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RESEARCH

Quercetin Supplementation Alleviates Cadmium Induced Genotoxicity-Mediated Apoptosis in Caprine Testicular Cells

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Abstract

Being a common environmental pollutant, cadmium causes detrimental health effects, including testicular injury. Herein, we document the ameliorative potential of quercetin, a potent antioxidant, against cadmium-induced geno-cytotoxicity and steroidogenic toxicity in goat testicular tissue. Cadmium induced different comet types (Type 0 – Type 4), indicating the varying degree of DNA-damage in testicular cells. The quantitative analysis at 50 and 100 µM cadmium concentration revealed the DNA damage with per cent tail DNA as 75.78 ± 1.49 and 94.65 ± 0.95, respectively, in comparison to the control group (8.87 ± 0.48) post 8 h exposure duration. Cadmium caused a substantial decrease in the activity of key steroidogenic enzymes' (3β-HSD and 17β-HSD) along with reduction of testosterone level in testicular tissue. Furthermore, cadmium treatment induced various types of deformities in sperm, altered the Bax/Bcl-2 expression ratio in testicular tissue and thus suggesting the apoptosis-mediated death of testicular cells. Simultaneous quercetin supplementation, however, significantly ($p < 0.05$) averted the aforementioned cadmium-mediated damage in testicular tissue. Conclusively, the cadmium-induced DNA-damage and decrease in steroidogenic potential results in death of testicular cells via apoptosis, which was significantly counteracted by quercetin co-supplementation, and thus preventing the cadmium-mediated cytotoxicity of testicular cells.

Keywords Apoptosis · Cadmium · Comet · Infertility

Introduction

Heavy metals-contaminated water and soil have rapidly increased during the recent decades as a result of electronic wastes, municipal wastes disposal, smelting and mining, fossil fuel burning [1], and application of pesticides, fertilizer, and sewage [2]. Heavy metals like lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are reproductive toxins commonly present in the environment and negatively impact the reproductive health of organisms [3]. Current research on the quality of human male sperm has revealed that environmental contaminants, not genetic defects, are the primary cause of observed defects in male reproductive function [4, 5]. A parallel reduction in fertility rates has been observed, which has also been related to toxic environmental exposures involving heavy metals [6]. Cadmium (Cd) has been documented as a ubiquitous industrial, agricultural, and environmental toxic heavy metal that adversely affects the male reproductive system by causing subfertility or infertility [7]. One more potential source of Cd exposure to the human population is smoking. However, after smoking, Cd content of smoker is found to be 4–5 times higher than non-smokers [8]. Nearly 15% of people worldwide are recognized as infertile due to smoking, with men accounting for 50–60% of the total, due to the high risk of impairment in male germ cells, which accumulate mutation in each spermatogenesis division [9, 10]. In recent studies, Cd has been shown to have a wide range of toxicity effects such as kidney malfunctioning, teratogenicity, oncogenicity, and toxicity of reproductive and endocrine system [11, 12]. The reproductive organs are one of the most important

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target organs of Cd accumulation and intoxication, which is important here. Studies have demonstrated that Cd gets accumulated in the reproductive system of *Xenopus laevis* after chronic treatment of Cd, in addition to the kidney and liver [13]. Acute as well as chronic studies on exposure of Cd have established that it affects negatively the structure and function of the testes. In this context, Cd causes numerous derangements that include diminishing the function of Sertoli cell, impairment of testicular steroidogenesis, lowering of serum testosterone, germ cells' death, and abolishing the quality of semen (sperm motility, quantity, morphology, etc.) [8, 9, 14]. Cadmium (Cd) induced reactive oxygen species (ROS) and oxidative stress have been associated with apoptosis in many cell types including the spermatogenic cells, where it has been shown to upregulate the expression of pro-apoptotic protein Bax and caspase 3 with simultaneous dampening of anti-apoptotic protein Bcl-2 [15]. Therefore, abnormal hormone production, enhanced oxidative stress, DNA damage, and resulting apoptosis of testicular germ cells presumed to play an important role in the pathogenesis of Cd-induced male infertility [16, 17]. However, in recent years, numerous efforts have been made by researchers to reduce the toxicity induced by Cd using various strategies, like chelation therapy and abolition by several agents [18]. Chelation therapy has not been effective in cases of low and chronic exposure to Cd, so there is a demand for the development of a more safe and cost-effective therapeutic approach to alleviate the Cd-elicited toxicity in testes. Quercetin (3, 39, 49, 5, 7-pentahydroxyflavone), an omnipresent flavonoid, is widely distributed in fruits, vegetables, and accounts for the majority of flavonoids found in daily foods [19]. Because of its ameliorative power, quercetin (Qcn) has the potential to protect the kidney [20], liver [21], brain [22] and other tissues [23] from damage caused by Cd and other factors. Our previous studies have also demonstrated the protective potential of Qcn against Cd-induced oxidative and ultrastructural damage in testicular tissue [24, 25]. Therefore, the present work aimed at further exploring the potential of Qcn to attenuate Cd-evoked testicular dysfunctions at molecular level. Particularly, the underlying mechanism of Qcn to counteract the Cd-induced DNA damage associated apoptosis of testicular cells and steroidogenic impairment in goat were examined under in vitro conditions.

