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Research article

Curative effects of tectochrysin on paraquat-instigated testicular toxicity in rats: A biochemical and histopathological based study

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ABSTRACT

Background: Paraquat (PQ) is a herbicide that is used globally in the agriculture sector to eradicate unwanted weeds, however it also induces significant damages in various organs of the body such as testes. Tectochrysin (TEC) is an important flavonoid that shows versatile therapeutic potentials. Currently, there is no established antidote to cure PQ-induced testicular toxicity.
Objective: The present study was conducted to evaluate the ameliorative effects of TEC against PQ prompted testicular damage.
Methods: Sprague-Dawley rats (n = 48) were used to conduct the trial. Rats were allocated in to 4 groups i.e., Control, PQ administrated group (5 $mgkg^{-1}$), PQ + TEC co-administrated group (5 $mgkg^{-1}$ + 2.5 $mgkg^{-1}$) and TEC only administrated group (2.5 $mgkg^{-1}$). The trial was conducted for 8 weeks. The activity of anti-oxidants and the levels of MDA and ROS were determined by spectrophotometric method. Steroidogenic enzymes as well as apoptotic markers expressions were evaluated by qRT-PCR. The level of hormones and inflammatory indices was quantified by enzyme-linked immunosorbent assay.
Results: PQ exposure markedly ($P < 0.05$) disturbed the biochemical, spermatogenic and histological profile in the rats. Nevertheless, TEC treatment considerably ($P < 0.05$) increased CAT, GPx GSR and SOD activity, besides decreasing MDA and ROS contents. TEC administration also increased sperm viability, count and motility. 17β-HSD, 3β-HSD, StAR and Bcl-2 expressions were also increased following TEC administration. The supplementation of TEC substantially ($P < 0.05$) decreased Bax, Caspase-3 expression and the levels of inflammatory markers i.e., interleukin-1β (IL-1β), interleukin-6 (IL-6), nuclear factor kappa-B (NF-κB), tumor necrosis factor-α (TNF-α) and cyclooxygenase-2 (COX-2) activity. Additionally, the levels of plasma testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were increased following TEC supplementation. Furthermore, TEC supplementation considerably decreased sperm structural abnormalities and histomorphological damages of the testes. The mitigative role of TEC might be due to its anti-inflammatory, anti-apoptotic, androgenic and anti-oxidant potentials.
Conclusion: Taken together, it is concluded that TEC can be used as a potential candidate to treat testicular toxicity.

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1. Introduction

Paraquat (PQ) acts as herbicide in agriculture to inhibit the growth of different weeds [1,2]. Due to its low cost and high efficiency, it has replaced other pesticides that became less effective due to plant resistance [3,4]. Therefore, PQ is used as a herbicide in many countries due to its low cost of production [5]. PQ shows low volatile potential and high-water solubility [6], therefore, its exposure can induce serious health issues [7]. Humans are generally subjected to PQ through dermal contact or mouth and its acute exposure can cause death within 3.5 hours [8]. The rate of PQ induced toxicity has been increased in developing countries [9,10]. PQ toxicity causes respiratory failure and induces damage in the hepatocytes, adrenal glands, kidneys, lungs, immune system and heart [11]. Moreover, neurological degenerative diseases in rats have also been reported following PQ exposure [12].

Testes are the vital organs that contain seminiferous tubules responsible for the sperm production [1]. Various exogenous chemicals may affect sperm production and maturation in testes [13]. PQ suppresses the endogenous anti-oxidant enzymes and generates free radicals [14]. These free radicals stimulate the ROS production in the testicular tissues, which eventually prompts oxidative stress (OS) that can impair the Leydig and Sertoli cells [15,16]. Any disturbance in these cells may interfere with spermatogenesis. Moreover, PQ exposure instigates sperm mitochondrial damage that eventually reduces the formation of ATP necessary for sperm motility [17]. Previous literature has reported that PQ intoxication loweres hormonal levels (FSH, LH and testosterone) and anti-oxidant activities [14,18,19].

Flavonoids belong to a family of hydroxylated phenolic compounds that are getting attention due to their multiple pharmacological properties [20,21]. They are reported to show anti-inflammatory, anti-cancer and anti-oxidant potentials [22,23]. Tectochrysin (TEC) is a flavone that is present in many plants [24], particularly, edible species such as propolis [25] *Lychnophora markgravii* [26] and *Alpinia oxyphylla Miq* [27]. TEC demonstrates anti-microbial, anti-cancer [26,28] anti-diarrheal [29] anti-oxidant and anti-inflammatory [24,30] properties. Moreover, the supplementation of TEC improves the spatial memory performance in mice [31].

To overcome PQ induced testicular toxicity, a detailed investigation on plant-based remedies was required. Therefore, based on the abovementioned pharmacological potentials of TEC, the current study was formulated to assess the attenuative role of TEC against PQ induced testicular impairments.

2. Materials and methods

2.1. Chemicals

PQ (Molecular Weight: 257.16, CAS No: 75365-73-0) and TEC (CAS No: 520-28-5, Molecular Weight: 268.26) were purchased from Sigma-Aldrich (Germany).

