Toxicology 282 (2011) 69–81

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Protective effects of nanostructures of hydrated C_{60} fullerene on reproductive function in streptozotocin-diabetic male rats

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ARTICLE INFO

Article history:
Received 14 September 2010
Received in revised form
28 November 2010
Accepted 6 December 2010
Available online 14 December 2010

Keywords:
Streptozotocin
Diabetes mellitus
Hydrated C_{60} fullerene
Testis
Oxidative stress
Apoptosis
Sperm characteristics
Fatty acid composition

ABSTRACT

Diabetes mellitus is a well-recognized cause of male sexual dysfunction and impairments of male fertility. Streptozotocin (STZ) is used for medical treatment of neoplastic islet β-cells of pancreas and producing of animal model of diabetes mellitus type 1 that is characterized by suppression of reproductive activity due to the hyperglycaemia-induced oxidative stress and histopathological alterations in testes. Seeking for the agents that could alleviate diabetes-induced damage to reproductive system is yet the important area of inquiry. The present study was designed to evaluate whether hydrated C_{60} fullerene (C_{60}HyFn), which is known to be powerful bioantioxidant, eliminate testicular dysfunction induced by STZ-diabetes in rats. Wistar strain male albino rats were divided into four groups of six animals each: (1) control group, (2) C_{60}HyFn-treated nondiabetic group, (3) STZ-diabetic group and (4) C_{60}HyFn-treated diabetic group. Once hyperglycaemia was induced by STZ, rats in the second and fourth groups were treated with C_{60}HyFn (in the form of drinking water) at the dose of 4 μg/kg daily for 5 weeks. In diabetic rats, relative weights of right cauda epididymis, seminal vesicles, prostate, sperm motility and epididymal sperm concentration were significantly less than those of control group, but which were restored in the fourth group treated with C_{60}HyFn ($p < 0.001$). In hematoxylin and eosin staining, marked histopathological changes including degeneration, desquamation, disorganisation and reduction in germinal cells, interstitial oedema and congestion were evident in the testis of diabetic rats, but C_{60}HyFn treatment resulted in recovery of histopathological changes and an increase in Johnsen's testicular score significantly ($p < 0.001$). C_{60}HyFn treatment restores the increased apoptosis induced by STZ-diabetes. In diabetic rats, levels of serum testosterone, testicular reduced glutathione (GSH) and alpha-tocopherol were significantly reduced and testicular lipid peroxidation level was increased ($p < 0.001$). Nevertheless, treatment of diabetic rats with C_{60}HyFn resulted in significant corrective effects on these parameters towards the control levels. C_{60}HyFn, applied alone, did not exert any toxic effects in testicular tissues. Furthermore, C_{60}HyFn treatment in diabetic and nondiabetic rats resulted in considerable elevations of some important polyunsaturated fatty acids. In conclusion, we have presented for the first time substantial evidence that administration of C_{60}HyFn significantly reduces diabetes-induced oxidative stress and associated complications such as testicular dysfunction and spermatogenic disruption.

1. Introduction

Streptozotocin (STZ) has long been used as a tool for creating experimental diabetes in animal. An advantage of the STZ-model is that it closely mimics type 1 diabetes in humans rather than type 2 (Schnedl et al., 1994). Due to its relatively specific β-cell

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doi:10.1016/j.tox.2010.12.003

cytotoxic effect STZ is used for treating metastatic cancer of the pancreatic islet cells. While it carries a substantial risk of toxicity and rarely cures the cancer, its use is generally limited to patients whose cancer cannot be removed by surgery (Brentjens and Saltz, 2001). STZ was found to be selectively toxic to the β-cells of the pancreatic islets, the cells that normally regulate blood glucose levels by producing the hormone insulin. Therefore, the main symptom of STZ intoxication is hyperglycaemia causing short and long term diabetic complications (Brownlee, 2005). It is well recognized that diabetes-induced hyperglycaemia leads to oxidative stress caused by impairment of mitochondrial electron transfer and activation of the polyol pathway resulting in the excessive generation of reactive oxygen species (ROS) (Sivitz and Yorek, 2010; Jang et al., 2010). ROS in concert with nitric oxide species (NOS) alter intracellular macromolecules, such as lipids, proteins, nucleic acids and carbohydrate as well as constituents of membranes (Halliwell and Gutteridge, 2007). The level of lipid peroxidation in cell is controlled by enzymatic and nonenzymatic antioxidant system, scavenger systems, whose levels are disturbed in diabetes. Thus, elevated ROS and NOS levels together with depleted endogenous antioxidant capacity aggravate oxidative stress (Pérez-Matute et al., 2009).

Although scientists and clinicians largely focus on common diabetic complications such as diabetic cardiomyopathy, nephropathy, neuropathy, retinopathy, and skin ulcer, diabetes has the detrimental effects on testis (Mallick et al., 2007; Zhao et al., 2010). It is well known that infertility is common complication in diabetic men and also in experimentally induced diabetes mellitus in rats, male reproductive activity are also suppressed. Diabetes is considered to produce erectile dysfunction, retrograde ejaculation and reduced levels of testicular hormone and seminal quality changes in diabetic men (Pitteloud et al., 2005). Serum testosterone impairment and varying degrees of testicular and epididymal structural lesions have also been demonstrated in STZ-induced diabetic animal models (Anderson and Thliveris, 1986; Cai et al., 2000). Besides, free radicals, which control sperm maturation, capacilitation and hyperactivation in health, can induce lipid peroxidation, DNA damage and apoptosis of spermatozoa when hyperglycaemia triggers a cascade of biochemical event resulting in oxidative stress (Kothari et al., 2010).

Both forms of diabetes are associated with long-term complications that affect function of reproductive organs. However, the vast majority of patients with type 1 diabetes are diagnosed before the age of 30 (Williams and Pickup, 2004), thus diabetes affects much more males prior to and during their reproductive years. On the other hand, the potential impact of diabetes in young males and its effects on their reproductive function remain poorly explored. Due to multiple action of STZ intoxication (and keeping in mind the growing prevalence of diabetes mellitus in the world) understanding how uncontrolled hyperglyceamia impacts the reproductive function and seeking for efficient drugs able to alleviate diabetes-induced complications, are yet important areas of inquiry. In this work, we focused our attention on C_{60} fullerene, the third allotrope of carbon, which is considered to be the most prominent member of the nanomaterial family (Kroto et al., 1985). Since their discovery in 1985, fullerenes have been investigated extensively due to their unique physicochemical properties and biological activities (Prato, 1997; Bosi et al., 2003; Satoh and Takayanag, 2006). C_{60} is practically insoluble in water that limits its biomedical application (Ruoff et al., 1993). Various methods of chemical derivatization and supramolecular complexes formation have been developed to make fullerene molecules water-soluble and increase their ease of use in biological systems (Nakamura and Isobe, 2003; Partha and Conyers, 2009). However, it is important to emphasize that C_{60} nanoparticles prepared by different solubilisation and modification methods exhibit highly distinct biological effects. Some functionalized derivates of fullerenes are reported to display pronounced toxic effects in vivo and in vitro indicating that biological responses highly depends on the chemical nature of attached functional groups but not C_{60} core itself (Kolosnjaj et al., 2007). Nevertheless, fullerenes appeared to be the potent antioxidants and being investigated as therapies for a wide range of diseases (Beuerle et al., 2008). C_{60} fullerene and some of its derivatives have been shown to exhibit effective protection against oxidative stress in vitro and in vivo (Tsai et al., 1997; Monti et al., 2000; Srdjenovic et al., 2010) without inducing acute or subacute toxicity (Gharbi et al., 2005; Mori et al., 2006). However, despite the growing body of effects noted in the literature, question remains regarding fullerene effects on mammal's reproductive function.

In contrary to the methods of chemical modifications of fullerene, the method for producing of water-soluble chemically unmodified C_{60} fullerene without using of any solubilizers or stabilizers has been developed (Andrievsky et al., 1995). The preparation obtained has been denoted as "hydrated C_{60} fullerene" (C_{60}HyFn). Stable aqueous solutions of C_{60}HyFn in water (C_{60} fullerene water solution – C_{60}FWS) contain single hydrated C_{60} fullerene molecules as well as their labile clusters (secondary associates) with the size of 3–36 nm. Chemically, C_{60}HyFn is highly hydrophilic and highly stable donor–acceptor complexes C_{60} with water molecules – $C_{60}@\{H_2O\}_n$, $n = 22–24$ (Andrievsky et al., 1995, 1999, 2002; Avdeev et al., 2004). Biological effects of C_{60}HyFn have been the subjects of intense research over the past decade, indicating its beneficial action as neuroprotectant, anticancer, antiinflammatory, antiatherogenic, and radioprotective agent (Andrievsky et al., 2005, 2009; Podolski et al., 2007; Tykhomyrov et al., 2008). It is proved that positive effects of C_{60}HyFn are mainly determined by its unique bio-antioxidative properties, which are unexpectedly manifested even at super-small concentrations and doses (Andrievsky et al., 2005, 2009, 2010).

Using of co-administration of toxin STZ, which is able to induce systemic metabolic disturbance in the whole organism and diabetic complications as well, and nanostructures of hydrated C_{60} fullerene, which are known to be the potent antioxidant and tissue-protective agent, we were focused on two general questions: (1) what are the effects of C_{60}HyFn on reproductive function of male rats, if any; (2) whether C_{60}HyFn administered to STZ-treated rats is capable of preventing of testicular damage and increasing sperm viability and quality? The answer on the second question could provide clinical implication of water-soluble fullerenes for developing novel antisterility drugs.

