Effects of Pyrroloquinoline Quinone and Vitamin C on Diabetes Associated Testicular Dysfunction and Oxidative Damages in Testis of Streptozotocin-Induced Diabetic Mice: Histopathological Study

Narendra Kumar* and Anand Kar

School of Life Sciences, Devi Ahilya University, Takshashila Campus, Indore, India

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*Corresponding author: Narendra Kumar, School of Life Sciences, Devi Ahilya University, Takshashila Campus, Indore, Madhya Pradesh, India, Tel: +91 9425481242; Fax +91 731 2360026; E-mail: narendrakumar93@gmail.com

Abstract

The aim of this study was to investigate the hitherto unknown potential of Pyrroloquinoline Quinone (PQQ) in regulating diabetes associated testicular dysfunctions and oxidative damages in testis of adult mice. Seven groups such as normoglycemic and PQQ treated controls; STZ-treated and STZ + PQQ treated (5, 10 and 20 mg/kg/day, separately) and STZ + Vit.C (50 mg/kg) were established. After 18 days of experimentation, alterations in the markers of oxidative stress, various antioxidants, lipid profile, serum insulin, testosterone and testicular histology were evaluated. Administration of single dose of STZ (150 mg/kg b.wt) enhanced not only testicular lipid peroxidation and lipid hydroperoxides levels; but also serum concentration of glucose, cholesterol, triglyceride, low density lipid and very low density lipid; with a parallel decrease in serum insulin, testosterone, and different antioxidants in diabetic mice. However, on simultaneous administration of PQQ, particularly at a dose of 20 mg/kg, most of these adverse effects were ameliorated. While the PQQ (20 mg/kg) decreased the serum glucose by 50%, it increased insulin and testosterone levels by 59% and 169% respectively in STZ-induced animals, these results suggest that PQQ may have potential to ameliorates diabetes-induced testicular dysfunction.

Keywords: Diabetes mellitus; PQQ; Insulin; Testosterone; Oxidative stress; Testis; Vitamin C

Introduction

Diabetes mellitus (DM) is primarily a metabolic disorder and is characterized by hyperglycemia [1]. Diabetic condition increases oxidative stress that is believed to be the result of increased production of reactive oxygen species (ROS) and decreased antioxidant defense system [2]. In fact, tissue injury due to free radical damage acts as an important factor in the pathogenesis and complication of DM. It is also believed that an increase in ROS production causes non-specific modifications in nucleic acids, protein and phospholipid structures that leads to damage in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) as well as changes in the levels of antioxidant enzymes [3].

Diabetes is also known to exert negative effects on male

fertility through hyperglycemia-induced testicular dysfunctions leading to atrophy of sex organs; decrease in testosterone level; loss in libido and in sperm count and motility [4, 5]. Agbaje, et al. [6] also reported that streptozotocin (STZ)-induced diabetic animals show a significant increase in the level of fragmented DNA in sperms. These adverse effects are thought to be due to DM associated oxidative stress [7-9]. These observations led us to assume that a potent antioxidant is crucial in reducing the damage caused by oxidative stress. Some experimental, epidemiological and clinical studies also emphasize that antioxidants might be helpful for treating diabetes and its complication [10-14].

Pyrroloquinoline Quinone (PQQ) is a ubiquitous molecule that is reported to be beneficial for growth and stress tolerance in both bacteria and higher organisms [15, 16]. In recent years, this has been found to act as an antioxidant [17-21]. Both *in vivo* and *in vitro* studies suggest that PQQ can protect against several types of oxidative damages and irradiation injury [22, 23]. Further, PQQ is found to be involved in regulating various physiological processes through its redox cycling property [16, 24-26]. In fact, PQQ is believed to possess potent antioxidant activity, much stronger than other quinones and enediols including ascorbic acid [27]. PQQ administration reverses the metabolic disorders and significantly improves lipid profile [28, 29]. Mechanistically, PQQ is involved in scavenging ROS, regulating calcium and insulin signaling pathways through PI3K/AKT cascade [30]. In fact, despite good amount of evidences on its antioxidative properties, so far nothing was investigated on its role in hyperglycemia-induced oxidative damages in testis of diabetic ones. Present one is an attempt in this direction.

