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Quercetin ameliorates polychlorinated biphenyls-induced testicular DNA damage in rats

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Summary

Polychlorinated biphenyls (PCBs) are a group of environmental contaminants widely reported to cause gonadal toxicity in both humans and animals. This study investigated the amelioratory role of quercetin in PCBs-induced DNA damage in male Wistar rats. Polychlorinated biphenyls were administered intraperitoneally at a dose of 2 mg kg^{-1} alone or in combination with quercetin (orally) at 50 mg kg^{-1} for 25 days. Quercetin modulation of PCBs-induced gonadal toxicity was evaluated using selected oxidative stress indices, comet assay, measurement of DNA concentration and histology of the testes. Administration of PCBs alone caused a significant ($P < 0.05$) depletion in the total thiol level in testes of treated rats. Conversely, the levels of reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) production were markedly elevated in testes of PCBs-treated rats compared with control. Further, PCBs exposure produced statistically significant increases in DNA tail migration, degraded double-stranded DNA (dsDNA) concentration and histological alterations of testes of the treated rats compared to control. Quercetin cotreatment significantly improved the testicular antioxidant status, decreased DNA fragmentation and restored the testicular histology, thus demonstrating the protective effect of quercetin in PCBs-treated rats.

Introduction

Polychlorinated biphenyls (PCBs), which are chemicals containing 1–10 chlorine atoms substituent on biphenyl, are of anthropogenic origin and belong to the group of persistent organic pollutants (Adeogun *et al.*, 2013). Despite the ban or restriction on the use of PCBs years ago, it is amazing that they are still found all over the world even where they have never been used (Dang *et al.*, 2010; Li *et al.*, 2010; Wu *et al.*, 2014). The persistence and ubiquitous distribution of PCBs potentially poses serious hazards to human and animal health especially the male reproductive system (Gray *et al.*, 1993; Kimbrough, 1995; Murgesan *et al.*, 2005).

Earlier studies on the adverse effects of PCBs on the male reproductive system implicated PCBs to interfere with hypothalamic–pituitary–testicular axis. PCBs have been reported to reduce the weight of testis and accessory sex organs, decrease epididymal sperm count, increase sperm abnormalities along with a marked testicular atrophy in rats (Senthilkumar *et al.*, 2004; Aly *et al.*, 2009; Elumalai *et al.*, 2009). Moreover, PCBs reportedly inhibited the activities of cytochrome P450 side chain cleavage enzyme, 3β- and 17β-hydroxysteroid dehydrogenases, consequently disrupting the Leydig cellular functions of steroidogenesis in exposed animals (Murugesan *et al.*, 2008). Furthermore, PCBs are well reported to exert their toxic effects by increasing free radicals generation, thus inducing oxidative stress (Murgesan *et al.*, 2005; Aly *et al.*, 2009; Atessahin *et al.*, 2010; Aly, 2013). Recent studies on PCBs toxicity demonstrated that PCB-induced genotoxicity in male gonads through DNA oxidative damage and inhibition of DNA repair gene expression (Attia *et al.*, 2014). Increased ROS production in the cellular environment can damage DNA and oxidise cellular proteins (Patlolla *et al.*, 2015).

Quercetin is one of the flavonoids, which has been reported to be a promising chemoprotective agent against

the noxious effects of PCBs in experimental animals. It is ubiquitously present in fruits, vegetables, plant-derived foods and medicines (Havsteen, 2002; Farombi *et al.*, 2012). Quercetin has been reported to mitigate PCBs-induced neurobehavioral changes, neuronal and hepatic oxidative damage as well as apoptosis adult rats (Sekaran *et al.*, 2012; Selvakumar *et al.*, 2012, 2013). Moreover, quercetin reportedly protected against PCBs-induced injury in endometrial cells of the pregnant rats through inhibition of cytokines secretion (Xu *et al.*, 2014). Quercetin is well known to possess multiple biological activities including antioxidant and free radical scavenging activities as well as a potential anticarcinogenic activity (Musonda & Chipman, 1998; Murakami *et al.*, 2008).