2. Materials and methods

2.1. Chemicals and C_{60} fullerene water solution (C_{60}FWS)

C_{60} fullerene samples with purity of more than 99.98% were obtained from "TERM" (Moscow State University, Russia). Sodium lauryl sulfate (SDS), acetic acid, thiobarbituric acid aqueous solution (TBA), n-butanol, pyridine, 1,1,3,3-tetramethoxypropane standard, trichloroacetic acid (TCA), phosphate buffer, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), and reduced glutathione (GSH) standard, reagents, were obtained from Fluka (Taufkirchen, Germany). All the other chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All the chemicals used were of analytical grade.

C_{60}FWS was produced without using any solubilizers and chemical modification. This method is based on transferring of fullerene from organic solution into the aqueous phase by ultrasonic treatment as described elsewhere (Andrievsky et al., 2005). Concentrated C_{60}FWS (up to 5.5×10^{-3} M ~ 4 mg/ml) contains both single C_{60}HyFn ($C_{60}@\{H_2O\}_n$ or $C_{60}@nH_2O$, where $n = 22–24$) and their labile nanoassociates with the size of 3–72 nm (Andrievsky et al., 1999, 2002; Avdeev et al., 2004). Concentrated C_{60}FWS solution was diluted in distilled water so that the final concentration of C_{60}HyFn in drinking water was 30 nM (Tykhomyrov et al., 2008).

2.2. Animals and experimental design

The experimental protocols were approved by the local Animal Use Committees of Firat University (Elazig, Turkey). Animal care and experimental protocols complied with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). Forty healthy adult male Wistar albino rats, aged

8–9 weeks and weight in the range of 180–210 g, were obtained and maintained from Firat University Experimental Research Centre (Elazig, Turkey). The animals were housed in polycarbonate cages in a room with a 12 h day–night cycle, temperature of 24 ± 3 °C, humidity of 45–65%. During the whole experimental period, animals were fed with a balanced commercial diet (Elazig Food Company, Elazig, Turkey) ad libitum and fresh distilled drinking water (with or without fullerene) was given ad libitum.

2.3. Experimental design and induction of diabetes

The animals were randomly divided into 4 groups with 6 animals in each group as in the following way:

(i) Control group: healthy rats received distilled water only;
(ii) C_{60}HyFn group: healthy rats received distilled water containing aqueous solutions of C_{60}HyFn at concentration of 30 nM (~22 ng/ml);
(iii) STZ-diabetes group: STZ-induced diabetic rats received distilled water only;
(iv) STZ-diabetes + C_{60}HyFn group: STZ-induced diabetic rats received aqueous solution of C_{60}HyFn at a concentration of 12 nM (~9 ng/ml).

Diabetes in overnight fasted rats of STZ-diabetes and STZ-diabetes + C_{60}HyFn groups was induced by a single intraperitoneal injection of a buffered solution (0.1 M citrate, pH 4.5) of STZ at a dose of 50 mg/kg body weight (Baydas et al., 2003). The blood glucose level was measured using reagent strips (Contour, Bayer, Leverkusen, Germany). The animals were considered diabetic if blood glucose values were more than 300 mg/dl at 72 h after STZ treatment, using a drop of blood obtained by a tail-vein puncture monitored at different time intervals throughout the study. Twenty rats were made diabetic in this way.

It should be noted that the daily consumption of C_{60}HyFn by the animals in both C_{60}HyFn and STZ-diabetes + C_{60}HyFngroups were equal to ~4 μg/kg. The animals in C_{60}HyFn group drank water containing C_{60}HyFn at a concentration of 30 nM (~22 ng/ml). Whereas, the rats in STZ-diabetes + C_{60}HyFn group drank water containing C_{60}HyFn at concentration of 12 nM (9 ng/ml), since the rats in this group drank approximately 2.5 times more water compared to C_{60}HyFn group animal. Therefore, the rats in STZ-diabetes + C_{60}HyFngroup drank water containing 2.5 times less concentration of C_{60}HyFn so that these animals of STZ-diabetes + C_{60}HyFngroup received equal amount of C_{60}HyFn to the rats in C_{60}HyFn group, as 4 μg/kg daily. The food and water intake were monitored daily throughout the study period (5 weeks).

2.4. Sample collection and homogenate preparation

After the animals were decapitated at the end of the 5th week, the blood was collected and testis, epididymides, seminal vesicles and ventral prostate were removed, cleared of adhering connective tissue and weighed. Right testicles were fixed with Bouin's fluid. Left testicles were frozen in liquid nitrogen and stored at −70 °C until use for malondialdehyde (MDA), GSH, fatty acids and alpha-tocopherol analysis. Serum was separated and also stored at −70 °C until use to estimate some biochemical parameters using the appropriate kits (Boehringer Mannheim, Germany).

2.5. Histopathology

After fixation in Bouin's solution, the tissues were embedded in paraffin for TUNEL histochemistry and haematoxylin and eosin staining. All assessment was performed in a blinded fashion. For histological examination, sections were stained with haematoxylin and eosin. Using light microscopy, diameters of seminiferous tubules and germinal cell layer thicknesses were measured and the damages in testicular tissue were evaluated. The images were viewed and analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan), and the images were transferred into the computer using a digital video camera (JVC TK-890E, Japan). For each animal thirty tubules were evaluated. Johnsen's testicular score system (Johnsen, 1970) was used for evaluation of degrees of the histopathological changes in control and treatment groups. All cross sectioned tubules were evaluated systematically, and a score between 1 (very poor) and 10 (excellent) was given to each tubule according to Johnsen's criteria.

2.6. Localization of apoptotic cells in the testis

The localization of apoptotic cell death in the spermatogenic cells was defined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay with theApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, the fixed testicular tissue was embedded in paraffin, and sectioned at 4 μm. The paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed in PBS. The sections were treated with 0.05% proteinase K for 5 min, which was followed by treatment with 3% hydrogen peroxide for 5 min to inhibit endogenous peroxidase. After washing in PBS, sections were then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-11-dUTP at 37 °C for 1 h in humidified chamber at 37 °C for 1 h, and then stop/wash buffer was applied for 30 min at 37 °C. Sections were visualized with diaminobenzidine (DAB) substrate. Negative controls were performed using distilled water in the place of the TdT enzyme. Sections were counterstained with Mayor's hematoxylin, dehydrated in graded alcohol, and cleared.

2.7. Sperm analyses

2.7.1. Epididymal sperm concentration

The epididymal sperm concentration was determined with a hemocytometer using a modified method (Türk et al., 2008). The right epididymis was finely minced by anatomical scissors within 1 ml of isotonic saline in a Petri dish. It was completely squashed by a tweezers for 2 min, and then allowed to incubate at room temperature for 4 h to provide the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue–fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid containing all epididymal spermatozoa was drawn into the capillary tube up to 0.5 lines of the pipette designed for counting red blood cells. The solution containing 0.595 M sodium bicarbonate, 1% formalin and 0.025% eosin was pulled into the bulb up to 101 lines of the pipette. This gave a dilution rate of 1:200 in this solution. Approximately 10 μl of the diluted sperm suspension was transferred to both counting chamber of Improved Neubauer (Deep 1/10 mm, LABART, Darmstadt, Germany) and allowed to stand for 5 min. The spermatozoa in both chambers were counted with the help of light microscope at 200× magnification.

2.8. Sperm motility

Freshly isolated left epididymal tissue was used for the analysis of sperm motility. The percentage sperm motility was evaluated using a light microscope with heated stage. For this process, a slide was placed on a light microscope with a heated stage warmed up to 37 °C, and then several droplets of Tris–buffer solution [0.3 M Tris (hydroxymethyl) aminomethane, 0.027 M glucose, 0.1 M citric acid] were dropped on the slide and a very small droplet of fluid obtained from left cauda epididymis with a pipette was added to the Tris–buffer solution and mixed by a cover-slip. The percentage of sperm motility was evaluated visually at 400× magnification. Motility estimates were performed from 3 different fields in each sample. The mean of the 3 successive estimations was used as the final motility score.

2.9. Sperm morphology

To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate) were prepared. The slides were then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide (3000 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage (Türk et al., 2008).

2.10. Determination of MDA–TBA level

The concentration of TBARS in the tissues samples was estimated by the method of Niehaus and Smuelson (1968). In brief, 1 ml of tissue homogenate (supernatant; Tris–HCl buffer, pH 7.5) was mixed with 2 ml of (1:1:1 ratio) TBA–TCA–HCl reagent (0.37% thiobarbituric acid, 0.25 N HCl, and 15% TCA) placed in water bath for 60 min, cooled, and centrifuged at room temperature for 10 min. Thiobarbituric acid-reactive substances (TBARS) were determined by reading the fluorescence detector set at λ (excitation) = 515 nm and λ (emission) = 543 nm. TBARS calculated from a calibration curve using 1,1,3,3-tetraethoxypropane as the standard. The MDA–TBA complex was analyzed using the HPLC equipment. The equipment consisted of a pump (LC-10 ADVP), a Fluorescence detector (RF–10_{XL}), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto, Japan). Inertsil ODS-3 column (15 mm × 4.6 mm, 5 μm) was used as the HPLC column. The column was eluted isocratically at 20 °C with a 5 mM sodium phosphate buffer (pH 7.0) and acetonitrile (85:15, v/v) at a rate of 1 ml/min (de las Heras et al., 2003). The values were expressed as mM/g tissues.