2.2. Experimental animals

Sprague-Dawley rats (body weight 200 ± 30g, n = 48) were kept in the research station of University of Agriculture, Faisalabad by using steel cages. The experiment was performed under standard environmental conditions i.e., 23–25 °C temperature and photo-period of 12 h day/night cycle were maintained. Balanced rodent diet and tap water was supplied to all the animals. Rats were handled in line with instructions of European Union of Animal experimentation and Care (CEE Council 86/609) that were further approved by UAF ethical committee (DGS/18657–60/19–05–2022).

2.3. Experimental protocol

Four groups were made, each consisting of 12 rats and exposed to different regiments for 8 weeks. These groups included control, PQ group (5 $mgkg^{-1}$), PQ + TEC group (5 $mgkg^{-1}$ + 2.5 $mgkg^{-1}$) and TEC group (2.5 $mgkg^{-1}$). The doses were administered with an oral gavage. After 8 weeks of trial, the rats were sedated, cardiac blood was gathered and centrifuged for 15 min (3000 rpm) to isolate the plasma. The plasma was stored at −20 °C for analysis. Testes were collected and right testis was kept in formalin for histological analysis. Left testis was kept at −80 °C for biochemical analysis. Testicles were homogenized in Na_3PO_4 buffer solution at 12000 rpm for 14–15 min. Finally, various parameters were evaluated using the supernatant.

2.4. Biochemical analysis

2.4.1. Catalase (CAT)

The method described by Aebi [32] was followed to evaluate CAT activity. 0.4 mL of 5.9 mM H_2O_2, 2.5 mL of 50 mM phosphate buffer (pH 5.0) and 0.1 mL of enzyme extract were used to make reaction mixture. At 240 nm, variations in the mixture's absorbance were recorded. A change in absorbance of 0.01 units per minute was considered to quantify CAT activity.

2.4.2. Superoxide dismutase (SOD)

SOD activity was evaluated by following the practice of Kakkar et al. [33]. The reaction mixture consisted of 0.1 mL of phenazine methosulphate (186 mM) and 1.2 mL of sodium pyrophosphate buffer (0.052 mM; pH 7.0). The reaction solution was mixed with 0.3 mL of supernatant obtained from centrifugation of the homogenate at 10,000×*g* for 15 min. Then, 0.2 mL of NADH (780 mM) was

poured to trigger reaction and it was terminated by pouring glacial acetic acid (1 mL). Lastly, the quantity of chromogen was determined by observing the color intensity change (at 560 nm). The activity of SOD was denoted as units/mg protein.

2.4.3. Glutathione peroxidase (GPx)

GPx activity was appraised by using Rotruck et al. [34] technique. The components of reaction mixture were 0.2 mL of 10 mM glutathione, 0.5 mL of 0.2 mM H_2O_2, 2.0 mL of 0.4 M Tris-HCl buffer (pH 7.0) and 0.01 mL of 10 mM sodium azide. After incubation for 10 min at 37 °C, 0.4 mL of 10 % (v/v) TCA was used to terminate the reaction. After centrifuging the mixture at 5000 rpm, for 5 min absorbance was noted at 430 nm. Values were presented as unit/mg protein.

Glutathione reductase (GSR)

Carlberg and Mannervik strategy [35] was employed to determine GSR activity. The reaction mixture (2 mL volume) contained: 1.65 mL of phosphate buffer (0.1 M, pH 7.6), 0.05 mL of oxidized glutathione (1 mM), 0.1 mL homogenate (10 %) as well as 0.1 mL NADPH (0.1 mM). GSR activity was quantified by observing the decrease in NADPH at 340 nm.

2.4.4. Assessment of MDA

MDA content was determined by using Afsar et al. method [36]. Thiobarbituric acid reactive compounds assessment was carried out by examining against standard curve of MDA (equivalents) generated by 3 tetramethoxy propane and acid-catalyzed hydrolysis of 1, 1, 3. The resultant values were denoted by standard unit (nmoL/mg protein).

2.4.5. Assessment of ROS

ROS level was ascertained by using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). 100 μL of 2 mg/mL DCFH-DA was reacted with 100 μL of cell lysates. Following the period (30 min) of incubation at 37 °C, fluorescence change was observed employing fluorescence plate reader. The wavelength of excitation was measured at 488 mm, while emission wavelength was determined at 525 mm when both the wavelengths were set accordingly. Result was displayed as relative fluorescence unit (RFU) per mg protein.

2.5. Assessment of inflammatory indices

ELISA kits (Cloud-crone Corp. USA) were used to assess IL-1β (CSB-E08055r), NF-kB (CSB-E13148r), IL-6 (CSB-E04640r), TNF-α (CSB-E07379r) levels and COX-2 (CSB-E13399r) activity, by following the company instructions. 50 μL of sample was dispensed in the microplate wells. Subsequently, 50 μL of an antibody cocktail were added to each well.Incubated was accomplished at room temperature for 1 h. After washing with the wash buffer, 100 μL of TMB substrates were added and allowed to incubate for 10 min. The color was produced when 100 μL of stop solution was mixed. Tecan Multimode Reader was used to quantify optical density at 450 nm.