However, the protective role of quercetin in PCBs-induced testicular damage has not been reported. Investigation into the influence of quercetin on gonadal genotoxicity is indispensable as some of the noxious effects produced by xenobiotic exposure could be transmitted to subsequent generation through reproduction. Considering the beneficial health effects of quercetin, we have investigated for the first time, the amelioratory role of quercetin in PCBs-induced testicular oxidative DNA damage using Wistar rats as an animal model.

Materials and methods

Chemicals

Quercetin, polychlorinated biphenyls (PCBs, Aroclor 1254), thiobarbituric acid (TBA), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), cysteine and 5′,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade and were obtained from local commercial sources.

Animal model

Thirty-two adult male Wistar rats (10 weeks old; 186 ± 5 g) obtained from Central Animal House of the Federal University of Santa Maria (UFSM) were housed in clean polypropylene cages and maintained on a 12-h light/dark cycle and a temperature of 20–25 °C with *ad libitum* access to food and water. All experimental procedures were carried out according to the guidelines and approval of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (Protocol number 116/2011).

Experimental protocol

The animals were randomly assigned into four groups consisting of eight rats each and were treated for 25 days as follows. Group I rats received corn oil intraperitoneally daily as a vehicle. Group II rats were orally treated with quercetin at 50 mg kg^{-1}. Group III rats were intraperitoneally treated with PCBs (Aroclor 1254) at 2 mg kg^{-1}. Group IV rats were cotreated with quercetin and PCBs in a similar manner as groups II and III respectively. The doses of PCBs and quercetin were selected from previous studies (Elumalai *et al.*, 2009; Tieppo *et al.*, 2009). We evaluated quercetin amelioratory effect on PCBs-induced testicular DNA damage at half of the time (25 days) required to achieve a cycle of spermatogenesis in rats (Cooke *et al.*, 1996; Chandra *et al.*, 2010). Intraperitoneal route of PCBs exposure was chosen because it is practically simple and allows relatively long periods of absorption from the repository site.

Preparation of homogenates

All the animals were sacrificed 24 h after the last treatment under ketamine and xylazine anaesthesia. The testes of the rats were rinsed in ice-cold physiological saline and weighed. Ten per cent testicular homogenate (w/v) was prepared in 0.05 M phosphate buffer (pH 7.4) using a motor driven Teflon pestle homogeniser and centrifugation at 500 ***g*** for 10 min at 4 °C. Subsequently, the supernatant was decanted and centrifuged at 2000 ***g*** for 60 min at 4 °C. The cellular fraction obtained, which was referred to as 'homogenate', was subsequently used for biochemical analysis.

Lipid peroxidation (TBARS) assay

Testicular lipid peroxidation was quantified as the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction according to previously published study (Ohkawa *et al.*, 1979). Briefly, the reaction mixture consisting 200 μl of testes homogenates or standard (0.03 mM MDA), 200 μl of 8.1% sodiumdodecyl sulphate (SDS), 500 μl of 0.8% TBA and 500 μl of acetic acid solution (2.5 M HCl, pH 3.4) was heated at 95 °C for 1 h. The absorbance was measured at 532 nm using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA). TBARS tissue levels were expressed as μmol MDA per mg of protein.

Reactive oxygen species detection

Reactive oxygen species production was quantified by the 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) method based on the ROS-dependent oxidation of DCFH-DA to DCF (Zapolska-Downar *et al.*, 2002). Briefly, 50 μl of testes homogenates, Tris-HCl buffer (10 mM; pH 7.4) and DCFH-DA solution at final concentration of 50 μM were incubated in the dark for 30 min to allow the probe to be incorporated into any mem-

brane-bound vesicles, and the diacetate groups cleaved by esterases. Fluorescence of the samples was monitored at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a SpectraMax plate reader (Molecular Devices). Background fluorescence was corrected by inclusion of parallel blanks. DCF levels were expressed as percentage of control.

Total thiol groups (T-SHs) determination

Total thiol content was determined according to the method previously described by Ellman (1959). Briefly, the reaction mixture consisted 40 µl of homogenate, 10 µl of 10 mM DTNB and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 200 µl. The mixture was incubated for 30 min at ambient temperature and then read the absorbance at 412 nm using a SpectraMax plate reader (Molecular Devices). A standard curve was plotted for each measurement using cysteine as a standard and the results expressed as µmol per mg protein.