2.11. Determination of GSH level in tissue samples

Reduced glutathione (GSH) was determined by the method of Ellman (1959). Briefly 1 ml of tissue homogenate was treated with 1 ml of 5 metaphosphoric acid (Sigma, St. Louis, MO), the mixtures were centrifuged in 5000 rpm and the supernatants were taken. After deproteinization, the supernatants were allowed to react with 1 ml of Ellman's reagent (30 mM 5,5′-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium citrate). The absorbance of the yellow product was read at 412 nm in spectrophotometer. Pure GSH was used as standard for establishing the calibration curve (Akerboom and Sies, 1981).

2.12. Lipid extraction

Lipids of tissue samples were extracted with hexane–isopropanol (3:2, v/v) by the method of Hara and Radin (1978). 1 g tissue sample was homogenized with 10 ml

hexane–isopropanol mixture. Fatty acids in the lipid extracts were converted into methyl esters including 2% sulphuric acid (v/v) in methanol (Christie, 1992). The fatty acid methyl esters were extracted with 5 ml n-hexane. Analysis of fatty acid methyl ester was performed in a Shimadzu GC-17A instrument gas chromatograph equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm i.d. Permabond fused-silica capillary column (Machery – Nagel, Germany). The oven temperature was programmed between 145 and 215 °C, 4 °C/min. Injector and FID temperatures were 240 and 280 °C, respectively. The nitrogen carrier gas flow was 1 ml/min. The methyl esters of fatty acids were identified by comparison with authentic external standard mixtures analyzed under the same conditions. Class GC 10 software version 2.01 was used to process the data. The results were expressed as μg/g tissue.

2.13. Saponification and extraction

Alpha-tocopherol, alpha-tocopherol acetate and cholesterol were extracted from the lipid extracts by the method elaborated by Sánchez-Machado et al. (2004), with minor modifications. 5 ml of n-hexane/isopropyl alcohol mixture treated with 5 ml of KOH solution (0.5 M in methanol) were added and immediately vortexed for 20 s. The tubes were placed in a water bath at 80 °C for 15 min. Then after cooling in iced water, 1 ml of distilled water and 5 ml of hexane was added, and the mixture was rapidly vortexed for 1 min, then centrifuged for 5 min at 5000 rpm. The supernatant phase were transferred to another test tube and dried under nitrogen. The residue was redissolved in 1 ml of the HPLC mobile phase (68:28:4 (v/v/v) methanol:acetonitrile:water). Finally, an aliquot of 20 μl was injected into the HPLC column. Before injection, the extracts were maintained at −20 °C away from light.

2.14. Chromatographic conditions

Chromatographic analysis was performed using an analytical scale (15 cm × 0.45 cm i.d.) Supelco LC 18 DB column with a particle size 5 μm (Sigma, USA). HPLC conditions were as follows: mobile phase 60:38:2 (v/v/v): acetonitrile:methanol:water; a flow rate of 1 ml/min; column temperature 30 °C. The detection was operated using two channels of a diode-array spectrophotometer, and 202 nm for alpha-tocopherol and cholesterol. Alpha-tocopherol, alpha-tocopherol acetate and cholesterol were identified by retention and spectral data (Sánchez-Machado et al., 2002; Lopez-Cervantes et al., 2006).

2.15. Serum testosterone

The plasma testosterone level was measured by ELISA method using DRG Elisa testosterone kit (ELISA EIA-1559, 96 Wells kit, DRG Instruments, GmbH, Marburg, Germany) according to the standard protocol supplied by the kit manufacturer.

2.16. Statistical analysis

One-way analysis of variance (ANOVA) and post hoc Tukey–HSD test were used to determine differences between groups in all parameters except serum testosterone level, whose statistical comparison was performed using the nonparametric Mann–Whitney *U* test. Results are presented as means ± S.E.M. Values were considered statistically significant if $p < 0.05$. The SPSS/PC program (Version 10.0; SPSS, Chicago, IL) was used for the statistical analysis.

3. Results

3.1. Reproductive organ weights

Absolute and relative organ weights of testis, epididymis, right cauda epididymis, seminal vesicules and prostate of all groups at the end of the study period are shown in Fig. 1 as bar graph. The relative organ weights were estimated by dividing the absolute reproductive organ weights to body weight. C_{60}HyFn administration to healthy animals had no significant effects on weights of the reproductive organs in comparison to the control group. However, in STZ-diabetes group, absolute weights of testis, epididymis, right cauda epididymis, seminal vesicules and prostate and relative weights of right cauda epididymis, seminal vesicules and prostate were significantly less than those of control group ($p < 0.001$). Although absolute weights of testis and epididymis were significantly decreased in diabetic rats compared to control and C_{60}HyFn administration caused the weight of epididymis to increase significantly when compared to STZ-diabetes group ($p < 0.001$), there were no significant differences between the groups in their relative weights. Hence, the weights of testis and epididymis are correlated to whole body weight. Administration of C_{60}HyFn to STZ-induced diabetes mellitus animals resulted in statistically significant increments in absolute weights of epididymis and seminal vesicules and relative weight of seminal vesicules ($p < 0.001$).

3.2. Epididymal sperm characteristics

Epididymal sperm characteristics of control, C_{60}HyFn, diabetic and C_{60}HyFn administered diabetic rats are presented in Table 1. There was no significant difference in sperm motility, epididymal sperm concentration and abnormal sperm rate between control and C_{60}HyFn groups, while there is a numerical increase in sperm motility and epididymal sperm concentration and a decrease in the rate of abnormal sperm rate in C_{60}HyFn group. In STZ-induced diabetic rats, there was a significantly less sperm motility and epididymal sperm concentration and higher rate of tail and head abnormality of sperm than those in control animals ($p < 0.001$). Administration of C_{60}HyFn to diabetic rats attenuated hyperglycaemia-induced effects on the sperm characteristics. The increase in sperm motility, epididymal sperm concentration and the decrease in abnormal sperm rate were statistically significant ($p < 0.001$). Furthermore, there was no significant difference in epididymal sperm concentration between control, C_{60}HyFn and STZ-diabetes + C_{60}HyFn groups.

3.3. Testicular histopathology

Histological sections from testis of control, C_{60}HyFn, STZ-diabetes and STZ-diabetes + C_{60}HyFn groups are presented in Fig. 2. Histological appearances of testicular tissues of control (Fig. 2A) and C_{60}HyFn groups (Fig. 2B) were similarly normal. Marked changes including degeneration, desquamation, disorganisation and reduction in germinal cells, interstitial oedema and congestion were evident in the testis of diabetic rats (Table 2, Fig. 2C). Administration of C_{60}HyFn to diabetic rats caused a marked amelioration in testicular histological view, reducing the given histopathological changes induced by diabetes mellitus except germinal disorganisation, interstitial oedema and congestion (Table 2, Fig. 2D). The diameter of seminiferous tubule, thickness of germinal cell layer and Johnsen's criteria for control, C_{60}HyFn, STZ-diabetes and STZ-diabetes + C_{60}HyFn groups are shown in Table 3. It was found that the diameter of seminiferous tubule and Johnsen's criteria were significantly decreased in STZ-diabetes group compared with the

Table 1
Sperm parameters of control and different treatment groups (mean ± SEM).

Groups	Parameters				
	Sperm motility (%)	Epididymal sperm concentration (million/cauda epididymis)	Abnormal sperm rate (%)		
			Head	Tail	Total
Control	76.7 ± 4.0[a]	99.8 ± 8.6[a]	3.2 ± 1.1[a,c]	2.5 ± 0.5[a]	5.7 ± 1.0[a]
C_{60}HyFn	83.3 ± 2.7[a]	103.2 ± 9.6[a]	1.8 ± 0.6[a]	3.0 ± 0.4[a]	4.8 ± 0.9[a]
STZ	30.6 ± 3.7[b]	42.5 ± 9.1[b]	18.2 ± 4.1[b]	12.4 ± 1.5[b]	30.6 ± 3.2[b]
STZ+C_{60}HyFn	57.8 ± 2.4[c]	93.8 ± 13.2[a]	7.3 ± 0.8[c]	7.0 ± 0.7[c]	14.3 ± 1.2[c]

The mean differences between the values bearing different superscript letters within the same column are statistically significant ($p < 0.001$).