2.6. Evaluation of sperm indices

Sperm count was appraised by using a hemocytometer, as demonstrated by Yokoi et al. [37]. The left epididymal region was gently crushed in 5 mL saline solution. To dilute the supernatant, a solution consisting of 1 mL of formalin, 25 mg eosin/100 mL of distilled water and 5g of sodium bicarbonate was taken at a ratio of 1:100. After that, 10 μL drops of the aforesaid combination was put in a sperm count chamber and analyzed microscopically (400X). Moreover, the semen samples were observed to quantify sperm motility by using the method delineated by Kenjale et al. [38]. The sperm viability was evaluated by staining the samples using eosin-nigrosin and then observed under a microscope [39]. Furthermore, the protocol of Correia et al. [40] was used to assess sperm anomalies.

2.7. Hypo-osmotic swelling test (HOS)

HOS test was employed to evaluate sperm integrity by using the method of Correa and Zavos [41]. HOS test was performed by adding 20 μL of semen in 180 μL of fructose solution and maintained at 70 mOsm/L osmotic pressure for 25 min. After incubation, the specimens were examined under a microscope at 400X and stained with eosin or nigrosin.

2.8. Assessment of hormonal level

Plasma testosterone (serial no. H090), LH (serial no. H206) and FSH (serial no. H101) levels were measured using (ELISA) kits by following the company's instructions (Los Angeles, CA, USA). ELISA plate was filled with assay diluent (50 μL) as well as plasma (10 μL). Incubation was carried out for 120 min at room temperature. Following through washing with deionized water, the plates were incubated for 2 h then 100 μL of peroxidase-conjugated immunoglobulin G (IgG) anti-LH, anti-FSH or anti-testosterone was added to each well. Plates were once again washed with deionized water, then wells were filled with substrate solution following the incubation period (25 min) at room temperature. To terminate the reaction, stop solution (50 μL) was used. Finally, the absorbance of plasma testosterone, LH, and FSH was measured at 450 nm.

2.9. Real-time polymerase chain reaction (qRT-PCR)

Steroidogenic enzymes and apoptotic markers expressions were estimated by RT-qPCR. Total RNA was separated employing TRIzol reagent and cDNA was generated by using a Fast Quant RT kit (China). The relative expressions of these parameters were determined

by $2^{-\Delta\Delta CT}$ and β-actin was considered as an internal control [42]. Primer sequence of targeted genes are presented in Table 1 [43].

2.10. Histopathology

The testicular tissues were washed using normal saline solution and fixed in formalin (10 %). After that testicular tissues were dehydrated by using alcohol and fixed in paraffin wax. Using a microtome 5 μm slices were made and slides were dipped in tape water for 1 min. Hematoxylin stain was poured onto the slides for 5 min. Slides were again dipped in tape water for 1 min and then transferred to a jar containing 1 % acid alcohol and washed in tape water. Finally, 1 % eosin was poured onto the slides for 30–60 seconds and washed with tape water and observed under a microscope. The analysis of microphotographs were carried out by using Image-J2X.

2.11. Statistics

Data were shown as Mean ± SEM. The assessment of normality was carried out by employing Shapiro-Wilk test. Minitab was used to perform statistical analysis followed by one-way ANOVA and Tukey's test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Protective potential of TEC on oxidants

Exposure to PQ noticeably ($P < 0.05$) lowered anti-oxidants activity and considerably increased MDA and ROS contents with respect to control rats. Nevertheless, PQ + TEC administration considerably ($P < 0.05$) improved anti-oxidants activity, while decreasing ROS and MDA levels with respect to PQ administered rats. The rats administered with TEC alone showed the values of these parameters comparable to control rats (Table 2).

3.2. Protective potential of TEC on inflammatory mediators

IL-6, NF-κB, IL-1β, TNF-α levels and COX-2 activity were markedly ($P < 0.05$) increased after exposure to PQ with respect to control rats. Nonetheless, a substantial decrease in the aforementioned markers was noticed in PQ + TEC rats as compared to PQ-treated rats. Moreover, inflammatory indices in TEC administered group were compareable to control group (Table 3).

3.3. Protective potential of TEC on spermatogenic profile

A remarkable ($P < 0.05$) decrease was observed in sperm motility, count and viability after PQ exposure. Conversely, PQ-treated rats showed more sperm abnormalities as compared to control group. The co-treated group (PQ + TEC) showed improvements in sperm viability, motility and count, while a decrease in sperm structural abnormalities was noticed with respect to PQ intoxicated group. Moreover, the semen profile of TEConly treated group was comparable to control (Table 4).

3.4. Protective potential of TEC on hormonal profile

The results of the hormonal assay revealed that PQ exposure prompted a notable ($P < 0.05$) reduction in FSH, LH and testosterone level with respect to control group. Conversely, in PQ + TEC group the hormonal levels were considerably increased with respect to PQ-treated group. Moreover, TEC only treated rats showed a normal hormonal profile as in the control group (Table 5).

Table 1
Primers sequences for qRT-PCR.

Gene	Primers 5′-3′	Accession number
3β-HSD	Forward: GCATCCTGAAAAATGGTGGC Reverse: GCCACATTGCCTACATACAC	NM_001007719
17β-HSD	Forward: CAGCTTCCAAGGCTTTTGTG Reverse: CAGGTTTCAGCTCCAATCGT	NM_054007
StAR	Forward: AAAAGGCCTTGGGCATACTC Reverse: CATAGAGTCTGTCCATGGGC	NM_031558
Bax	Forward: GGCCTTTTTGCTACAGGGTT Reverse: AGCTCCATGTTGTTGTCCAG	NM_017059.2
Bcl-2	Forward: ACAACATCGCTCTGTGGAT Reverse: TCAGAGACAGCCAGGAGAA	NM_016993.1
Caspase-3	Forward: ATCCATGGAAGCAAGTCGAT Reverse: CCTTTTGCTGTGATCTTCCT	NM_012922.2
β-actin	Forward: TACAGCTTCACCACCACAGC Reverse: GGAACCGCTCATTGCCGATA	NM_031144

Table 2
Effects of PQ + TEC on the biochemical markers.