Materials and Methods

Chemicals

STZ was procured from Sigma-Aldrich chemicals (St. Louis, MO, USA); Ellman's reagent, m-phosphoric acid, Thio-Barbituric Acid (TBA), sodium dodecyl sulphate, Tricarboxylic Acid (TCA) and Hydrogen Peroxide (H₂O₂) were obtained from E. Merck Ltd.,

Mumbai, India. Assay kits for the estimation of different lipids, glucose, urea, and creatinine were procured from Transasia Bio-Medicals Ltd., Solan, India. PQQ was purchased from Quality of Life Lab, USA. While testosterone kit (CLIA) was from Autobio Diagnostics Co., Ltd Zhengzhou, China; insulin estimation was done with ELISA kit from IRI Research Inc, lake view, Canada. All other chemicals were of reagent grade and obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

Animals

Swiss albino male mice (7-8 weeks old), weighing 30 ± 2 g were housed in polypropylene cages in a standard photoperiod (14 h light:10 h dark) and temperature ($27 \pm 1^\circ\text{C}$) controlled room with the provision of laboratory feed (Gold Mohur Feed, Hindustan Lever Limited, Mumbai, India) and water *ad libitum*. Animals were maintained in accordance with the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment, Forest and Climate Change, New Delhi, Govt. of India. (Reg. No. 779/Po/Ere/S/03/CPCSEA)

Induction of diabetes in mice

Diabetes was induced in 24 hours fasted mice by a single intraperitoneal (i.p.) injection of STZ at 150 mg/kg, dissolved in citrate buffer [31] (0.1 M citrate, pH 4.5) solution. After 72 h of STZ administration, the tail vein blood was collected to determine fasting blood glucose levels using a glucometer and mice with blood glucose more than 225 mg/dl were considered diabetic and included in the experiments.

Experimental design

Forty-nine healthy male mice were divided into seven groups of seven each. Group I animals receiving single (i.p.) injection of citrate buffer (0.1ml, 0.1 M citrate, pH 4.5) solution served as control. Group II, injected with single dose of citrate buffer and PQQ at 20 mg/kg for 15 days served as PQQ control; whereas those of group III, IV, V, VI and VII received single dose of STZ (150 mg/kg, i.p.). After rendering DM / hyperglycemia, animals of group IV, V and VI were treated (i.p.) with three different doses (5, 10 and 20 mg/kg/day, respectively) of PQQ [32,33] and group VII received vitamin C (50 mg/kg, i.p.) for 15 days [16]. The dose concentrations were selected from previous studies [32, 33]. Total time duration of experimentation was 18 days (1st 3 days/ 72 hours for rendering diabetes/ hyperglycemia and 4th to 18th day for PQQ and Vit.C treatments).

Preparation of serum and testis homogenate

Mice were sacrificed by a mild ether anesthesia and blood from each animal was collected. Blood samples were centrifuged at 3000 rpm for 5 min; serum was separated and stored at -20°C until estimation of different biochemical parameters including serum concentrations of insulin, testosterone, glucose, total cholesterol, and triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL). Testis homogenates were prepared in cold phosphate buffered saline (PBS, 0.1M, pH 7.4) using a homogenizer, which

were then centrifuged at 15,000 g for 30 min at 4°C and the supernatant was used for the estimation of lipid peroxidation (LPO), super-oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities as well as reduced glutathione (GSH) content and lipid hydroperoxide (LOOH).

Histopathological study of testis

Immediately after exsanguinations, testis was washed with ice chilled phosphate buffer (0.1M, pH 7.4) and fixed in 10% formaldehyde for 24 hours. The tissues were dehydrated in the descending grades of isopropanol, finally cleared in xylene, and then embedded in molten paraffin wax [34]. Sections were cut at 5 μm thicknesses, stained with toluidine blue and scrutinized under Nikon microscope with digital camera system (Nikon ECLIPSE 50i).