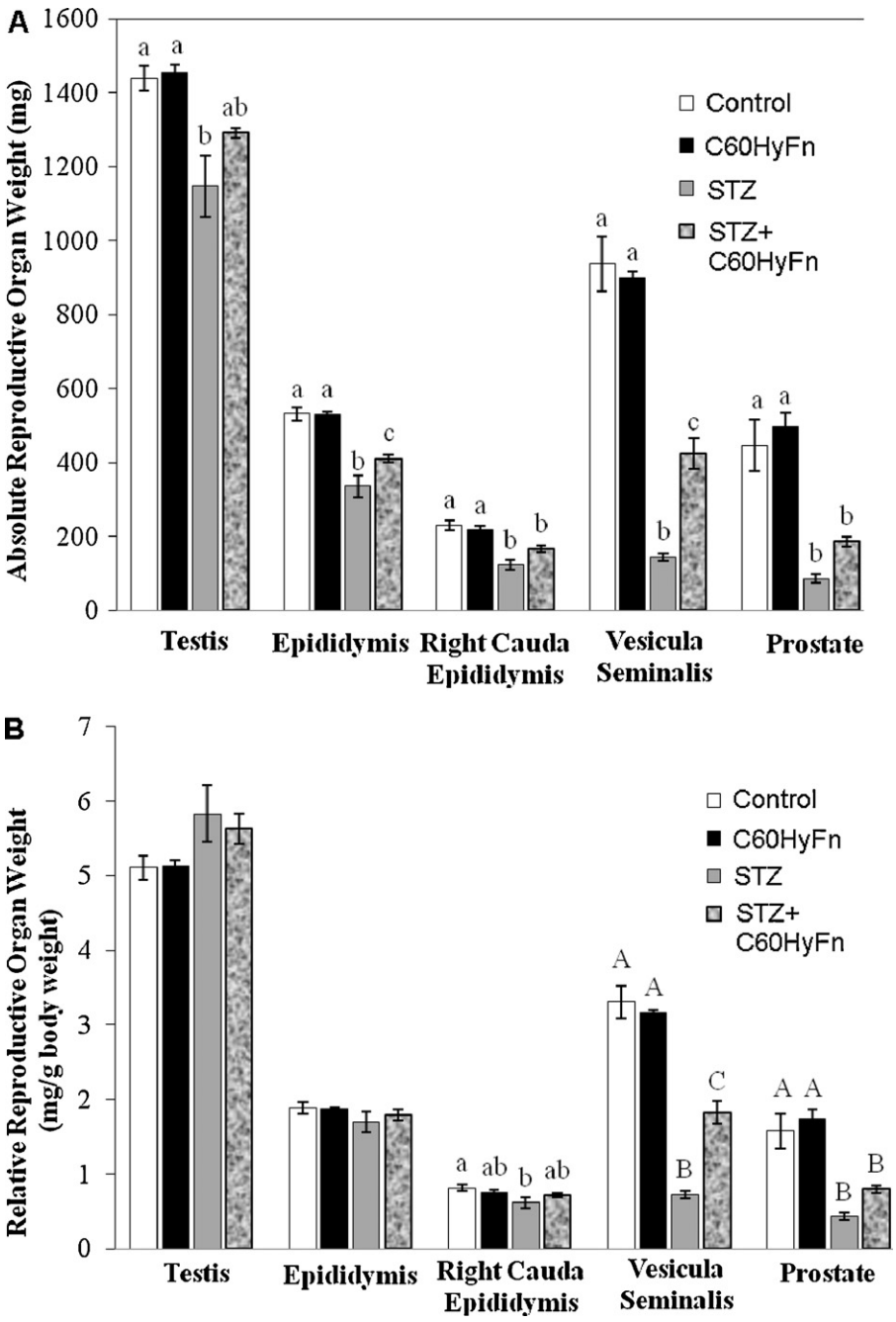

Fig. 1. Absolute (A) and relative (B) weights of reproductive organs including testis, epididymis, right cauda epididymis, vesicular seminalis and prostate (mean ± SEM). The mean differences between the values bearing different letters for the same organs are statistically significant ($p < 0.001$).

control and C_{60}HyFn ($p < 0.001$) groups. However, these values were significantly increased in the C_{60}HyFn-treated diabetic rats compared with the diabetic rats ($p < 0.001$). Though not significant a similar pattern was observed in the thickness of germinal cell layer.

3.4. Evaluation of TUNEL staining

Apoptosis in testis of control, C_{60}HyFn, STZ-diabetes and STZ-diabetes + C_{60}HyFn groups of rats, demonstrated by TUNEL staining, are shown in Fig. 3. In the current study, TUNEL-positive cells had

Table 2
The existence of some pathological lesions in rat testicular tissues of control and different experimental groups. Severety of the changes was scored between 0 and 3 as in the following: 0 – normal; 1 – mild; 2 – moderate; 3 – severe.

	Control	C_{60}HyFn	STZ	STZ + C_{60}HyFn
Degeneration in Sertoli cells	0	0.1	0.3	0.4
Degeneration in germinal cells	0	0.1	1.2	0.3
Disorganisation in germinal cells	0.1	0	1.1	0.3
Interstitial oedema and capillary congestion	0.1	0.3	1.2	0.35

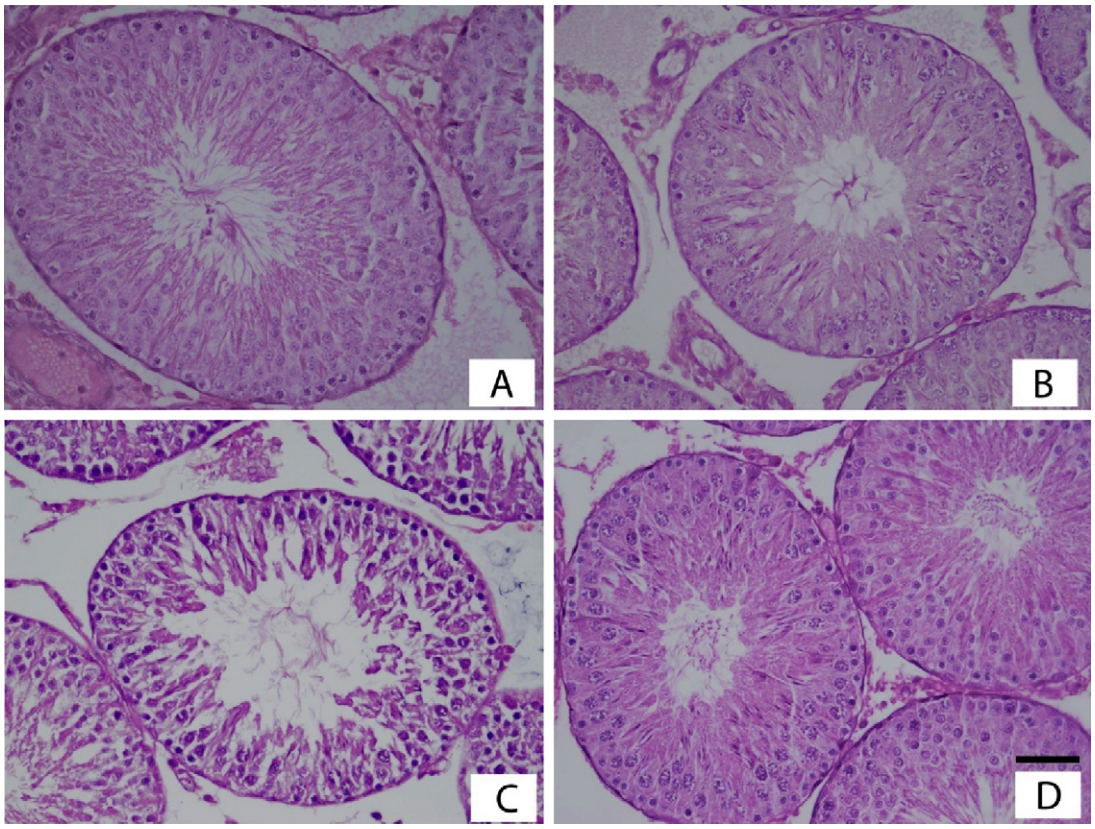

Fig. 2. Representative photomicrographs of hematoxylin-eosin-stained sections in the testes of rats. (A) Normal histological appearance of seminiferous tubules in control group. (B) Normal histological appearance of seminiferous tubules in C_{60}HyFn group. (C) Disorganisation, degeneration and interstitial oedema and capillary congestion in STZ-diabetes group. (D) Histological appearance of seminiferous tubules. Note the amelioration in histopathological changes observed in STZ-diabetes + C_{60}HyFn group. Calibration bar: 50 μm.

the typical morphological features of apoptosis, including chromatin condensation, cytoplasmic budding and apoptotic bodies. TUNEL-positive cells were occasionally observed in the testis of control and C_{60}HyFn-administered rats (Fig. 3A and B). Whereas the number of positive cells were significantly increased in the testis of diabetic rats ($p < 0.001$) (Fig. 3C). TUNEL-positive cells were detected in spermatogenetic cells. However, a significant decrease in the number of TUNEL-positive cells was observed in the testis of hydrated C_{60} fullerene-treated diabetic rats (Fig. 3D) with respect to the STZ-diabetes group ($p < 0.001$).

In order to estimate the apoptotic index, TUNEL-positive cells in seminiferous tubules (100 per animal) in 20 randomly chosen fields were counted. The apoptotic index was calculated as the percentage of cells with TUNEL positivity. In the apoptotic index, a significant increase was striking in the STZ-diabetes group ($11.4 \pm 2.7\%$) when compared to that of control ($0.5 \pm 0.2\%$) and C_{60}HyFn ($0.6 \pm 0.3\%$) groups. C_{60}HyFn-treated diabetic rats exhibited significantly lower apoptotic index ($1.1 \pm 0.4\%$).

3.5. Biochemical parameters

In terms of toxicity assessment of C_{60}HyFn, serum level of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, creatinine kinase, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, urea, creatinine and uric acid and concentrations of sodium, chloride and inorganic phosphate ions were also measured (data not shown). No significant difference in any of these biochemical parameters tested was observed after the administration of C_{60}HyFn for 35 days between groups.

Levels of glucose and testosterone in serum, levels of lipid peroxidation (TBARS) and antioxidant (GSH), some fatty acids (palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid and docosapentaenoic acid), cholesterol, alpha-tocopherol and alpha-tocopherol acetate in testicular tissue are presented in Table 4. Induction of diabetes by STZ resulted in a significant elevation in plasma glucose in comparison to control

Table 3
Diameter of seminiferous tubules, germinal cell layer thickness and Johnsen's testicular score.