Parameters	Groups			
	Control	PQ	PQ + TEC	TEC
CAT (U/mg protein)	10.39 ± 0.27	4.97 ± 0.08#	8.67 ± 0.18*	10.43 ± 0.26*
GPx (U/mg protein)	26.49 ± 0.38	11.10 ± 0.30#	20.23 ± 0.78*	26.61 ± 0.47*
SOD (U/mg protein)	8.45 ± 0.17	3.61 ± 0.05#	7.04 ± 0.19*	8.49 ± 0.16*
GSR (nM NADPH oxidized/min/mg tissue)	6.52 ± 0.12	2.56 ± 0.21#	5.32 ± 0.19*	6.61 ± 0.12*
MDA (nmol/mL)	0.59 ± 0.07	3.51 ± 0.13#	1.57 ± 0.08*	0.56 ± 0.08*
ROS (RFU/ mg protein)	0.65 ± 0.04	5.29 ± 0.18#	1.21 ± 0.15*	0.62 ± 0.07*

Values are shown on the basis of Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the values in this table are based on 12 biological replicates per group with 3 technical replicates of each.

Table 3
Effects of PQ + TEC on the inflammatory markers.

Parameters	Groups			
	Control	PQ	PQ + TEC	TEC
NF-κB (ng/g tissue)	15.46 ± 1.33	85.67 ± 2.09#	33.59 ± 1.12*	15.39 ± 1.33*
TNF-α (ng/g tissue)	11.62 ± 0.18	43.70 ± 2.40#	19.51 ± 1.09*	10.81 ± 0.18*
1L-1β (ng/g tissue)	17.40 ± 1.22	82.66 ± 0.57#	38.84 ± 1.39*	17.33 ± 1.21*
IL-6 (ng/g tissue)	5.85 ± 1.06	61.58 ± 1.64#	16.51 ± 1.58*	5.72 ± 0.96*
COX-2 (ng/g tissue)	24.39 ± 1.22	75.94 ± 1.35#	32.55 ± 0.68*	24.30 ± 1.26*

Values are shown on the basis of Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the values in this table are based on 12 biological replicates per group with 3 technical replicates of each.

Table 4
Effects of PQ + TEC on sperm parameters.

Parameters	Groups			
	Control	PQ	PQ + TEC	TEC
Epididymal sperm count (million/mL)	21.96 ± 0.86	7.12 ± 0.20#	16.62 ± 0.71*	22.21 ± 0.92*
Sperm motility (%)	87.37 ± 1.15	29.78 ± 0.79#	71.05 ± 1.10*	87.48 ± 1.21*
Dead sperm (%)	8.40 ± 0.15	80.40 ± 0.55#	23.93 ± 0.86*	8.30 ± 0.09*
Head abnormality (U/mg protein)	4.35 ± 0.10	34.20 ± 1.29#	8.75 ± 0.46*	4.32 ± 0.09*
Mid sperm abnormality (%)	0.69 ± 0.03	6.74 ± 0.11#	2.71 ± 0.08*	0.67 ± 0.04*
Tail abnormality (%)	3.39 ± 0.12	25.26 ± 0.76#	6.76 ± 0.35*	3.36 ± 0.13*
Hypo- osmotic swelled sperm count (HOS) (%)	84.10 ± 1.30	23.44 ± 1.21#	71.19 ± 1.39*	84.36 ± 1.48*

Values are shown on the basis of Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the values in this table are based on 12 biological replicates per group with 3 technical replicates of each.

3.5. Protective potential of TEC on anti-apoptotic and apoptotic markers

The results of apoptotic and anti-apoptotic assay demonstrated that PQ exposure prompted a substantial ($P < 0.05$) up-regulation in Caspase-3, Bax expressions and a decline in Bcl-2 expression with respect to control group. However, PQ + TEC group showed upsurge in Bcl-2 expression and a notable downregulation in Caspase-3 and Bax expression as compared to PQ treated group. Furthermore, in TEC only treated rats the aforementioned expressions were comparable to control group [Fig. 1(a-c)].

Table 5
Effects of PQ + TEC on hormonal level.

Parameters	Groups			
	Control	PQ	PQ + TEC	TEC
Plasma Testosterone (ng/mL)	5.07 ± 0.10	2.47 ± 0.09#	4.22 ± 0.09*	5.12 ± 0.10*
LH (ng/mL)	2.60 ± 0.07	0.58 ± 0.11#	1.99 ± 0.12*	2.64 ± 0.09*
FSH (ng/mL)	4.13 ± 0.11	1.06 ± 0.12#	3.78 ± 0.07*	4.27 ± 0.12*

Values are shown on the basis of Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the values in this table are based on 12 biological replicates per group with 3 technical replicates of each.