Hormone estimation

The serum testosterone was measured through ARCHITECT system by using a human Chemiluminescent Micro-Particle Immunoassay (CMIA) kit (ABBOTT Max-Planck-Ring 2, Wiesbaden, Germany) and following manufacturer's instructions. Estimations of serum insulin were done by commercially available kit and protocol manufactured by IRI Research Inc. lake view, Canada. IRI insulin is a solid phase ELISA.

Analytical Procedures

After completion of treatment, animals were sacrificed by mild ether anesthesia and testis were removed quickly, washed in PBS and processed for different parameters such as LPO, LOOH, SOD, CAT, GPx activities, GSH content and protein as well as serum was separated and stored at -20°C until estimations of different biochemical parameters including glucose, total cholesterol, HDL, LDL and VLDL concentrations [35-45].

Statistical analysis

Data are expressed as means $\pm$ SEM ($n=7$). For the statistical evaluation, analysis of variance (ANOVA), followed by post hoc Newman-Keul's Multiple Comparison Test using the trial version of Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft Office 2003 for windows. A "P" value of 0.05 or less is considered to be significant.

Results

Alterations in the body weight (g) of different experimental groups have been depicted in table 1. After comparing the body weight of 1st day, 4th day (the day when PQQ administration was started) and that of last day in the respective group, body weight (b.wt.) of the control animals as well as in PQQ (20 mg/kg) treated normoglycemic animals showed a normal growth at the end of experiment, while there was a loss in body weight in all the STZ treated of animals on the 4th day, which is the possible indications of rendering diabetes as compared with the 1st day body weight of respective group of animals. While after administration of PQQ (5,10 & 20 mg) and Vit. C (50 mg/kg for 15 days) there was no further body weight loss observed in respective group of animals as compared with 4th and 18th days

body weight, which confirmed that the PQQ and Vit.C treatment in STZ-treated animals had protective role in body weight loss (Table 1).

With respect to different biochemical analyses a significant increase in the level of serum glucose was found in STZ treated animals ($P < 0.001$) as compared to normal control animals. However, on administration of PQQ at a dose of 5, 10 and 20 mg/kg to diabetic animals; a significant decrease in the serum glucose level was observed with last 2 doses ($P < 0.05$ and $P < 0.001$ respectively, as compared to that of STZ-induced diabetic mice). Decreased glucose level was maximum (50%) by 20 mg/kg of PQQ, while administration of Vit.C at 50 mg/kg to STZ-treated mice also decreased its level significantly ($P < 0.001$) with a percentage decrease of 41 %. However, following administration of 20 mg/kg PQQ to normoglycemic mice, there was no significant alteration in glucose level as compared to that of normal animals (Figure 1). On changes in serum insulin level, it was significantly decreased in the STZ-induced diabetic mice (P

< 0.001) as compared to normal animals. While administration of 20 mg/kg PQQ, to normoglycemic mice, did not alter insulin level significantly, its administration at 5, 10 and 20 mg/kg to diabetic animals enhanced the hormone level in dose dependent manner. In fact, the highest dose of PQQ (20 mg/kg) increased its level by 59 %; while administration of Vit.C 50 mg/kg did not increase insulin level significantly (Figure 2). Serum testosterone level was also significantly decreased in the STZ-induced diabetic mice ($P < 0.001$), while administration of PQQ at 10 and 20 mg/kg to STZ-treated mice, increased its level significantly ($P < 0.05$ and $P < 0.001$ respectively; as compared to that of STZ-induced mice) showing a 169% increase in the highest dose. No significant alteration was observed when 20 mg/kg of PQQ was treated to normoglycemic animals. Interestingly, on administration of Vit.C 50 mg/kg in STZ-treated diabetic animals a significant increase ($P < 0.05$) in the testosterone level was observed with a percentage of 62% as compared to STZ-treated diabetic animals (Figure 3).