Double-stranded DNA assay

The concentration of double-stranded DNA (dsDNA) was determined according to Abdalla *et al.* (2014) using a Quant-IT TM PicoGreen® dsDNA kit (Invitrogen-Life Technologies, Grand Island, NY, USA) in accordance with the manufacturer's instructions. The DNA PicoGreen® dye presents high affinity for the dsDNA and therefore able to quantify the dsDNA fractions released by dead cells into the extracellular medium. Briefly, the reaction mixture consisting 50 µl of the sample and 50 µl of the PicoGreen® working solution was incubated for 5 min in a dark room. The fluorescence was measured at an excitation of 485 nm and an emission of 520 nm using a SpectraMax plate reader (Molecular Devices). The dsDNA concentrations of the groups were expressed as ng ml^{-1}.

Comet assay

The alkaline comet assay was performed to according to established procedure (Tice *et al.*, 2000; Spiazzi *et al.*, 2013). Briefly, 100 µl of single-cell suspensions derived from the testes was mixed with 900 µl 1% low melting-point agarose in PBS, and 200 µl of the resulting suspensions was applied to microscope slides (Fisher Scientific, St. Louis, MO, USA) previously coated with 1% agarose and was allowed to set at 4 °C for 5 min. Subsequently, the slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1% triton X-100 with 10% DMSO) to remove cell proteins, leaving DNA as 'nucleoids'. The slides were then placed on a horizontal electrophoresis unit, covered with a fresh solution (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V cm^{-1}). Following neutralisation, the slides were washed in double distilled water and stained using a silver staining protocol (Maluf and Erdtmann, 2000; Nadin *et al.*, 2001) and air-dried at room temperature overnight. The gels were thereafter analysed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides of five animals per group) were selected and analysed. Cells were visually scored according to tail length and receive scores from 0 (no migration) to 4 (maximal migration) according to tail intensity (Tice *et al.*, 2000). The slides were analysed at least by two different individuals who were blinded to the treatment groups. The extent of DNA damage was expressed in arbitrary units (AU) and calculated using the following formula:

$$\text{A.U.} = \frac{0\,(n \text{ level } 0) + 1(n \text{ level } 1) + 2(n \text{ level } 2) + 3(n \text{ level } 3) + 4(n \text{ level } 4)}{\text{Total comets analysed}} \times 100$$

where n is the number of cells.

Histology and histomorphometry of the testes

Biopsies of the testes were carefully removed and fixed with 10% formaldehyde. Subsequently, the tissues were dehydrated in graded concentrations of alcohol, cleared using xylene and embedded in paraffin wax. Sections of 4–5 µm were prepared by a microtome and stained with haematoxylin and eosine (H & E). All slides were coded before examination with light microscope (Olympus CX21FS1, Tokyo, Japan) and photographed using a digital camera. Histomorphometry was performed using image analysis software (Image J; NHI, Bethesda, MD, USA). Each microscopic slide was evaluated in 50 random histological fields during the measurement of seminiferous epithelium and tunica albuginea thickness.

Statistical analysis

All values were expressed as mean ± SD. Data were analysed using one-way analysis of variance (ANOVA) follo-

wed by Dunnett's *t*-test for post hoc evaluation using GraphPad Prism 5 software. (version 4; GraphPad Software, La Jolla, California, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Oxidative stress indices

As depicted in Fig. 1, there was a significant decrease in testicular total thiol (T-SHs) level, whereas the TBARS and ROS generation levels were increased significantly in PCBs-treated rats when compared with the control group. However, co-administration of quercetin reversed the PCBs-mediated decrease in T-SHs and elevation of ROS and LPO levels to near normal in the treated rats. Administration of PCBs decreased the testicular T-SHs by 45%, whereas it caused 66% and 148% elevation in ROS and TBARS levels respectively. Interestingly, quercetin co-treatment resulted in 57%, 66% and 91% restoration in the levels of T-SHs, ROS and TBARS respectively.

Comet assay

Comet assay demonstrates the level of damage to the DNA strand. A higher DNA strand migration through the electrophoresis gel indicates a marked DNA fragmentation. As depicted in Fig. 2, PCBs-treated rats showed a significantly higher DNA damage index when compared with the control group. However, the level of DNA fragmentation was reversed to near control level following cotreatment with quercetin when compared with the PCBs-treated rats.