Materials and Methods

Chemicals

Quercetin (Qcn) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA (purity ≥ 95%). Cadmium chloride ($CdCl_2$) was acquired from Sigma-Aldrich (St. Louis, MO, USA, purity 99.99%). Unless otherwise stated, all other reagents used were of highest purity (analytical grade) and obtained from HiMedia Laboratories Pvt., India.

Procurement of Goat Tetes

The testes of mature goat (*Capra hircus*; Jamnapari breed) were procured from the slaughterhouses around Kurukshetra (29°6′ N, 76°50′ E) and Chandigarh (30°43′ N, 76°12 ′E), placed in ice-cold normal saline (0.9%) and brought to the laboratory. The testis was properly washed in normal saline followed by its decapsulation. However, the goat testes were obtained as a by-product of routine castration and thus did not cause any harm to the animal.

In-Vitro Treatment

- Post washing, the decapsulated testicular tissue was cut into small pieces having size of approximately $1mm^3$.
- These testicular tissues were incubated with $CdCl_2$ along with Qcn co-supplementation at 10, 50, and 100 µM (concentrations were based on previous studies [26, 27]).
- Testicular tissues were cultured in media-TCM199 supplemented with antibiotics (100 IU/mL each of penicillin and streptomycin) in a CO_2 incubator (5% CO_2, 95% humidity, 38°C temperature) for 4 and 8 h, respectively.
- However, tissues in the control group, on the other hand, were not treated with $CdCl_2$ and Qcn. Tissues were simply cultured in media-TCM199 for respective time durations (Fig. 1).

- Post incubation, the testicular tissues from both control and treatment groups were subjected to additional testing for a variety of assays.

Preparation of Testicular Cell Suspension

After the relevant treatment for respective time durations, the goat testicular tissue from treated and untreated control groups was kept in a petri dish containing phosphate-buffered-saline (PBS, pH 7.2) and chopped with a sharp blade. Suspension of testicular cells was aspirated and centrifuged at 3000 rpm using a GeNeI™ centrifuge for 5 min. The supernatant was removed, and the pellet was resuspended in PBS. The procedure was repeated three times to obtain a debris-free cell suspension [24].