Testicular LPO was increased significantly ($P < 0.001$ as

Table 1: Alterations in the body weight (g) on 1st, 4th, and on 18th (last) day of experimentation in different experimental groups of animals

Groups	Alteration in body weight (g)		
	1 st Day	4 th Day	18 th Day
Control	29.00 ± 1.632	30.50 ± 1.002	35.00 ± 1.154
PQQ 20 mg	29.65 ± 1.754	31.40 ± 0.914	35.95 ± 1.250
STZ	28.35 ± 1.463	26.00 ± 0.925	22.00 ± 1.290
STZ + PQQ 5mg	28.85 ± 1.069	26.00 ± 1.121	27.00 ± 1.112
STZ + PQQ 10mg	29.00 ± 1.290	26.50 ± 1.231	27.51 ± 0.975
STZ + PQQ 20mg	29.50 ± 0.816	27.00 ± 0.936	28.50 ± 0.487
STZ + Vit.C	29.20 ± 1.519	26.00 ± 1.315	26.80 ± 1.451

Data are expressed in mean ± SEM (n=7)

Figure 1: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in serum glucose. Data are mean ± S.E.M. (n=7). ^x $P < 0.001$ as compared to the respective control values. ^a $P < 0.001$ and ^c $P < 0.05$ as compared to the respective STZ treated values. STZ, Streptozotocin, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

Figure 2: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in serum insulin (uU/ml) level. Data are mean ± S.E.M. (n=7). ^x $P < 0.001$ as compared to the respective control values. ^a $P < 0.0001$ and ^c $P < 0.05$ as compared to the STZ treated value. STZ, Streptozotocin, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

Figure 3: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in serum testosterone (ng/ml) level. Data are mean $\pm$ S.E.M. (n=7). * $P < 0.001$ as compared to the respective control values. ^ $P < 0.001$ and ^ $P < 0.05$ as compared to the STZ treated value. STZ, Streptozotocin, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

compared to that of control ones) following the STZ administration. When PQQ (5, 10 and 20 mg/kg) was administered to STZ-treated mice, a significant decrease ($P < 0.001$, for all) in LPO level was observed as compared to that of STZ-induced diabetic mice. However, out of the three doses of PQQ, 20 mg/kg was again found to be most effective exhibiting the highest percentage decrease, i.e., 79%. Administration of 20 mg/kg PQQ to normoglycemic mice, did not significantly alter LPO level as compared to normal control ones, while administration of Vit.C at 50 mg/kg to STZ-treated mice decreased the LPO level significantly ($P < 0.001$), with a percentage decrease 49% (Figure 4). Similarly with respect to testicular LOOH level, STZ administration increased its level significantly ($P < 0.001$ as compared to that of control ones). When PQQ (5, 10 and 20 mg/kg) was administered to STZ-treated mice, a significant decrease ($P < 0.001$, for all) in LOOH level was observed in all the tested doses as compared to that of STZ-induced diabetic mice. However, out of the three doses of PQQ, 20 mg/kg was found to be most effective, with a 59% decrease, while administration of Vit.C at 50 mg/kg to STZ-treated mice decreased the LOOH level significantly ($P < 0.001$), with a percentage decrease 35% (Figure 5).

With respect to different antioxidants status of testis, STZ administration decreased the SOD activity significantly ($P < 0.05$ as compared to that of control ones), after PQQ (20 mg/kg) administration in normoglycemic mice, no significant alteration was observed. However, when three different test doses of PQQ were administered to STZ-treated mice, a marked increase in SOD level was observed only with 10 and 20 mg/kg of PQQ ($P < 0.05$ and $P < 0.001$ respectively, as compared to that of STZ-induced diabetic mice). Of course the higher dose increased SOD activity to a greater extent with a percentage increase of 91 %. However, A significant increase ($P < 0.05$) with a percentage of 47% was

Figure 4: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in LPO (nm of MDA formed/h/mg protein) level in the testis. Data are mean $\pm$ S.E.M. (n=7). * $P < 0.001$ as compared to the respective control values. ^ $P < 0.001$ as compared to the respective STZ treated values. STZ, Streptozotocin, LPO, Lipid Peroxidation, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