Table 1 shows the sum of cells and the degrees (0–4) of damage according to tail migration of DNA strands in comet assay. PCBs-treated rats showed a higher number of cells in classes 1–4, whereas the number of cells exhibiting no migration (class 0) was lower compared with control. Considering class 4 (maximal migration) which represents apoptotic cells, the comet assay evidenced the presence of apoptotic cells in the PCBs-treated testes. However, rats co-administered with quercetin and PCB showed no apoptotic cells and the number of migrating cells was decreased to near normal.

Histology and histomorphometry of the testes

The histomorphometry analysis of the testes is presented in Fig. 3. The result indicated that the thickness of tunica albuginea was not statistically altered in all the treatment groups. However, the thickness of seminiferous epithelium was significantly decreased in PCBs-treated testes than control. However, co-administration of quercetin restored the thickness of the seminiferous epithelium to normalcy. Photomicrographs of the testes are depicted in Fig. 4. The testes of control rats appeared structurally and functionally normal. The testes of rats exposed to PCBs alone showed some interstitial congestion, oedema and haemorrhage (yellow arrow). Conversely, the testes of rats cotreated with quercetin appeared normal and similar to the control.

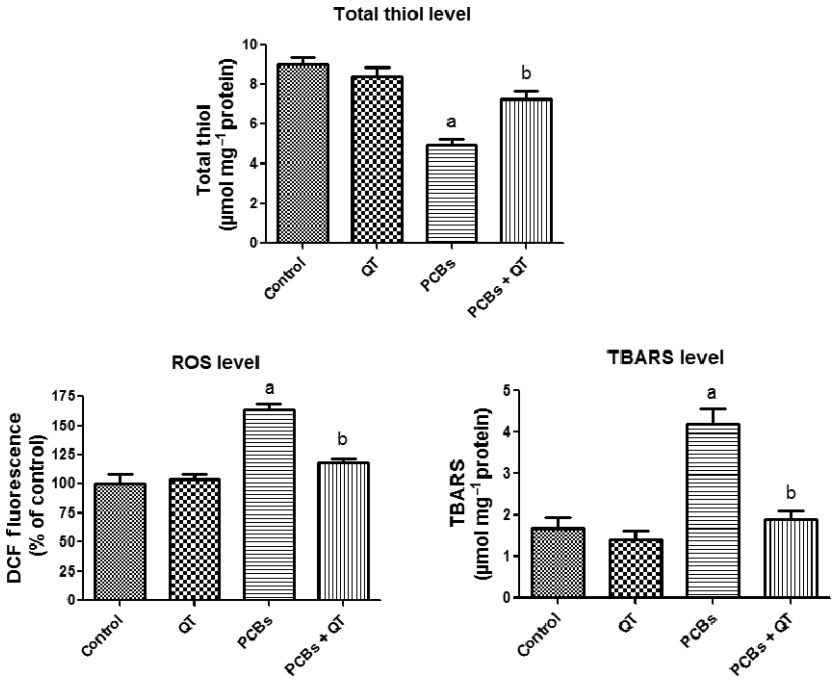

Fig. 1 Effects of quercetin and PCBs on total thiol, reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) levels in testes of rats. Each bar represents mean ± SD of eight rats. $^{a}P < 0.05$ against control. $^{b}P < 0.05$ against PCBs.

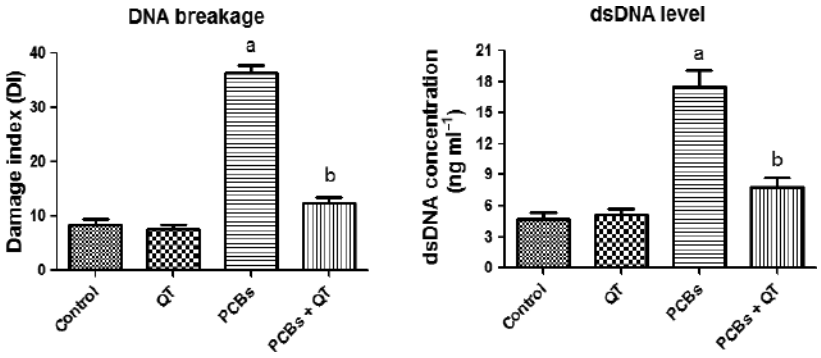

Fig. 2 Effects of quercetin and PCBs on levels of DNA breakage and dsDNA in testes of rats as revealed by comet and PicoGreen assays respectively. Each bar represents mean ± SD of eight rats. $^{a}P < 0.05$ against control. $^{b}P < 0.05$ against PCBs.