3.6. Protective potential of TEC on steroidogenic enzymes profile

PQ exposure noticeably ($P < 0.05$) decreased the expressions of 3β-HSD, StAR and 17β-HSD. Whereas, a substantial increase in these expressions was observed in co-treated (PQ + TEC) group as compared to PQ intoxicated group. Moreover, in TEC only treated group the expression of these markers was compareable to the control group [Fig. 2(a-c)].

3.7. Protective potential of TEC on testicular histology

PQ treatment induced severe histological damage in the testicular tissues, including lowered seminiferous epithelial height, tunica propria width along with seminiferous tubular diameter. On the other hand, interstitial spaces (IS) along with tubular lumen (TL) showed a substantial increase with respect to control group. Conversely, PQ + TEC co-administration markedly regained tunica propria thickness, epithelial height as well as seminiferous tubular diameter, besides IS and TL were lowered in comparison to the PQ intoxicated rats. Additionally, in the TEC treated rats the histological profile was close to control rats [Table 6, Fig. 3(a-d)].

4. Discussion

Environmental and industrial toxicants can potentially cause male infertility. These toxic chemicals disrupt endocrine system that leads to reproductive abnormalities or infertility [44]. PQ is a herbicide that deteriorates semen quality and induces germ cell apoptosis by increasing OS [45,46]. Moreover, PQ administration may trigger cytotoxic damage to the male reproductive tract [47]. Plant-based compounds are getting attention owing to their potential pharmacological effects [22,23]. TEC is a phytochemical that shows anti-apoptotic, anti-oxidant and anti-inflammatory activities [24,26,28]. Therefore, the current study was intended to appraise the sheilding effect of TEC on PQ-prompted testicular impairments.

PQ intoxication remarkably lowered CAT, GPx, GSR and SOD activities, whereas the levels of MDA and ROS were elevated in the testicular tissues. ROS induces adverse effects on biological molecules (DNA, lipid and protein) by inducing OS [48,49]. Hydroxyl free radicals, hydrogen peroxide along with superoxide anion are the main oxidative species [50]. These free radicals attack the testicular

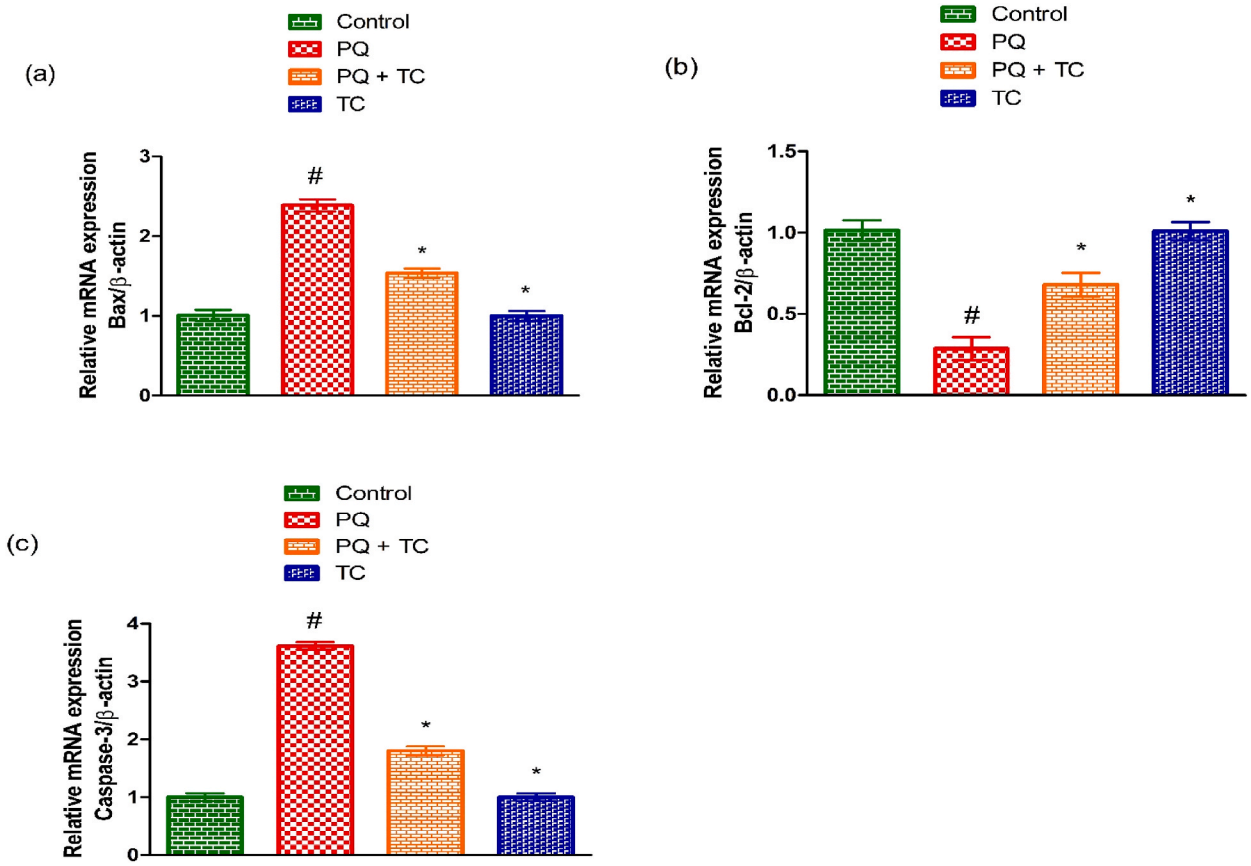

Fig. 1. The effects of PQ and TEC on (a) Bax (b) Bcl-2 and (c) Caspase-3 expression. Bars are based on Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the graphs in this figure are based on 12 biological replicates per group with 3 technical replicates of each.

