells and also cytokine expression in diabetic testis could therefore be a suitable candidate for further investigations that involve the therapeutic treatment of post diabetic testicular dysfunction.

ACKNOWLEDGEMENT

DISCLOSURE

The authors declare that there is no conflict of interest.

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