Figure 5: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in LOOH (nm of LOOH formed /h /mg protein) level in the testis. Data are mean $\pm$ S.E.M. (n=7). * $P < 0.001$ as compared to the respective control values. ^ $P < 0.001$ and ^ $P < 0.01$ as compared to the respective STZ treated values. STZ, Streptozotocin, LOOH, Lipid Hydroperoxide, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

observed after administration of Vit.C 50 mg/kg in diabetic animals (Figure 6). STZ administration also decreased the CAT activity significantly ($P < 0.001$), While PQQ at 20 mg/kg did not alter its activity significantly in normoglycemic animals, PQQ (5, 10 and 20 mg/kg) in STZ-treated mice, significantly increased its activity only at 20 mg/kg of PQQ ($P < 0.001$, as compared to

that of control ones) by 59%. Administration of Vit.C in diabetic animals also increased SOD level significantly ($P < 0.05$) with a percentage of 29% (Figure 7). Changes in tissue GPx activity are illustrated in Fig. 8, GPx was significantly decreased in the STZ-induced diabetic mice ($P < 0.01$). While, following the administration of 20 mg/kg of PQQ to normoglycemic mice, no significant alteration in GPx activity was observed, when PQQ (5, 10 and 20 mg/kg) was administered to STZ-treated mice, there was a significant increase in its activity at the dose of 10 and 20 mg/kg ($P < 0.01$ and $P < 0.001$ respectively), of course the higher dose could increase to a greater extent with an increase of 78% , while after administration of Vit.C to STZ-treated animals, significant increase in GPx level was observed ($P < 0.01$) with a percentage of 64% (Figure 8). GSH level was also significantly decreased by STZ ($P < 0.01$). While administration of 20 mg/kg PQQ to normoglycemic mice, did not significantly alter its level, when PQQ (5, 10 and 20 mg/kg) was administered to STZ-treated animals, a significant decrease in its levels was observed in all the tested doses as compared to that of STZ-induced diabetic mice ($P < 0.05$, $P < 0.01$ and $P < 0.01$ respectively). However, out of the three doses of PQQ, 20 mg/kg was found to be most effective with 62% increase. After administration of Vit.C to STZ-treated animals, significant increase with a percentage of 14% in GSH level was observed ($P < 0.05$), as compared to the respective value of STZ-induced diabetic animals (Figure 9).

With respect to alteration in different lipid levels of testis, a

Figure 7: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in CAT activity ($\mu\text{M H}_2\text{O}_2$ decomposed/min/mg protein) in the testis. Data are mean $\pm$ S.E.M. ($n=7$). x , $P < 0.001$ as compared to the respective control values. a , $P < 0.001$ as compared to the STZ treated values. STZ, Streptozotocin, ^{CAT}, Catalase, ^{PQQ}, Pyrroloquinoline quinone and ^{Vit.C}, Vitamin C

Figure 6: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in SOD activity (units/mg protein) in the testis. Data are mean $\pm$ S.E.M. ($n=7$). z , $P < 0.05$ as compared to the respective control values. b , $P < 0.01$ and c , $P < 0.05$ as compared to the STZ treated values. STZ, Streptozotocin, ^{SOD}, Superoxide dismutase, ^{QQ}, Pyrroloquinoline quinone and ^{Vit.C}, Vitamin C

Figure 8: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in GPx activity ($\mu\text{M GSH}$ consumed/mg protein) in the testis. Data are mean $\pm$ S.E.M. ($n=7$). y , $P < 0.001$ as compared to the respective control values. a , $P < 0.001$ and c , $P < 0.05$ as compared to the STZ treated values. STZ, Streptozotocin, ^{GPx}, Glutathine peroxidase, ^{PQQ}, Pyrroloquinoline quinone and ^{Vit.C}, Vitamin C

Figure 9: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in GSH content (μM GSH/mg protein) in the testis. Data are mean $\pm$ S.E.M. ($n=7$). ^a $P < 0.05$ as compared to the respective control values. ^b $P < 0.01$ and ^c $P < 0.05$ as compared to the STZ treated values. STZ, Streptozotocin, GSH, Reduced glutathione, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