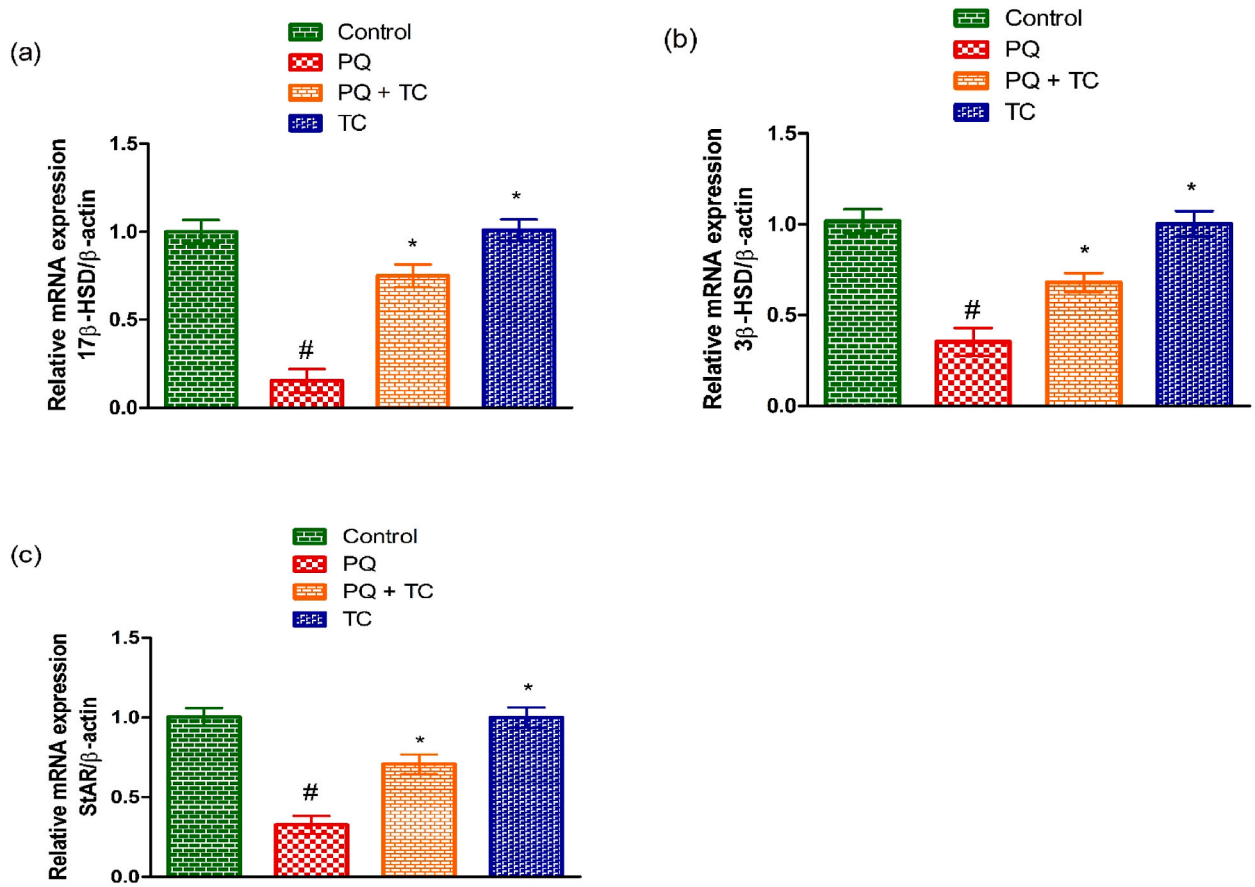

Fig. 2. The effects of PQ and TEC on steroidogenic enzymes expression: (a) 17β-HSD (b) 3β-HSD (c) StAR. Bars are based on Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the graphs in this figure are based on 12 biological replicates per group with 3 technical replicates of each.

Table 6
Effects of PQ + TEC on testicular histology.

Parameters	Groups			
	Control	PQ	PQ + TEC	TEC
Interstitial Space (μm)	7.89 ± 0.54	75.39 ± 1.79#	18.94 ± 1.33*	7.79 ± 0.45*
Tunica propria (μm)	64.77 ± 1.05	12.82 ± 0.77#	35.47 ± 1.27*	65.25 ± 1.01*
Diameter of tubules (μm)	386.01 ± 6.81	110.38 ± 7.72#	278.10 ± 8.98*	388.49 ± 7.16*
Seminiferous tubule epithelial height (μm)	89.47 ± 1.46	22.62 ± 1.01#	78.72 ± 1.73*	89.94 ± 1.78*
Tubular lumen (μm)	18.74 ± 1.44	74.32 ± 1.27#	35.60 ± 1.07*	18.58 ± 1.43*
Spermatogonia (n)	64.65 ± 1.23	19.67 ± 1.12#	57.63 ± 1.02*	64.72 ± 1.27*
Primary spermatocytes (n)	59.83 ± 1.43	21.69 ± 0.79#	45.37 ± 1.26*	59.97 ± 1.47*
Secondary spermatocytes (n)	55.52 ± 1.26	18.48 ± 0.63#	47.34 ± 1.09*	55.59 ± 1.27*
Spermatids (n)	51.53 ± 1.42	12.74 ± 1.51#	42.22 ± 1.57*	51.74 ± 1.52*