Groups	Diameter of seminiferous tubules (μm)	Germinal cell layer thickness (μm)	Johnsen's testicular score
Control	$261.8 \pm 2.02^{a,*}$	73.46 ± 1.12^{a}	$9.9 \pm 0.1^{a,\dagger}$
C_{60}HyFn	$258.2 \pm 3.00^{a,**}$	72.26 ± 1.34^{a}	$9.8 \pm 0.1^{a,\dagger}$
STZ	246.6 ± 3.01^{b}	68.93 ± 1.38^{a}	7.6 ± 0.2^{b}
STZ + C_{60}HyFn	$256.4 \pm 2.24^{a,***}$	71.17 ± 2.24^{a}	$9.1 \pm 0.2^{a,\dagger}$

The mean differences between the values bearing different superscript letters within the same column are statistically significant.
* $p < 0.001$.
** $p < 0.01$ compared with STZ-diabetes group;
*** $p < 0.05$ compared with STZ-diabetes group;
† $p < 0.001$ compared with STZ-diabetes group.

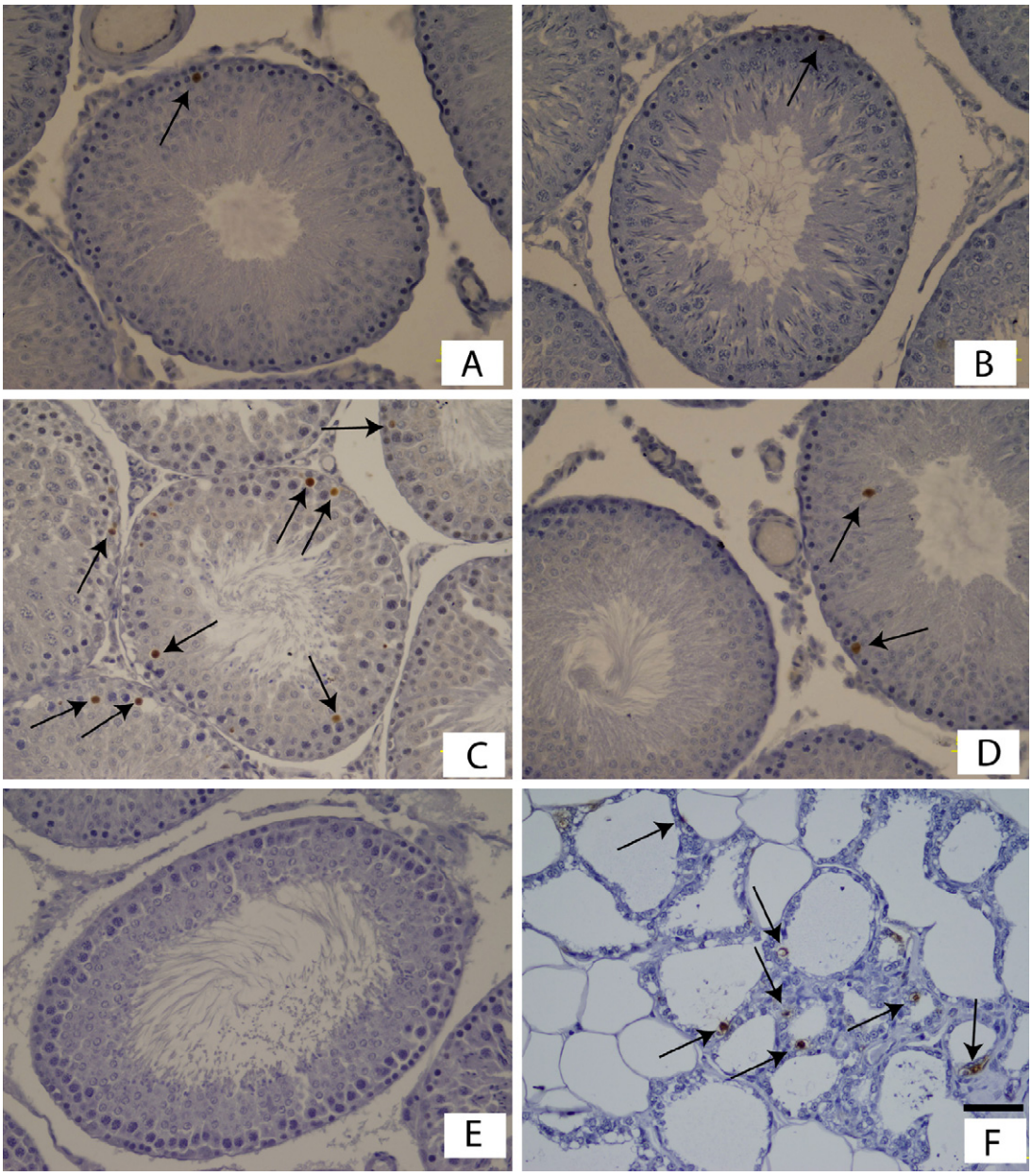

Fig. 3. Representative photomicrographs of TUNEL-stainings in the testes of control (A), C_{60}HyFn (B), STZ-diabetes (C) and STZ-diabetes + C_{60}HyFn (D) groups. (E) Negative staining control is also illustrated to ensure the staining methods are working well. Note that there were no detectable signals in the negative control. (F) Positive control: TUNEL-stained cells im breast tissue where continous apoptosis takes place. Arrows indicate candidate apoptotic cells. Calibration bar: 50 μm.

and C_{60}HyFn groups ($p < 0.001$) and administration of C_{60}HyFn to the diabetic animals did not make any significant change in blood glucose level (Table 4).

In diabetic rats, levels of alpha-tocopherol and alpha-tocopherol acetate were significantly reduced ($p < 0.001$). However, treating diabetic rats with C_{60}HyFn resulted in significant corrective effects on these parameters towards the control levels ($p < 0.001$).

While administration of C_{60}HyFn to nondiabetic rats did not significantly change the LPO measured as TBARS levels, a significant increase in LPO measured was evident in testis of diabetic rats (Table 4). C_{60}HyFn administrations to diabetic rats provided numerical but not statistically significant decrease in LPO level as compared to STZ-diabetes group. Testicular tissue GSH level was significantly lower in the diabetic rats than that in the control ($p < 0.001$). Although administration of C_{60}HyFn did not increase GSH level when compared to the control group, C_{60}HyFn administration to diabetic rats provided a statistically significant increase in GSH level as compared to diabetic group ($p < 0.05$).

In STZ-induced diabetic rats, levels of palmitic acid, palmitoleic acid, oleic acid and linoleic acid were significantly decreased when compared to control group ($p < 0.001$) (Table 4). C_{60}HyFn treatment of diabetic animals increased palmitic acid ($p < 0.001$), palmitoleic acid ($p < 0.001$), stearic acid ($p < 0.05$), arachidonic acid and docosapentaenoic acid significantly. No significant differences in stearic acid (18:0) were found between any of these groups.

In streptozotocin-induced diabetic rats, cholesterol level was significantly decreased when compared with controls, but C_{60}HyFn treatment resulted in a significant elevation of cholesterol level ($p < 0.001$) so that there was no significant difference between control and STZ-diabetes + C_{60}HyFn groups.

There was no significant difference in serum testosterone level between control and C_{60}HyFn groups. Serum levels of testosterone

Table 4
Levels of serum glucose and testosterone and testicular tissue thiobarbituric acid reactive substances (TBARS), glutathione (GSH), cholesterole, α-tocopherol, α-tocopherol acetate and some fatty acids including palmitic, palmitoleic, stearic, oleic, linoleic, arachidonic and docosapentaenoic acids (mean ± SEM).