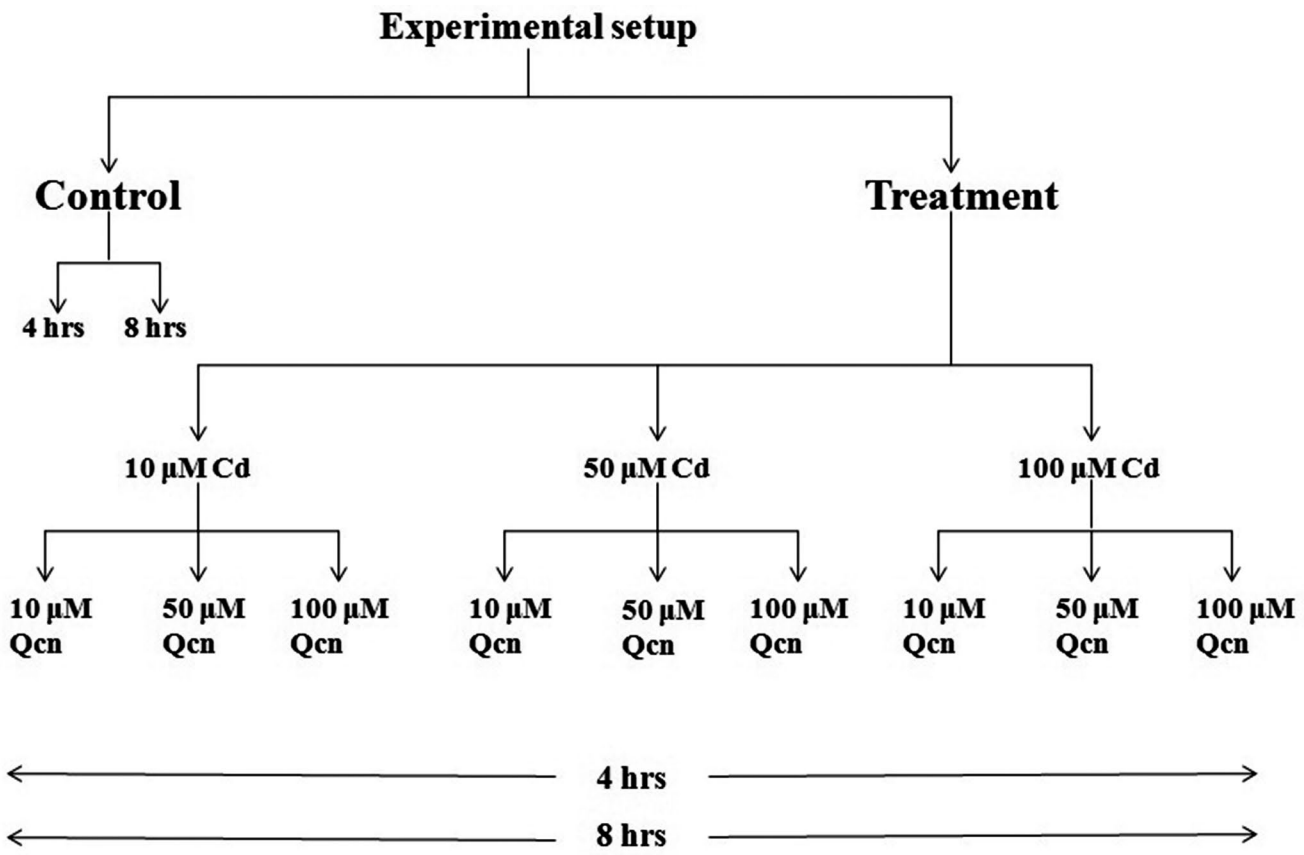

Fig. 1 Experimental setup to study the effects of Cd and quercetin on testicular germ cells in a dose-and time-dependent manner

MTT Assay

The testicular cells (~1×10^6) from the freshly prepared cell suspension were seeded in each well containing 100 µL culture media supplemented with respective doses of Cd and Qcn (10, 50 and 100 µM) into microplates (tissue culture grade, 96 wells, flat bottom) and cultured for respective time durations (4 and 8 h). After respective durations, MTT solution (10 µL) from the stock (5 mg/mL) was added in each well followed by incubation at 37ºC for 2 h. Post incubation, DMSO (200 µL) was added into each well to terminate the reaction. Finally, absorbance was taken at 570 nm on a microtitre plate reader (Bio-Rad, California, USA) to assess viability percentage of cells.