Values are shown on the basis of Mean ± SEM. Significant differences are displayed as # $P < 0.05$ compared to control, * $P < 0.05$ compared to PQ treated group. All the values in this table are based on 12 biological replicates per group with 3 technical replicates of each.

antioxidant enzymes and impair the whole organ [51]. Moreover, ROS can attack PUAFs in the plasma membrane of sperm and triggers lipid peroxidation (LP) [52]. During LP, MDA is released as a byproduct and its level can indirectly reveal the damage that results due to LP and ROS [53]. CAT is a major enzyme of the antioxidant system that is involved in H_2O_2 catabolism [54]. SOD is another free radical-scavenging enzyme that neutralizes the superoxide radicals by reducing them into O_2 and H_2O_2 [55]. GSR maintains GSH concentration, which is essential for the continue activity of GPx [56]. However, TEC + PQ co-treatment significantly increased the anti-oxidants activities, besides lowering MDA and ROS contents. Flavonoids display anti-oxidant activity due to the presence of phenolic rings and OH groups in their structural formula [57]. The results of our experiment showed compatibility with the study of Lee et al. [58], who performed an *in vivo* experiment and reported that TEC significantly increased the activity of anti-oxidant

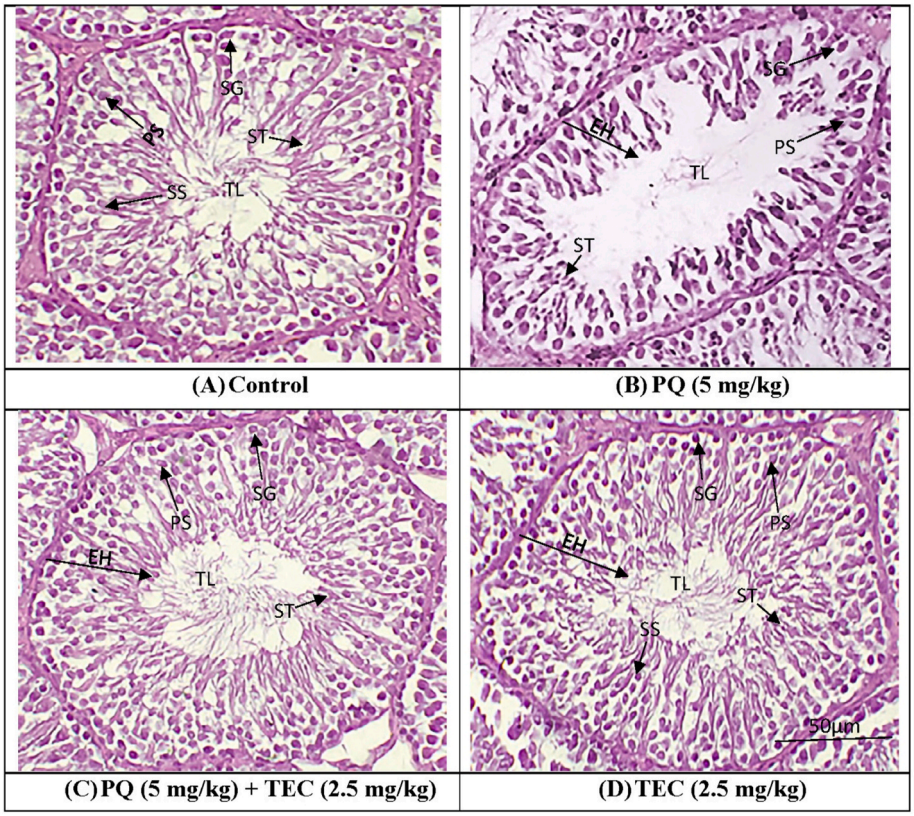

Fig. 3. (a) Control group displaying normal histopathology (b) PQ treated group showing epithelial cells sloughing, lessened the tunica propria thickness as well as augmented IS and TL. (c) PQ + TEC co-treated group showing improvement in the process of spermatogenesis by increased spermatid number, reduced epithelial cell sloughing and interstitial space. (d) TEC-treated group presenting typical seminiferous tubules with a tiny IS and luminal section contained germ cells, indicating regained spermatogenesis.

enzymes and lowered the level of MDA.

PQ exposure significantly reduced sperm quantity, motility along with sperm viability. Besides the morphological abnormalities of sperms i.e., head, midpiece and tail were increased. According to previous literature, sperms are highly susceptible to free radicals [59]. Excessive ROS can disrupt the PUAFs of sperm membrane by inducing LP. This process adversely effects the permeability and fluidity of the plasma membrane of male germ cells [60]. Our results are in line with the study of Wasiu and Abdulfatai [61], who reported that PQ administration resulted in oxidative stress which eventually reduced sperm motility and sperm count. Furthermore, OS produced due to high ROS concentration adversely affects the mitochondria of sperm and disturbs the production of ATP. Reduced production of ATP decreases flagellar activity of spermatozoa that induces immobility and apoptotic death of male gametes [60]. However, TEC treatment significantly mitigated all these impairments due to its ROS-scavenging property.