Table 1 Levels of damage according to migration of DNA strands in comet assay

	Levels of damage (Σ cells counted)				
Treatment	0	1	2	3	4
Control	459	41	0	0	0
Qt	464	36	0	0	0
PCBs	383	71	31	12	3
PCBs + Qt	446	46	8	0	0

Qt, quercetin; Level 0 (intact comets without migration); Level 1 (1–25% damage); Level 2 (25–45% damage); Level 3 (45–70% damage) and Level 4 (more than 70% damage with maximal migration).

Discussion

The present investigation evaluated the amelioratory role of quercetin in PCBs-induced testicular DNA damage by determining the morphological, biochemical and molecular alterations in testes of the treated rats. The results of this study showed that PCBs exposure caused a significant decrease in the testicular T-SHs whereas it increased the ROS and TBARS levels, thus evidencing a state of oxidative stress. The reduction in the intracellular T-SHs level in PCBs-treated rats could indicate its overutilisation and/or oxidation following PCBs exposure (Chan *et al.*, 2010; Aly, 2013). Pro-oxidative alterations in the intracellular thiol status are well known to adversely affect cellular processes (Fourquet *et al.*, 2008; Kumar *et al.*, 2011; Toledano *et al.*, 2013; Adedara & Farombi, 2014). The observed elevation in the TBARS level, an index of lipid peroxidation, in PCBs-exposed rats is attributed to the increased ROS production as well as the depletion of testicular antioxidant status. However, the results of the present study showed that quercetin co-administration significantly mitigated against PCBs induction of oxidative stress evidenced by the restoration of testicular total thiol, ROS and TBARS levels in the treated rats. The protective effects of quercetin may be related to its antioxidant nature.

Standard comet assay is generally considered as a highly sensitive and reliable method for evaluating the DNA fragmentation due to single- and double-strand breaks. PicoGreen®, a commercially available fluorescent dye, intercalates into the double helix of DNA with a frequency of about one dye molecule per four base pairs of dsDNA as well as the phosphate backbone surface using electrostatic interactions along with groove binding. PicoGreen® reagent exhibits an emission maximum at 530 nm when bound specifically to dsDNA. Interestingly, the observed biochemical alterations in antioxidant status in the present study were accompanied by a remarkable DNA damage, which was detected in the testes of PCBs-treated rats with the aid of comet assay and PicoGreen® dsDNA kit. Excessive intracellular ROS production is well known to attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids which eventually leads to DNA, RNA, proteins and lipids damage (Wu *et al.*, 2002; Fedato & Maistro, 2014; Patlolla *et al.*, 2015). Moreover, oxidative damage to DNA has been suggested to be one of the most important factors responsible for PCBs-induced gonadal cell genotoxicity and carcinogenic modes of action (Attia *et al.*, 2014). However, co-treatment of quercetin with PCBs significantly ameliorated the fragmentation of DNA in the treated rats. The recovery of DNA integrity in testes of PCBs plus quercetin-treated rats is attributable to its ability to scavenge the deleterious free radicals in the testicular milieu.

In addition, the present investigation demonstrated that exposure to PCBs caused some morphological changes such as interstitial congestion, oedema and haemorrhage in testes of the treated rats. Histomorphometry analysis indicated that PCBs exposure decreased seminiferous epithelium thickness without affecting the