Single Cell Gel Electrophoresis/Comet Assay

Single cell gel electrophoresis assay (SCGE, also called Comet assay) was used for the detection and quantification of DNA damage in testicular tissue at the level of individual cell. The assay was performed according to the protocol of Ahuja and Saran (1999) [28] with slight modifications. Briefly, first layer of normal melting point agarose (NMPA) was made by applying 150–200 µL of NMPA (1%) on the poly-l-lysine coated slides and allowed to dry at 37°C in oven. After drying, 100 µL of low melting point agarose (LMPA, 0.5%) was mixed with 25 µL of testicular germ cell suspension and layered on the first layer of NMPA as a second layer followed by cooling at 4 °C for 10 – 15 min. Post solidification, 200 µL LMPA was applied over it as a third layer to sandwich the sample containing middle layer. Post solidification of gel, slides was placed in freshly prepared chilled lysis solution for duration of 10–12 h. After that, slides were kept in freshly prepared electrophoresis buffer (pH > 13) prior to electrophoresis for 20–40 min with no gap between them. Electrophoresis was performed at 25 V and 300 mA for 30 min. Post electrophoresis, the slides were coated with neutralization buffer followed by their staining with ethidium bromide. However, whole procedure was carried out in dim light to prevent artificial DNA damage and photolysis. The different types of comets were visualized and photographed using a Nikon Eclipse 90i Trinocular microscope (Nikon Instruments Inc, New York, U.S.A.) and DS-QiMC camera (Nikon Instruments Inc, New York, U.S.A.), respectively. The scoring of various types of comet parameters was done using OpenComet software.

Sperm Deformities Analysis

- Semen sample was collected from the goat testis my making incision with the help of a sharp blade in the epididymis (cauda) of goat testis.
- An appropriate volume of semen sample was diluted with PBS and checked under microscope to prevent clustering of spermatozoa.
- Diluted semen sample was incubated with respective concentrations of $CdCl_2$ and Qcn in media-TCM199 for 4 h in a CO_2 incubator (5% CO_2, 95% humidity, 38°C temperature).
- Post incubation, one semen sample's drop was placed on a cleaned glass slide and a smear was prepared followed by its staining with eosin stain.
- Slides were observed under the light microscope (Olympus, Japan) for various sperm deformities.
- However, the quantification of different types of sperm deformities was done by analyzing the 100 sperm cell in each treated group.
- The percentage of different sperm deformities were calculated by dividing the numbers of particular deformities with the total number of sperm cell (100) counted in each treatment slide.

Steroidogenic Enzyme Analysis

The activity of two key steroidogenic enzyme i.e., 3β-Hydroxysteroid dehydrogenase (3β-HSD) and 17β-Hydroxysteroid dehydrogenase (17β-HSD) in testicular tissue were measured according to the protocol of Bergmeyer, (1974) [29] with few alterations. Briefly, the freshly prepared testicular tissue extract (0.1 mL) was mixed with 100 mM sodium pyrophosphate buffer (0.6 mL, pH-8.9), 0.1 mM dehydroepiandrosterone (0.1 mL), 0.5 mM NAD (0.2 mL), and 2 mL of redistilled water, making the total reaction mixture to 3 mL. After that absorption of reaction mixture was taken at 340 nm against blank using IMPLEN nanospectrophotometer (Munchen, Germany). Activity of 3β-HSD was measured using the extinction coefficient 6.22 M^{-1} cm^{-1} and calculated as nmol NAD reduced/min/mg protein. Similarly, for 17β-HSD, testicular tissue extract (0.1 mL) was mixed with 100 mM sodium pyrophosphate buffer (0.6 mL, pH-8.9), 0.1 mM androsterone (0.1 mL), 1.1 mM NADPH (0.2 mL), and 2 mL of redistilled water, making the total reaction mixture to 3 mL. After that absorption of reaction mixture was taken at 340 nm against blank using IMPLEN nanospectrophotometer (Munchen, Germany). Activity of 17β-HSD was measured using the extinction coefficient 6.22 M^{-1} cm^{-1} and calculated as nmol NADPH oxidized/min/mg protein.

Hormonal Assay

The ADVIA Centaur® Testosterone II (TSTII) assay kit was used for the quantitative determination of testosterone in testicular tissue treated with different concentrations of Cd and Qcn for 8 h according to the manufacturer instructions using the SIEMENS automated chemiluminescence immunoassay systems (ADVIA Centaur®XP; Fernwald, Germany).