PQ intoxication considerably increased the levels of inflammatory markers. Various oxidizing agents stimulate the activation of NF-κB, a crucial redox-responsive transcriptional factor that has a primary role in the expression of other pro-inflammatory mediators (IL-1β, TNF-α, IL-6) [62]. A high level of pro-inflammatory mediator promotes the process of inflammation [63]. Moreover, COX-2 also performs a pivotal role in cellular inflammation [64]. However, TEC supplementation significantly reduced the levels of NF-κB, IL-1β, TNF-α, IL-6 and the activity of COX-2. Hou et al. [24] revealed that the chemical strcture of TEC such as double bond at C2–C3 and 7-OCH_3 on its A-ring are responsible for its anti-inflammatory property. Our results are also confirmed by a previous study [65], which reported that TEC treatment reduced the inflammatory indices in the liver of rats.

According to our findings, exposure to PQ dramatically lowered plasma testosterone, FSH, and LH levels. The hypothalamic-pituitary-gonadal (HPG) axis controls fertility and reproduction [66]. The production of testosterone is very crucial for male reproductive potential [67]. FSH is a glycoprotein polypeptide hormone that enhances sperm maturation and indirectly regulates testicular function [68]. LH mediates the synthesis of testosterone by Leydig cells, which is essential for sperm production [69]. Thus, the process of spermatogenesis depends on an appropriate ratio of LH, FSH and testosterone in testicular tissues [70]. According to Soni et al. [71], PQ affects the HPG axis that eventually disturbs the reproductive system. Nevertheless, TEC treatment significantly improved the hormonal levels that may be due to its regulatory effects on HPG axis.

3β-HSD, 17β-HSD and StAR expressions were markedly reduced following the PQ exposure. These enzymes play a vital role in the production of steroid hormone (testosterone) [72]. In males, testosterone is responsible for gametes production and secondary sexual characteristics. Cholesterol is used as a substrate by Leydig cells to produce testosterone [73]. StAR is a protein that performs important

role in transportation of cholesterol into mitochondria [74]. However, the two main enzymes that are essential for androgenesis are 3β-HSD and 17β-HSD [75,76]. These enzymes catalyze the conversion of cholesterol into testosterone [77]. The expressions of abovementioned enzyme were significantly increased in rats co-treated with PQ + TEC. Upregulated expression of the steroidogenic enzymes may be due to the fact that chemical configuration of flavonoids closely resembles with cholesterol and many other steroids that significantly regulates the androgen production [78].

The administration of PQ significantly increased the expressions of Caspase-3 and Bax, besides lowering the Bcl-2 expressions [79]. Moreover, Li et al. [1] demonstrated that Bcl-2 expressions were decreased in the rats following PQ exposure. Apoptosis is primarily activated by mitochondria, a central hub of cellular respiration and energy source [80]. Mitochondria normally release ROS as a by-product of mitochondrial respiration but high production of ROS due to certain chemical exposure can induce adverse effects such as swelling and rupturing of mitochondrial membrane [81]. Ruptured mitochondrial membrane encourages cytochrome C discharge into the cytosol [82]. This immense liberation of cytochrome C triggers Caspase-3 that leads to apoptosis [83]. The co-treatment of PQ + TEC lowered Caspase-3 and Bax expression and upsurged Bcl-2 expression owing to its anti-apoptotic property.

PQ administration induced significant testicular histopathological damages i.e., decrease in seminiferous epithelial height, diameter and tunica propria width. Tubular lumen (TL) along with interstitial spaces (IS) were increased following the PQ exposure. According to the findings of Wasiu and Abdulfatai [61], PQ exposure increases the interstitial space, damages the morphology of male germ cells, and distortes seminiferous tubules in rat testes. Nevertheless, these histopathological damages were significantly alleviated followed by PQ + TEC co-treatment. In conclusion, TEC improved the histological profile due to its ROS-scavenging, anti-inflammatory, anti-apoptotic and androgenic properties.

5. Conclusion

Our findings demonstrated that TEC showed protective effects against testicular damage prompted by PQ. TEC administration significantly recovered the activities of anti-oxidant enzymes and the levels of MDA and ROS. Moreover it restored the levels of inflamatory markers and regulated the expressions of apoptotic markers and steroidogenic enzymes. TEC administration also recovered the PQ induced histological impairments. Therefore, it is concluded that TEC has tendency to cure PQ prompted testicular impairments.

Limitations and future perspective

The study was performed using rats as experimental animal, however its clinical trials on human are imperative in future.

Ethical approval

Rats were handled by following the instructions of European Union of Animal Experimentation and Care (CEE Council 86/609) that were further approved by UAF ethical committee (DGS/18657–60/19–05–2022).

Data availability

The corresponding authors will provide the data when requested reasonably.

CRediT authorship contribution statement

Muhammad Umar Ijaz: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Kaynat Alvi:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Ali Hamza:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Haseeb Anwar:** Visualization, Validation, Software. **Khalid A. Al-Ghanim:** Writing – review & editing, Software, Resources, Investigation, Funding acquisition, Data curation. **Mian Nadeem Riaz:** Writing – review & editing, Writing – original draft, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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