Compound	Control ($n=6$)	C_{60}HyFn ($n=6$)	STZ ($n=6$)	STZ + C_{60}HyFn ($n=6$)
Glucose (mg/dl)	118.5 ± 3.4^{a}	113.4 ± 3.9^{a}	$481.5 \pm 18.0^{b,\#}$	$418.0 \pm 41.1^{b,\#}$
TBARS (nmol/ml)	74.9 ± 3.1^{a}	56.6 ± 5.9^{a}	$192.8 \pm 21.5^{b,\#}$	$170.9 \pm 5.3^{b,\#}$
GSH (nmol/ml)	57.9 ± 1.4^{a}	$49.2 \pm 2.4^{a,b}$	$46.1 \pm 2.4^{b,\bullet}$	$54.5 \pm 3.2^{a,\dagger}$
α-Tocopherol (μg/g)	$28.6 \pm 0.9^{a,d}$	$31.8 \pm 2.4^{a,b}$	$13.6 \pm 0.7^{c,\#}$	$25.2 \pm 1.7^{d}\blacklozenge$
α-Tocopherol acetate (μg/g)	5.26 ± 0.27^{a}	7.04 ± 0.49^{b}	$2.08 \pm 0.21^{c,\#}$	$2.69 \pm 0.09^{c,\#}$
Cholesterole (mM/g)	1.70 ± 0.06^{a}	1.70 ± 0.08^{a}	$1.33 \pm 0.03^{b,\#}$	$1.58 \pm 0.08^{a,\dagger}$
Testosterone (ng/dl)	102.9 ± 13.3^{a}	107.7 ± 11.9^{b}	$29.0 \pm 3.6^{c,\#}$	$44.0 \pm 4.6^{d,\#,\dagger}$
Palmitic acid (16:0) (μg/g)	$990.0 \pm 32.6^{a,+}$	$797.3 \pm 23.2^{b,+}$	$551.7 \pm 19.1^{c,+}$	$677.5 \pm 31.6^{d,+}$
Palmitoleic acid (16:1) (μg/g)	$237.0 \pm 32.6^{a,+}$	$101.9 \pm 8.3^{b,+}$	$35.6 \pm 7.5^{c,+}$	$141.7 \pm 2.9^{d,+}$
Stearic acid (18:0) (μg/g)	$221.5 \pm 7.8^{a,b}$	$212.0 \pm 13.6^{a,b}$	180.9 ± 9.4^{a}	$243.9 \pm 13.9^{b,\dagger}$
Oleic Acid (18:1) (μg/g)	761.0 ± 30.8^{a}	392.0 ± 33.0^{b}	$186.9 \pm 22.7^{c,\#}$	$217.8 \pm 13.7^{c,\#}$
Linoleic acid (18:2 n6) (μg/g)	999.5 ± 33.0^{a}	$476.3 \pm 30.9^{b,*,\dagger}$	$155.1 \pm 6.1^{c,*}$	$158.6 \pm 14.6^{b,c}$
Arachidonic acid (20:4 *n*6) (μg/g)	341.8 ± 13.9^{a}	$465.6 \pm 31.8^{b,*}$	$348.9 \pm 14.9^{a,b}$	$468.8 \pm 18.1^{c,\#}$
Docosapentaenoic acid (22:5 *n*6) (μg/g)	249.1 ± 6.0^{a}	$330.6 \pm 14.1^{b,c,*}$	$289.5 \pm 11.9^{a,b}$	$366.1 \pm 13.7^{c,\blacklozenge,\bullet}$

The mean differences between the values bearing different superscript letters within the same line are statistically significant ($p<0.05$).
* $p<0.01$ vs. control.
† $p<0.05$ compared with STZ-diabetes group.
# $p<0.001$ vs. control and C_{60}HyFn group.
+ $p<0.001$ compared with other groups.
♦ $p<0.001$ compared with STZ-diabetes group.
• $p<0.001$ compared with control.

in STZ-diabetes group were significantly lower than that in the control group ($p<0.001$) (Table 4). Administration of C_{60}HyFn to the diabetic rats resulted in a slight but significant corrective effect on the testosterone level towards the control ($p<0.05$).

4. Discussion

In the present study, we have elaborated the protective effects of C_{60}HyFn against STZ-diabetes-induced damages of the reproductive functions of male rats, evaluating histological changes, apoptosis identified by TUNEL staining in testicular tissue and levels of serum testosterone and testicular MDA, GSH, alpha-tocopherol and alpha-tocopherol acetate and some fatty acids. Our results confirmed that STZ-induced diabetes mellitus caused testicular dysfunctions in male rats as reported by others (Hakim and Goldstein, 1996) and for the first time showed that administration of C_{60}HyFn to diabetic rats attenuated the testicular dysfunction and spermatogenic disruption in the testis induced by uncontrolled hyperglyceamia in male rats.

4.1. Effects of C_{60}HyFn in nondiabetic animals

The treatment with C_{60}HyFn did not result in an acute or sub-acute toxicity in testis as evidenced histologically and biochemically. No tissue necrosis, fibrosis, and other degenerative signs were observed and no significant differences were observed in serum level of LDH, AST, ALT, alkaline phosphatase, creatinine kinase, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, urea, creatinine and uric acid and concentrations of sodium, chloride and inorganic phosphate ions at the given concentration (data not shown). Our findings on non-toxicity of pristine C_{60} are consistent with those of previous studies (Gharbi et al., 2005; Mori et al., 2006; Tykhomyrov et al., 2008).

4.2. Reproductive organ weights

There are some studies reporting that the relative weights of epididymis, seminal vesicle and prostate were reduced in STZ-treated rats (Scarano et al., 2006; Shrilatha and Muralidhara, 2007; Navarro-Casado et al., 2010), which are in agreement with our data obtained in the present study. The significant reductions in the weights of right cauda epididymis, seminal vesicles and prostate in diabetic rats (Fig. 1) are likely to be due to low serum testosterone level, since testosterone is the major regulator of normal growth of these organs (Mallick et al., 2007). The decreased testosterone level has been associated with absence or diminution of serum insulin levels, since it has been shown that insulin therapy restores body weight and the weight of most reproductive organs (Seethalakshmi et al., 1987). The relative weight of the testis increased in the STZ-treated group in the current study, it is not significant though. This finding is consistent with the reports by Scarano et al. (2006) and Shrilatha and Muralidhara (2007). Navarro-Casado et al. (2010) demonstrated a decrease in testicular weight after 6 weeks of diabetes inducted, which was but not evident over time. After administration of C_{60}HyFn to diabetic rats, the weights of these reproductive organs were recovered towards the control level.

4.3. Testicular histopathology

In our study, diabetes-induced changes in testis consisted of decreased seminiferous tubular diameter (STD), degeneration in germinal cells, disorganisation in germinal cells, interstitial oedema and capillary congestion. In the present study, Johnsen's scores of diabetic rats were about 8/9, which indicates the presence of mild testicular damage (Johnsen, 1970). Consistently, earlier workers have demonstrated similar alterations in the morphology of the testis (Cameron et al., 1985; Seethalakshmi et al., 1987). The reduction in tubular diameter has also been demonstrated by some other researchers (Anderson and Thliveris, 1986). Whereas Scarano et al. (2006) did not find any alterations in the testicular structure, which is likely to be that they used very short duration of diabetic conditions before terminating the experiments.

Testicular tissue damage can primarily be accounted for by decreased glucose utilization due to lack of insulin in STZ-induced diabetic rat (Mallick et al., 2007). The decreased seminiferous tubular diameter (STD) in diabetic rats may be due to low gonadotrophins (Ballester et al., 2004) and testosterone (Castro et al., 2002). Ballester et al. (2004) explained the decreased seminiferous tubular diameter in diabetes by the decreased level of FSH, which in turn, will diminish the response of the epithelium of seminiferous tubules to FSH stimulation. Because of insulin insufficiency insulin-mediated cell proliferation decreases in seminiferous tubules, resulting in a decrease in the number

of spermatogonia in seminiferous tubules and thus leading to a decrease in seminiferous tubule diameter. Seminiferous diameter has also been reported to be reduced in STZ-induced diabetes mellitus (Guneli et al., 2008). However, administration of C_{60}HyFn to the diabetic rats ameliorated these histopathological deficits by providing protection against the impairment of seminiferous tubules. Johnsen's scores of diabetic rats treated with C_{60}HyFn were nearly10, as good as control group.

4.4. Epididymal sperm characteristics

Our observation on the lower epididymal sperm concentration and sperm motility and a higher abnormal sperm rate are consistent with those of previously reported studies on the male reproductive disorders in diabetic state (Cai et al., 2000; Ghosh et al., 2002). Scarano et al. (2006) have found higher rate of sperm motility (73.3%) in the STZ-treated animals when compared to what we have found in our study (30.6%). Yet, Navarro-Casado et al. (2010) found that sperm motility was uneffected by STZ intoxication. This can be accounted for by the difference in composition of dilution medium used. The rate of the decrease in epididymal sperm concentration in the current study (~40%) were comparable to that reported by Shrilatha and Muralidhara (2007) (~20%), but lesser than that reported by Scarano et al. (2006) (~80%) and that by Navarro-Casado et al. (2010) (~70%). The decrease in epididymal sperm concentration can be explained by alterations in the spermatogenesis process (Scarano et al., 2006). Abnormal sperm morphology observed in our study was also observed in the study by Navarro-Casado et al. (2010). However, Scarano et al. (2006) did not find abnormal morphology in sperm morphology. This might be due to that the duration of their study was too short (2–3 weeks) for the abnormalities to develop.

Reduced epididymal sperm concentration and sperm motility and higher rate of abnormal sperm in diabetic rats could be interpreted as a combined effect of decreased Leydig cell function (as evidenced by reduced testosterone levels in serum/testis) (Tanaka et al., 2001) and the oxidative stress induced by diabetes (Ghosh et al., 2002). It is demonstrated that oxidative damaging effect of free radicals diminishes sperm motility and viability (Hsu et al., 1998). Shrilatha and Muralidhara (2007) reported that the susceptibility of spermatozoa to oxidative stress in the epididymal milieu was increasingly evident after 2 weeks of STZ injection. Cummins et al. (1994) suggested that spermatozoa produced in the testis are reasonably well protected by the microenvironment of Sertoli cells, but they are less protected against oxidation in the epididymis, which apparently worsen the figure in epididymis where sperm is stored. It is reported that oxidative damage to sperm can lead to DNA damage, altered membrane functions, impaired motility, decreased fertilization capacity (Lopes et al., 1998). Alternatively, the marked decrease in epididymal sperm concentration and increase in the abnormal sperm rate in diabetic rats could also possibly be explained by the inhibition in spermatogenesis (Dawson et al., 1992).