Real-Time PCR Using SYBR Green

Quantitative real-time PCR (qRT-PCR) was performed to analyze the relative changes in mRNA levels of apoptotic (Bax) and anti-apoptotic (Bcl-2) genes in testicular germ cells after 8 h treatment with Cd and Qcn at different concentrations. Total RNA from the testicular germ cells was isolated using the Qiagen RNeasy Kit (Cat. no.-74,106) according to manufacturer's protocol with slight modifications. Purified RNA testing is done by qiaExpert for quality and quantity. 250 ng purified and good quality RNA from each sample was used for synthesis of cDNA using Bio-Rad iScript™ cDNA sysnthesis Kit (Cat. no.- 1,708,891) as per their instructions. Quantitative real-time PCR (qRT-PCR) was performed using the QuantiTect PCR Kits (qPCR kits, Catalogue no.- 204,343) as per the manufacturer's instructions. Quantitative real-time PCR was carried out in 10 µL reaction volume, which contains 0.5 µL of cDNA (template), 5 µL SYBER Green (2X), 0.5 µL of each forward and reverse primer, and 3.5 µL of Milli-Q water. The two-step amplification protocol included 10 min at 95ºC and then fluorescence collection through 40 cycles at 95ºC for 15 s and 58ºC for 60s. PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal positive-control standard. The—BLAST tool (NCBI, www.ncbi.nlm.nih.gov/blast) was used for designing the Bax, Bcl-2, and GAPDH primers.

Table 1 given below represents the sequences of primers utilized for RT-PCR during the current study. The mRNAs expression levels between different groups were measured by comparative delta-delta cycle threshold (ΔΔCt) technique.

Statistical Analysis

Statistical analysis was performed using SPSS 16 statistical software (SPSS, Inc., Chicago, IL, USA). Each experiment was performed in triplicates to ensure biological reproducibility and data are expressed as mean ± standard error of

Table 1 Gene specific primers used in RT-PCR

Gene	Sequence	Accession no.
Bax	F: 5'-GCGCATTGGAGATGAATTGG-3' R: 5'-CAGTTTGCTGGCAAAGTAGAAA-3'	XM_013971446.2
Bcl-2	F: 5'-ATGTGTGTGGAGAGCGTCA-3' R: 5'-AGAGACAGCCAGGAGAAATC-3'	XM_018039337.1
GAPDH	F: 5'-TGAACGGGAAGCTCACTGG-3' R: 5'-TCCACCACCCTGTTGCTGTA-3'	XM_005680968.3

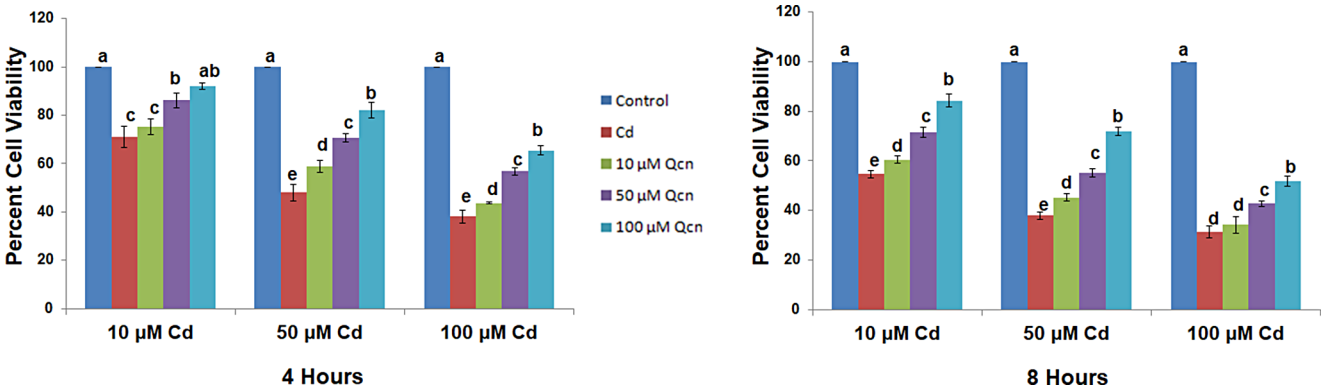

Fig. 2 Graphs depicting viability percentage of testicular cells assessed by MTT assay after Cd treatment and simultaneous Qcn supplementation at different doses and time durations. Data are presented as mean ± SEM, ANOVA (one-way analysis of variance) between different groups; different small letters on bars depicts significant ($p < 0.05$) differences whereas similar small letters denote non-significant ($p > 0.05$) difference post Duncan's test. Experiment was performed in triplicates to ensure statistical validity