significant increase in the level of total cholesterol, triglyceride, LDL and VLDL was found in STZ treated animals ($P < 0.001$ for all); the administration of PQQ at a dose of 20 mg/kg in STZ-induced diabetic animals, markedly reduced all these indices with a percentage decrease of 30%, 39%, 30% and 39% in total cholesterol, triglyceride, LDL and VLDL respectively; as compared to that of STZ-induced diabetic mice (Figure 3). By the administration of STZ, level of serum HDL decreased significantly ($P < 0.001$). However, simultaneous administration of PQQ in STZ - induced mice; 20 mg/kg of PQQ could significantly increase the HDL level with a 21% increase ($P < 0.05$). Following the test drug administration at 20 mg/kg to normoglycemic mice, there was no significant alteration in all these indices. Administration of Vit.C to STZ - treated animals reduced significantly the levels of total cholesterol, triglyceride, LDL, VLDL in STZ-induced diabetic animals with percentage decrease of 17%, 20%, 18% and 23%. While, administration of Vit.C 50 mg/kg did not alter HDL level significantly in STZ-induced diabetic animals (Figure 10). A comparison of the effects of PQQ and Vit.C; PQQ (20 mg/kg) showed more effectiveness than that of Vit.C (50 mg/kg) in cholesterol, triglyceride, LDL and VLDL with percentage decrease of 30%, 39%, 30% and 40% respectively for PQQ and 17%, 20%, 18% and 23% respectively for Vit.C (Figure 10), as compared with STZ-induced diabetic animals.

With respect to histopathological observations of testis of control animals showed normal testicular architecture with normal lumen of seminiferous tubules (LST), normal thickness of the basement membrane (Bm), spermatids (S), spermatogonia

Figure 10: Effects of PQQ (5, 10 and 20 mg/kg/d, i.p.) for 15 days, in serum cholesterol, triglyceride, HDL, LDL and VLDL (mg/dl). Data are mean $\pm$ S.E.M. ($n=7$). ^x $P < 0.001$ as compared to the respective control values. ^a $P < 0.001$, ^b $P < 0.01$ and ^c $P < 0.05$ as compared to the respective STZ treated values. HDL, High density lipoprotein, LDL, low density lipoprotein, VLDL, Very low density lipoprotein, STZ, Streptozotocin, PQQ, Pyrroloquinoline quinone and Vit.C, Vitamin C

(Sg), Leydig cells (L) and interstitial space (I); in STZ treated group, it showed marked alterations in these normal cellular architecture as evidenced by extensive atrophy and loss in spherical shape, with narrow and elongated seminiferous tubules and LST, loss of centrally located spermatogonial cells, spermatocytes and spermatids (S) and disappearance of the connections between cells. Also increased thickness of basement membrane was observed and widening of the interstitial spaces between seminiferous tubules was quite clear. Atrophy in Leydig cells was observed in the diabetic mice. However, administration of PQQ to STZ treated animals ameliorated these deleterious effects in a dose dependent manner. Similar ameliorative effects were observed on administration of Vit.C (50 mg/kg) in STZ-treated animal. No significant histological changes were observed by the administration of PQQ (20 mg/kg) to normoglycemic animals (Figure 11).

Discussion

From the results it is clearly evident that the test compound, PQQ has the potential to ameliorate the STZ-induced diabetes mellitus and oxidative damage in testis of mice as evidenced by a decrease in serum glucose, tissue LPO, LOOH, the levels of different serum lipids, insulin and testosterone. In fact, PQQ could nearly maintain the normal physiological values of almost all the indices in STZ-treated diabetic mice except with respect to CAT activity and HDL concentration, which were still less as compared to their respective normoglycemic control values. The antidiabetic effects of PQQ were supported by its antioxidative properties, as it not only inhibited tissue LPO, but also enhanced all the tested cellular antioxidants.

Earlier nothing was known on the role of PQQ in STZ-induced

Fig. 11

Figure 11: Photomicrographs of testis histology of representative samples from each experimental group (control mice; control treated with PQQ (20 mg/kg); STZ treated; and STZ + PQQ treated at 5, 10 and 20 mg/kg body weight. The staining was done with toluidine blue and original magnification is $\times 10$ and $\times 40$ respectively of (A & B). Testicular section of normal mouse testis; (C & D) testicular section of normal animals treated only with 20 mg of PQQ; (E-F), testicular section of the STZ treated diabetic group; (G & H) testicular section of the animals treated with PQQ at a dose of 5mg; (I-J) PQQ 10mg; (K & L), PQQ 20 mg; and (M & N) Vit. C 50mg. Control testis shows normal spermatogonia (Sg), spermatids (S), Leydig cells (L), lumen of seminiferous tubule (LST). The STZ-treated group shows, loss of normal cellular architecture, elongated and narrow seminiferous tubule and lumen of seminiferous tubules, loss of leydig cells, loss of centrally located spermatozoa, whereas PQQ treatment shows nearly normal morphology