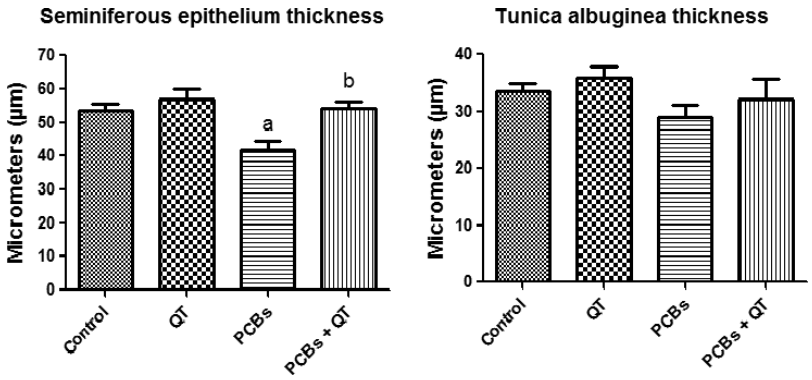

Fig. 3 Effects of quercetin and PCBs on histomorphometry endpoints in testes of rats. Each bar represents mean ± SD of eight rats. $^{a}P < 0.05$ against control. $^{b}P < 0.05$ against PCBs.

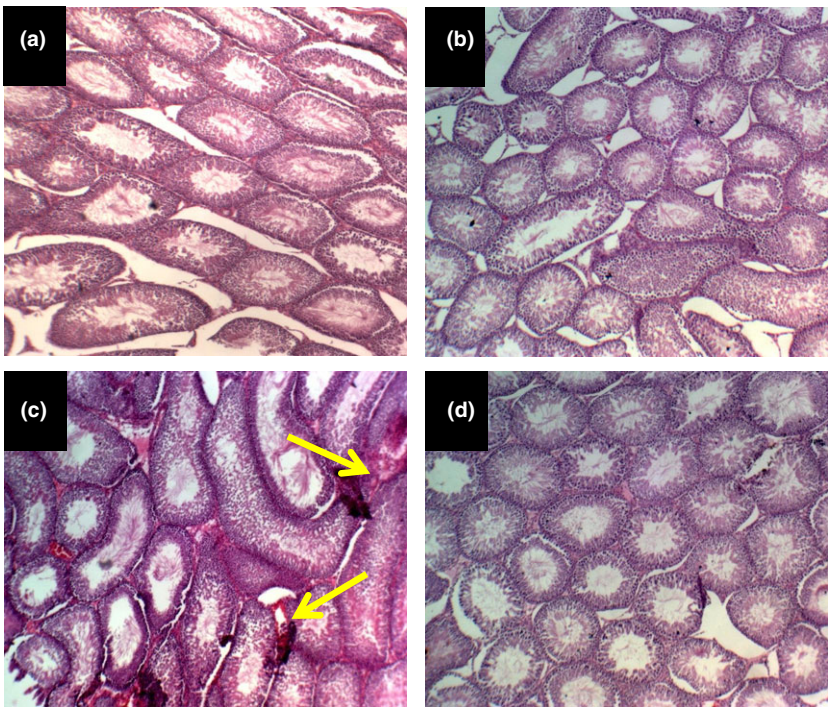

Fig. 4 Photomicrographs of testes from the experimental animals. The testes of control (a) and quercetin alone (b) rats showing normal histology. The testes of PCBs-treated rats (c) showing interstitial congestion, oedema and haemorrhage (yellow arrow). The testes of rats cotreated with quercetin (d) appeared structurally and functionally normal and comparable to control. Original magnification: 160×.

tunica albuginea thickness thus suggesting the susceptibility of the seminiferous epithelium to PCBs. The obvious restoration of the testicular histology and seminiferous epithelium thickness following cotreatment with quercetin indicates its protective effect in the PCBs-treated rats. In mechanistic term, the structure–activity relationship activities of quercetin is due to the presence of the *O*-dihydroxyl structure at the 3′- and 4′-positions in the B-ring, referred to as catechol group, is largely responsible for this activity (Bors *et al.*, 1990). This property is essential for exerting antioxidant activity by scavenging free radicals, such as superoxide anions, perhydroxyl radicals and chain propagating lipid peroxyl radicals (Rice-Evans *et al.*, 1996; Murakami *et al.*, 2008).

In conclusion, the results presented herein establish for the first time a beneficial role of quercetin in PCBs-mediated testicular antioxidant status depletion, ROS production and DNA damage, thus providing novel insights into the molecular mechanisms underlying the chemoprotective effects of quercetin in the testes. The findings in the present study highlight that quercetin as a promising compound may be developed to novel therapeutic agent against testicular toxicity resulting from PCBs exposure.

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