the mean (SEM). One-way variance analysis (ANOVA) followed by the Duncan post-hoc test and t-test were used to compare the significant differences between the means of different treatment groups. The statistically significant difference was considered to be $p < 0.05$. The Pearson rank correlation method was also used to determine the correlation between two experimental groups ($p < 0.01$).

Results

Cadmium-Induced DNA Damage and Cytotoxicity

Results indicated that after the acute exposure of testicular cells' culture to varied concentration of $CdCl_2$, a statistically significant ($p < 0.05$) decline in cell viability was noticed in comparison to the untreated control group (Fig. 2). Meanwhile, the simultaneous quercetin supplementation results in a significant increment in per cent cell viability in a concentration- and duration-dependent approach. Along this side, the genotoxicity analysis by comet assay revealed the occurrence of different types of comets in Cd-treated testicular cells (Fig. 3). In the untreated control groups, mostly type 0 comets were observed, indicating less or no DNA damage in testicular cells. However, in the Cd-treated groups, comets ranging from type 1 to type 4 were observed, indicating a greater damage to DNA of testicular cells (Fig. 4).

At highest selected concentration (100 µM) and exposure duration (8 h), mostly type 3 and type 4 comets were detected, signifying that with increasing concentration and duration of Cd exposure, the fragmentation of DNA was increased in testicular cells. Furthermore, the quantitative analysis also demonstrated the dose-time relationship of Cd-induced toxicity, i.e., with increasing Cd concentration and time of exposure, the extent of DNA damage in testicular cells was increased (Tables 2 and 3). Therefore, the percent tail DNA, indicating more DNA damage, was more in groups treated with Cd in comparison to control groups. However, the antioxidant Qcn significantly reduced the Cd-induced DNA damage in testicular cells as evidenced by presence of fewer numbers of comets in Qcn supplemented groups (Fig. 4; Tables 2 and 3). Moreover, a positive correlation has been observed between per cent tail DNA and apoptosis percentage in testicular cells (Table 4), suggesting the genotoxicity-mediated apoptosis in testicular cells.

Cadmium-Induced Sperm Deformities

Cadmium induced several morphological abnormalities or deformities in goat sperm in a concentration-dependent fashion when compared to the control group. In control group, majority of the sperm were present with normal morphology. However, in Cd-treated groups, various morphological deformities such as sperm with coiled tail, bent tail, headless sperm, and tailless sperm were observed (Fig. 5A).