oxidative damage in testicular tissues. Our results for the first time revealed that PQQ treatment for 15 days could improve the adverse effects in the male primary reproductive organ of diabetic animals. STZ-induced DM was supported by significant increase in glucose and decrease in serum insulin levels, which caused marked oxidative impairments, as evidenced by the increase in MDA and LOOH levels in the testicular tissues with a parallel decrease in the activity of antioxidants. A decline in serum testosterone and HDL and an increase in serum cholesterol, triglyceride, LDL and VLDL were also observed in these animals. These observed alterations are in accordance with some earlier reports, made on the oxidative impairments in the male reproductive system of diabetic subjects [6, 7, 14, 46].

Untreated hyperglycemia very often leads to a number of complications including over-production of ROS such as superoxide anion (O_2^-), nitric oxide (NO^-) and hydrogen peroxide (H_2O_2); which in turn damages the beta cells through the induction of apoptosis and the suppression of insulin biosynthesis [47-54]. In-fact, STZ is known to decrease insulin biosynthesis through alkylation of DNA and overproduction of nitric oxide (NO) and other free radicals [55]. Interestingly PQQ reversed these adverse effects in the DM-induced animals by increasing the antioxidants in testis suggesting that the test drug has the antioxidant potential and to ameliorate DM-induced oxidative stress in testis.

Determination of malondialdehyde (MDA), a product of LPO is used as an important indicator of tissue peroxidation [56]. The increased level of MDA observed in STZ-induced animals similar to an earlier report [46] suggests that hyperglycemia induces peroxidative reactions in lipids [57]. According to Selvakumar et al. [58] LPO and the resultant oxidative stress, play a crucial role in the development of complications in diabetes. The common free radicals that occur in this diseased state are superoxide ($O_2^\bullet$), hydroxyl ($OH^\bullet$) and peroxy ($LOO^\bullet$) radicals, that cause DNA damage, glycation and protein modification reactions in diabetes [59]. On the other hand certain enzymes play crucial role in antioxidant defense, to maintain viable reproductive ability, by providing a protective mechanism against oxidative stress. SOD and GPx are major enzymes that scavenge harmful ROS in male reproductive organs [32, 60]. Therefore, in our study, an increase in these enzymes by PQQ, as observed earlier in other tissues [20, 21] might have protected the STZ-induced testicular damages.

Glutathione, a well-known antioxidant provides the major protection against cellular oxidative damages as it maintains SH level in proteins. It forms reduced GSH from oxidized glutathione that in turn reduces hydrogen peroxide, lipid peroxides, disulfides, ascorbate and free radicals. In this study STZ-treated animals exhibited reduced GSH level as was seen earlier in DM [61, 62]. However, administration of PQQ increased the GSH level in diabetic animals, further consolidating the potential of test compound to enhance the state of this cellular antioxidant status.

Similarly, GPx is a cytosolic enzyme that complements with CAT in order to detoxify H_2O_2 and organic hydroperoxide [63]. Reduction of hydroperoxide by glutathione in the presence of GPx protects mammalian cells from oxidative damages. In the

male reproductive system, GPx is abundantly found in the testis and reduction in GPx activity contributes to male infertility [64]. Interestingly, in the present investigation decrease in GPx activity in STZ-induced diabetic animals was corrected following the PQQ administration, further supporting the antioxidative and protective nature of PQQ in testis.

PQQ is highly electrophilic in nature and it forms stable adducts with carbonyl reagents. These characteristics provide it the ability to oxidize the redox modulatory site, thus conferring protection against ROS-mediated cell injury [65]. PQQ-induced reduction in intracellular ROS levels hypothesizes that it acts directly or indirectly as a potent free radical scavenger [66]. Probably for this reason LPO level was normalized in PQQ treated animals. Whether its site of action is intra-mitochondrial or in the cytoplasm, or both, is yet not clear [66].