In the current study, we demonstrated that administration of C_{60}HyFn to diabetic rats resulted in marked recoveries in the epididymal sperm concentration, sperm motility and rate of abnormal sperm rate towards the control levels. It might be interesting to note that administration of C_{60}HyFn to non-diabetic animals resulted in numerical but not statistically significant improvements in these sperm characteristics. Therefore, based on literature resources (Gharbi et al., 2005; Beuerle et al., 2008; Andrievsky et al., 2009; Srdjenovic et al., 2010), we state that C_{60}HyFn treatment enhances testicular antioxidant capacity and reduce oxidative stress and thereby reduce diabetes-induced oxidative damage, leading to an in improvement of epididymal sperm characteristic in nondiabetic rats and restoration of epididymal sperm characteristic in diabetic rats. The protective effects of C_{60}HyFn as an antioxidant on the testis in the current study are consistent with similar earlier findings in diabetic models (Dawson et al., 1992; Shrilatha and Muralidhara, 2007). Consistently, as antioxidants, both ascorbic acid and melatonin have been shown to prevent the loss of sperm viability, membrane integrity and motility in vitro and to improve the suppressed sperm quality in vivo (Dawson et al., 1992; Tan and Reiter, 2005). Similarly Zhao et al. (2010) demonstrated the complete prevention of diabetes-induced testicular oxidative damage and apoptotic cell death by antioxidant n-acetyl cysteine (NAC).

4.5. TUNEL staining

The number of TUNEL-positive cells was significantly increased in the germinal epithelium of diabetic testis (Fig. 3C), which indicates that germinal cell apoptosis was induced by diabetes mellitus. There are reports demonstrating increases in germ cell apoptosis in the testis of STZ-induced diabetic animals (Cai et al., 2000; Shrilatha and Muralidhara, 2007; Guneli et al., 2008). Increased apoptotic cell death is reported to be implicated in the pathogenesis of testicular dysfunction in diabetic males and results from increased status of oxidative stress (Cai et al., 2000; Wang et al., 2003). The increased oxidative stress in the testis of diabetic rats induces release of cytochrome c and then activation of caspase, leading to apoptotic cell death (Wang et al., 2003; Zhao et al., 2010).

We demonstrated that administration of C_{60}HyFn in drinking water after induction of diabetes by STZ significantly reduced hyperglycaemia-induced apoptotic cell death in the testis. The protective properties of water-soluble fullerene derivatives against apoptotic cellular death have been demonstrated in different models of diseases (Misirkic et al., 2009). The preventive effect of C_{60}HyFn on diabetes-induced apoptotic cell death is most likely mediated by its antioxidant activity to suppress oxidative stress and damage. Similarly Zhao et al. (2010) demonstrated complete prevention of the diabetes-induced apoptotic cell death by of antioxidant n-acetyl cysteine (NAC).

4.6. Biochemical parameters

After STZ injection, fasting blood glucose level was increased, which are in line of early studies (Scarano et al., 2006). It is likely that the testicular dysfunction and spermatogenic disruption may partly be associated with the decreased testicular glucose utilization due to the lack of insulin as suggested by Mallick et al. (2007).

4.7. MDA

Significant reduction in the levels of enzymatic and nonenzymatic antioxidants and an increase in ROS production are well established features of the testicular tissues of diabetic animals, which are suggestive of the ongoing oxidative disturbances (Pigeolet et al., 1990; Chandrashekar and Muralidhara, 2009; Jelodar et al., 2009). There was a numerical but insignificant decrease in MDA level in the testis of the rats treated with C_{60}HyFn, suggesting that hydrated C_{60} fullerene exerts a slight antiradical activity even in the nondiabetic rats. Testicular tissue of diabetic rats showed nearly three fold elevation in MDA levels indicating a clear persistence of oxidative damage. Shrilatha and Muralidhara (2007) reported roughly double increase in MDA level in testicular tissue. MDA is an aldehyde resulting from peroxidation of plasma membrane phospholipids, level of which is used as a marker of lipid peroxidation extent. ROS can inactivate or alter the biological activity of molecules including lipids and protein or DNA that are essential for cell function. Increase in oxidative stress in the testicular tissue is demonstrated to have profound effect on testicular physiology and sperm function (Shrilatha and Muralidhara,

2007; Agarwal et al., 2008; Chandrashekar and Muralidhara, 2009). Plasma membrane of testicular cells including mature spermatozoa contain phospholipids composed of extremely high proportions of polyunsaturated fatty acids (PUFAs), but their cytoplasm includes relatively low concentrations of scavenging antioxidant enzymes (Yin et al., 1999; Valk and Hornstra, 2000; Gavazza and Catalá, 2001). Therefore, this high concentration of PUFAs in cellular membranes makes it prone to lipid peroxidative degradation (Agarwal et al., 2008). The structural and functional alterations may indicate that oxidative stress is highly likely to contribute to the development of pathophysiological dysfunctions in the testis of STZ-induced diabetic rats.

It is demonstrated that supplementation of C_{60}HyFn helped to reduce MDA level. In early diabetic phase, STZ-induced oxidative damage in testis is reported to be reversible by antioxidant supplements (Shrilatha and Muralidhara, 2007). Authors showed that ascorbic acid offset the STZ-induced oxidative damage in testis and epididymal sperm as evident from diminished MDA and ROS levels in cytosol and mitochondria and restoration of antioxidant enzymes.

4.8. Testicular antioxidants (GSH, vitamin E levels)

In the current study we determined a significant reduction in GSH level in diabetic testis, which confirm previous studies (Pigeolet et al., 1990; Guneli et al., 2008; Jelodar et al., 2009; Zhao et al., 2010). But the decrease in GSH in diabetic testis was much less than that reported by Shrilatha and Muralidhara (2007). However, the rats treated with C_{60}HyFn restored the level of GSH to the control level.

In the current study, the decline in the levels of alpha-tocopherol and alpha-tocopherol acetate was a striking finding for diabetic rats. The observed low level of alpha-tocopherol in the testis of diabetic rats corroborate with similar earlier findings in diabetic models (Shrilatha and Muralidhara, 2007). Furthermore, a decrease in the levels of alpha-tocopherol and alpha-tocopherol acetate in testicular tissue of diabetic rats might also contribute to a decline in GSH level or to the increased level of ROS in testis, because alpha-tocopherol has antioxidant property (Fuller et al., 1996).

However, we observed a significant elevation of alpha-tocopherol in diabetic and even in nondiabetic rats treated with C_{60}HyFn. The elevation of alpha-tocopherol by C_{60}HyFn might be another potential mechanism that C_{60}HyFn exerts, restoring the testicular dysfunction and spermatogenic disruption in the testis induced by diabetes mellitus in male rats, since alpha-tocopherol is known for its antioxidant, immune-enhancing, anti-inflammatory, and antiplatelet aggregation effects and is used clinically to prevent complications of diabetes (Fuller et al., 1996). Specifically, tocopherols are reported to detoxify lipid peroxyl radicals, as well as block the reactivity of singlet oxygen radicals (Kamal-Eldin and Appelqvist, 1996). It is interesting that alpha-tocopherol accumulates in the membranes of mitochondria and endoplasmic reticulum within the cell where oxygen radical production is greatest and thus where it is most required (Duthie et al., 1991). The antioxidant function of alpha-tocopherol is critical for the prevention of oxidation of tissue PUFAs, reducing the susceptibility to lipid peroxidation of tissue PUFAs (Fuller et al., 1996; Valk and Hornstra, 2000). Polyunsaturated fatty acids are essential for the maintenance of normal testicular function in rats. It is fair to state that C_{60}HyFn up-regulated enzymatic and nonenzymatic antioxidants such as GSH and alpha-tocopherol.

Cai et al. (2008) suggested that C_{60} fullerene displays not only direct antioxidant properties towards free radicals, but also indirect antioxidant pathways, such as induction of phase 2 enzymes. They shown that fullerenol (polyhydroxylated derivate of C_{60} fullerene) pretreatment significantly elevated the Nrf 2 expressions, which is a key regulator of antioxidant response element-mediated gene expression and the induction of phase 2 detoxifying enzymes and elevated activity of g-glutamylcysteine ligase (GCL), which is the rate-limiting a phase 2 enzyme for GSH synthesis, resulting in an increase in GSH level.

4.9. Testosterone and cholesterol levels

In the present study, testosterone levels in diabetic rats were significantly lower compared with control. Our observation on testosterone level is in agreement with those of previously reported studies. A decreased level of serum testosterone is reported to be associated with a decrease in LH, a decrease in the number of Leydig cells or both in male diabetic rats. Consistently, it is reported that serum level of LH, responsible for normal Leydig cell function (testosterone secretion), decreases in diabetes. It was reported that total Leydig cell number decreases and Leydig cell function is impaired in diabetes, since due to the lack of insulin, the insulin-mediated stimulation of androgen biosynthesis and cell proliferation declines in testicular tissues of diabetic rats, which is consistent with the histopathological changes in testis observed (Cameron et al., 1985; Zhao et al., 2010). Mallick et al. (2007) proposed an alternative explanation for low level of testosterone in diabetes, suggesting that the decrease in serum testosterone in diabetes may also be associated with the reduction in the activities of androgenic key enzymes i.e., Δ^5,3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase.

The decreased testosterone level measured in STZ-diabetes group may also partly be associated with the low level of cholesterol observed in the current study, since testosterone is derived from cholesterol (Waterman and Keeney, 1992). Administration of C_{60}HyFn resulted in a significant elevation of cholesterol level. This may indicate that hydrated C_{60} fullerene might promote testosterone production by increasing the conversion from cholesterol to testosterone by Leydig cells.