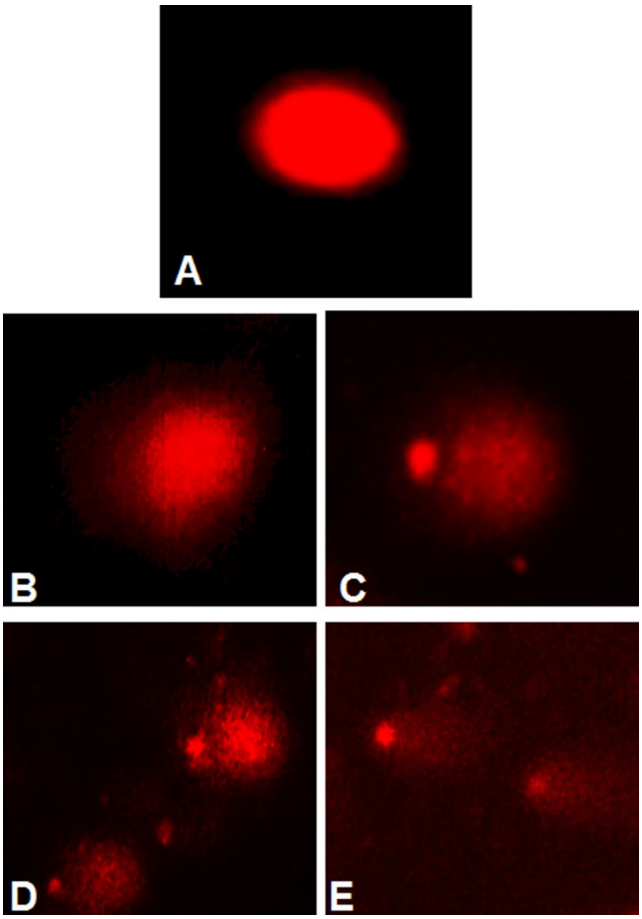

Fig. 3 Microphotographs showing different types of comets. (**A**) Type 0 (**B**) Type 1 (**C**) Type 2 (**D**) Type 3 (**E**) Type 4

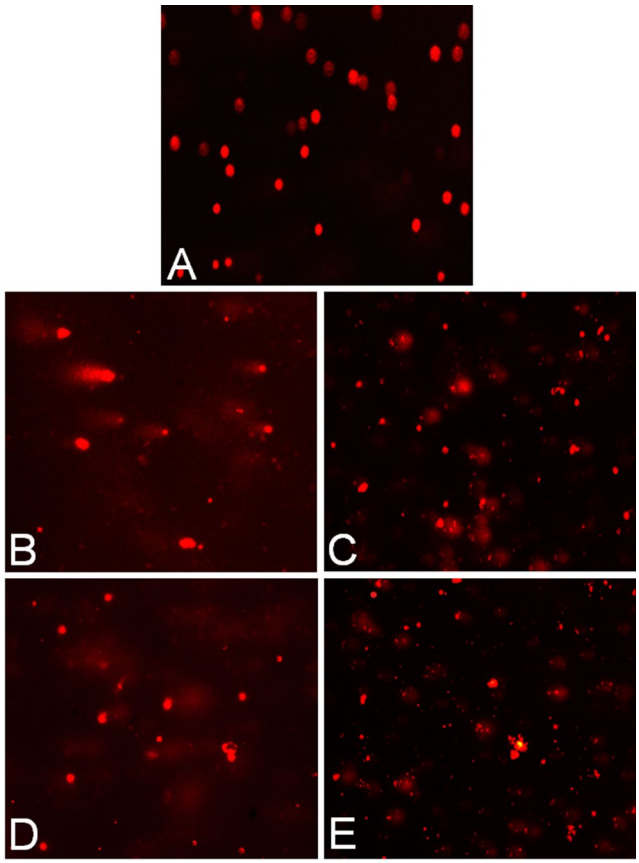

Fig. 4 Microphotographs showing different types of comets in testicular cells of goat post Cd treatmentǁ at 100 µM (**B**, **D**, & **E**) and 50 µM (**C**) along with ameliorative effect of quercetin at 10 µM (**B**), 50 µM (**C**), and 100 µM (**D** & **E**) concentration after 4 h (**A**, **C**) and 8 h (**B**, **D**, & **E**) exposure duration, x400

The simultaneous supplementation of Qcn, however, significantly reduced the incidence of abnormal sperm in a dose-dependent fashion (Fig. 5B; Table 5).

Steroidogenic Toxicity

Cadmium treatment causes a significant reduction in the activity of 3β-HSD within testicular tissue with increasing concentration and exposure durations in comparison to the control groups (Table 6). Similarly, the activity of another steroidogenic enzyme i.e., 17β-HSD was also reduced markedly with increasing concentration and exposure duration of Cd (Table 7). However, simultaneous supplementation of Qcn at all the selected concentrations significantly restored the Cd-mediated decrease in activity of both steroidogenic enzymes in a time- and dose-dependent manner (Tables 6 and 7).