The beneficial effect of PQQ was also reflected by the alterations in the level of different lipids and glucose. While, STZ administration increased the levels of serum glucose, cholesterol, triglyceride, LDL and VLDL; simultaneous PQQ administration reduced them. An increase in the serum total cholesterol is very often seen in DM in which alteration of cholesterol biosynthesis takes place, thereby increasing most of the serum lipids [67-69]. It was also reported earlier that there is a negative correlation between serum levels of triglycerides and VLDL to serum testosterone concentration [70]. Interestingly in our study, simultaneous PQQ administration decreased the cholesterol and triglyceride levels as was observed earlier by us and others [20, 29]. Since hyperlipidemia is thought to be ameliorated by the administration of antioxidants [71], PQQ might have reversed the STZ-induced adverse effects in testis by its strong antioxidant potential.

PQQ was also found to increase HDL level in STZ-induced diabetic animals. HDL, a good plasma lipoprotein, is utilized by testicular cells for androgen synthesis [72]. In fact, a good number of HDL receptors are found in the testicular tissue which may be related to its role in the testosterone synthesis [73]. Therefore, in the present study, STZ induced decrease in the HDL level appears to adversely affect the testicular function and it is increased by simultaneous administration of PQQ that maintains its normal level. Changes in the histopathological features of testis in PQQ treated animals, showing normal secretory function of the sertoli and leydig cells also support the positive effects of PQQ in spermatogenesis. Our results do corroborate with earlier reports where HDL level was directly correlated with testicular functions [69].

STZ-induced hyperglycemia leads to reduced leydig cell function, decreased testosterone level and the alterations in the seminiferous epithelium [74-76]. This is also known that increased level of free radicals and oxidative stress reduces the level of testosterone in diabetic animals [77, 78]. As testosterone is required for germinal cell health and for their mitotic division, the enhanced testosterone level in this study in PQQ treated diabetic animals might have prevented the testicular damages as supported by its positive effects on testicular histology and

on testosterone level in diabetic mice, suggesting its potential to correct the STZ-induced testicular dysfunction [79].

The possible mechanism of PQQ ameliorating STZ-induced testicular oxidative stress could be due to its strong antioxidant properties, as suggested in an earlier study, in which the free radical scavenging activity of PQQ has been clearly shown [16]. Although the exact mechanism of action of PQQ in the diabetic testis is not clear, from the present study, it appears that the beneficial effects are associated with its antioxidant properties, as we suggested earlier in hypothyroid mice [21]. Another possible mechanism for hypoglycemic action of PQQ in the present study could be through the regulation of phosphatidylinositol 3-kinase (PI3K) /Akt signaling pathway, which is known to control the glucose homeostasis [80]. This concept is further supported by a recent report in which it was stated that PQQ exhibits its protective effects via activating the PI3K / AKT pathway [81]. Although in the present investigation also the effects of PQQ in regulating DM-induced oxidative damages might have been mediated through activation of PI3K/ AKT pathway; exact mechanism remains to be worked out.

This may be emphasized that nothing was known till date on the role of PQQ in regulating STZ-induced oxidative stress in testis of diabetic ones, despite the fact that PQQ regulates liver, kidney and heart which are often associated with DM [32, 20]. Therefore, the present report appears to be the first one that clearly indicates the efficacy of PQQ in regulating STZ-induced oxidative damages in testis. Interestingly, the ameliorative effects were better expressed by PQQ as compared to that of Vit.C.

Conclusion

In conclusion, to the best of our knowledge, this work is the first report documenting the potential of PQQ to ameliorate diabetes mediated testicular damages through its effective regulation of glucose and lipid metabolism. Our findings provide a substantial basis for future investigations for assessing new PQQ based therapies for the treatment of diabetes associated reproductive problems. Interestingly, the ameliorative effects were better expressed by PQQ as compared to that of Vit.C. However, the detailed molecular mechanism underlying the effects requires further intensive investigations.

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