There is still a debate which type of diabetes has the strongest impact on the reproductive function in males. Despite adverse reproductive outcomes of type 1 diabetes have been acknowledged, the research has recently shown that hypogonadism is also common in middle-aged patients with type 2. It should be noted that young type 2 diabetic patients have significantly lower plasma concentrations of total and free testosterone (Chandel et al., 2008). Considering that the C_{60}HyFn contributed to the normalization of testosterone level in the blood of STZ-diabetic rats, it is believed that hydrated fullerene could be an effective tool for correction of reproductive dysfunction in type 2 diabetes as well.

4.10. Fatty acids

The phospholipids of testis composed of extremely high proportions of PUFAs (approximately 70% of the fatty acids) with a prevalence of linoleic (C18:2 n6), arachidonic (C20:4 n6) and docosapentaenoic (C22:5 n6) acids (Yin et al., 1999; Gavazza and Catalá, 2001). Many studies suggest that polyenoic acids, particularly C22:5 n−6 have an important role in the process of spermatogenesis in rats. Altered lipid metabolism is usually associated with testicular dysfunctions (Coniglio et al., 1977). In the present study, we observed significant changes in lipid constituents in testis of diabetic rats. In diabetic testis, biosynthesis of PUFAs is impaired due to the inhibition of desaturases involved in the desaturation of fatty acids (Huang et al., 1984). It is also likely that alteration in fatty acid composition might be associated with low testosterone level in diabetic animals, since it is reported that testosterone may be involved in the regulation of biosynthesis of PUFAs and lipids containing these polyenoic acids (Sheriff, 1980).

The low level of palmitic acid (16:0) observed in the current study in STZ-diabetes group may be associated with insulin insufficiency, since both activities of acetyl CoA carboxylase and fatty acid synthase are dependent upon the presence of insulin. C_{60}HyFn appear to have effects on these enzymes, since treatment of diabetic rats with hydrated C_{60} fullerene increased significantly when compared with those of STZ-diabetes group.

Palmitoleic (16:1 n−7) and oleic acids (18:1 n−9) are monounsaturated fatty acids, which are biosynthesized from 16:0 and 18:0 as substrate by stearoyl-CoA desaturase (SCD) (Ntambi, 1995). Activity of SCD enzyme increases in the presence of insulin. These fatty acids serve as mediators of signal transduction, cellular differentiation and regulator of apoptosis (Miyazaki et al., 2002).

While levels of arachidonic acid (20:4 n6) and docosapentaenoic acid (22:5 n6) were comparable in control and diabetic rats, C_{60}HyFn treatment in diabetic and nondiabetic rats resulted in significant elevations of these fatty acids. There are several other studies on measurement of arachidonic acid in testis of diabetic rats, which reports a slight but insignificant decrease in level of arachidonic acid (Wilder and Coniglio, 1984). Docosapentaenoic acid (C22:5 n6) has an important role in the process of spermatogenesis in the rat (Coniglio et al., 1977). It is known that arachidonic acid (AA) has an important role in a variety of biological processes, such as signal transduction, chemotaxis, cell proliferation, differentiation, apoptosis and testicular steroidogenesis (Romanelli et al., 1995). While free fatty acids such as palmitic acids and stearic acids induced apoptosis of the Leydig cells by ceramide production, AA can partly prevent the apoptotic effect induced by free fatty acids (FFAs) (Lu et al., 2003). Furthermore, exogenous AA causes a dose-dependent increase of testosterone production by rat Leydig cells (Romanelli et al., 1995). It appears that elevations of arachidonic acid (20:4 n6) and docosapentaenoic acid (22:5 n6) by C_{60}HyFn treatment are likely to be due to the increase of alpha-tocopherol as a consequence of C_{60}HyFn administration, which is reported to suppress the ascorbate–Fe^{2+}-induced lipid peroxidation in rat testis microsomes and mitochondria, protecting the most PUFA, arachidonic [C20:4 n6] and docosapentaenoic [C22:5 n6] acids from oxidative damage (Gavazza and Catalá, 2001). Therefore, the increases of arachidonic acid and docosapentaenoic acid by C_{60}HyFn treatment in the present study may partly explain why fullerene attenuates the testicular dysfunction and spermatogenic disruption in the testis induced by diabetes mellitus and why sperm parameters were much better than that in control.

Results described herein represent novel biological activity of hydrated neat C_{60}, suggesting that this agent protects male reproductive system against detrimental influence of uncontrolled hyperglycaemia. Hence, data obtained sufficiently expand our knowledge concerning involvement of fullerene nanostructures in processes occurring in living matter in health and disease. Because of their antioxidant properties, fullerenes have been shown to be involved in the inhibition of apoptosis in various cell types including neuronal cells (Dugan et al., 1996), hepatoma cells (Huang et al., 1998), blood mononuclear cells (Monti et al., 2000) and keratinocytes (Fumelli et al., 2000). The biological effects of fullerene and its water-soluble derivatives include neuroprotection (Dugan et al., 2001), radioprotection (Lin et al., 2001), antiproliferative (Lu et al., 1998), antitumor (Zhu et al., 2008), antiamyloidosis (Podolski et al., 2007), hepatoprotective (Gharbi et al., 2005), antiarthritis (Yudoh et al., 2009), and antiinflammatory activities (Dellinger et al., 2009). Besides they inhibit allergic response (Ryan et al., 2007), potentiate hair growth (Zhou et al., 2009), improve cognitive function and increase lifespan of mammals (Quick et al., 2008). Pristine C_{60} fullerene has prominent potential to attenuate toxicity and to eliminate the increase in the HO-radicals induced by the coadministration of methamphetamine plus morphine as an antiradical agent (Mori et al., 2007). Efficacy of carboxyfullerene to suppress acrilamide toxicity in human neuroblastoma cells in vitro (Sumizawa and Igisu, 2009) as well as protective effects of polyhydroxyfullerene (fullerenol) against doxorubicin-induced nephro-, pulmo-, cardio-, and hepatotoxicity (Srdjenovic et al., 2010) in rats have been also attributed to their antioxidant activity. It is worth to be mentioned that C_{60}HyFn has been recently demonstrated to be able to inactivate OH-radicals in the number that substantially exceeds theoretically expected level. It is postulated that C_{60}HyFn acts as peculiar antioxidant that does not react with free radicals directly but initiates and catalyzes the reactions of ROS recombination (dismutation) occurring in ordered interfacial water shells in the vicinity of fullerene' surface (thus preserving stability of its own chemical structure against ROS-induced oxidation) (Andrievsky et al., 2009). This assumption could explain why C_{60}HyFn exhibits prolonged bioactive effects, including antioxidant capacity, even in small and super-small concentrations and doses that has been observed and described elsewhere (Tykhomyrov et al., 2008; Andrievsky et al., 2005, 2010).

Thanks to the antioxidant properties of fullerenes, there is a tendency to believe that their biomedical application could be beneficial in the disorders, in which oxidative injury plays a crucial role in the pathogenesis. To the best of our knowledge currently there are no reports regarding the effects of non-modified C_{60} fullerene on reproductive function in mammals. It has been primarily reported that C_{60} fullerene prevents Fe^{2+}/ascorbate-induced oxidative stress in goat epididymal spermatozoa in vitro (Murugan et al., 2002). Also, Andrievsky et al. has been earlier observed that C_{60}HyFn at concentration range of 1–3 μM protects bovine spermatozoa against cold shock and increases essentially their viability following cryoconservation (data not published). Pretreatment of adult Wistar rats with fullerenol nanoparticles prevented the NO-induced alterations in the activities of antioxidative enzymes in interstitial cells of testes (Mirkov et al., 2004). Fullerenol has been recently observed to exert pronounced testicular-protective efficacy in preventing oxidative stress induced by doxorubicin (Srdjenovic et al., 2010). Noteworthy that we have not observed any signs of fullerene's spermatoxicity or gonadotoxicity during experimental period. This observation is in accordance with the results obtained by Mori et al. (2006) who have revealed that pristine fullerene does not induce acute toxicity in rats even being administered per os in the dose of 2000 mg/kg. Because of the involvement of free radicals in diabetes-induced testicular dysfunction and spermatogenic disruption in the testis in STZ-induced animal models of diabetes mellitus type 1 and in human of both type 1 and type 2, and administration of C_{60}HyFn to diabetic rats ameliorated the testicular dysfunction and spermatogenic disruption in the testis induced by diabetes mellitus in male rats in the current study, hydrated nanoparticles of unmodified C_{60} fullerene manifested the superior antioxidant activity can be used to treat in order to relieve testicular dysfunction induced by hyperglicaemia in human diabetic patients.

In conclusion, our results provide preliminary indication that hydrated nanoparticles of chemically non-modified C_{60} fullerene display the superior antioxidant activity necessary to effectively alleviate diabetic complications by elimination of testicular apoptosis and consequent histopathological abnormalities, thus resulting in elevation of testosterone level and increase of sperm content and motility. This protection is most likely mediated by C_{60}HyFn-induced up-regulation of antioxidant capability of tissues that, in turn, systemically prevent oxidative damage and maintain sex hormone levels in diabetic animals. Although further studies are required to provide new insights into the subtle mechanisms of tissue-protective effects of water-soluble fullerene nanostructures, our data persuasively demonstrate for the first time that fullerene-based nanopharmaceutical can be promising approach

for the treatment of diabetes-associated complications, in particular, for development of effective antisterility drugs.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

This work was supported by a grant from FÜBAP, 1811 (Turkey).

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