Table 2 Various comet parameters of testicular cells post Cd and Qcn treatment at different concentrations after 4 h exposure duration

TREATMENT		Head DNA %	Tail DNA%	Tail length	Tail moment	Olive moment
10 µM Cd	Control	95.95 ± 0.73^{a}	6.13 ± 0.99^{d}	0.55 ± 0.28^{d}	0.21 ± 0.13^{d}	0.90 ± 0.06^{c}
	Cd	58.43 ± 0.87^{d}	41.20 ± 0.82^{a}	19.04 ± 1.46^{a}	14.31 ± 0.81^{a}	5.25 ± 0.66^{a}
	50 µM Qcn	68.74 ± 2.00^{c}	33.93 ± 1.38^{b}	11.25 ± 1.03^{b}	8.18 ± 0.78^{b}	3.82 ± 0.30^{b}
	100 µM Qcn	75.29 ± 1.00^{b}	29.22 ± 1.35^{c}	7.00 ± 1.15^{c}	4.80 ± 0.55^{c}	3.27 ± 0.09^{b}
50 µM Cd	Cd	36.72 ± 1.37^{b}	64.31 ± 1.69^{a}	19.06 ± 1.38^{a}	17.31 ± 1.40^{a}	7.79 ± 1.12^{a}
	10 µM Qcn	39.27 ± 1.24^{b}	65.66 ± 2.08^{a}	22.88 ± 0.89^{a}	17.13 ± 2.23^{a}	10.66 ± 0.98^{a}
	50 µM Qcn	50.12 ± 1.55^{a}	55.01 ± 1.41^{b}	23.10 ± 1.73^{a}	17.39 ± 0.96^{a}	8.00 ± 0.79^{a}
	100 µM Qcn	53.71 ± 0.81^{a}	45.76 ± 2.52^{c}	12.52 ± 1.54^{b}	8.11 ± 0.63^{b}	4.06 ± 0.58^{b}

Data are presented as mean ± SEM, ANOVA (one-way analysis of variance) between different groups; different small letters in superscripts depicts significant ($p<0.05$) differences post Duncan's test

Table 3 Various Comet parameters of testicular cells post Cd and Qcn treatment at different concentrations after 8 h exposure duration

TREATMENT		Head DNA %	Tail DNA%	Tail length	Tail moment	Olive moment
50 µM Cd	Control	93.51 ± 0.96^{a}	8.87 ± 0.48^{c}	1.42 ± 0.27^{c}	0.63 ± 0.28^{c}	4.29 ± 0.60^{d}
	Cd	29.22 ± 1.19^{c}	75.78 ± 1.49^{a}	27.43 ± 1.65^{a}	20.97 ± 2.08^{a}	13.90 ± 0.61^{a}
	50µM Qcn	46.01 ± 1.23^{b}	57.03 ± 2.34^{b}	15.38 ± 1.63^{b}	14.82 ± 1.85^{b}	10.43 ± 0.42^{b}
	100µM Qcn	46.35 ± 1.85^{b}	60.69 ± 2.11^{b}	18.62 ± 1.39^{b}	12.78 ± 1.28^{b}	7.99 ± 0.97^{c}
100 µM Cd	Cd	8.67 ± 1.35^{d}	94.65 ± 0.95	41.29 ± 2.21^{a}	35.62 ± 2.46^{a}	20.29 ± 1.05^{a}
	10µM Qcn	24.87 ± 1.60^{c}	72.45 ± 1.78^{b}	32.97 ± 1.70^{b}	29.77 ± 1.11^{a}	14.14 ± 1.46^{b}
	50µM Qcn	39.93 ± 3.23^{b}	65.79 ± 0.90^{b}	35.07 ± 1.61^{ab}	29.70 ± 1.51^{a}	21.89 ± 0.90^{a}
	100µM Qcn	72.58 ± 373^{a}	34.78 ± 4.09^{c}	14.10 ± 3.62^{c}	8.06 ± 2.03^{b}	8.37 ± 2.51^{b}

Data are presented as mean ± SEM, ANOVA (one-way analysis of variance) between different groups; different small letters in superscripts depicts significant ($p<0.05$) differences post Duncan's test

Table 4 Correlation between different Comet parameters and percent apoptosis in testicular cells at 4 h (black) and 8 h (red)

	Head DNA %	Tail DNA %	Tail length	Tail moment	Olive moment	% Apoptosis
Head DNA %	1	-0.97** -0.97**	-0.87** -0.88**	-0.88** -0.90**	-0.85** -0.73**	-0.94** -0.84**
Tail DNA %		1	0.89** 0.91**	0.90** 0.87**	0.86** 0.77**	0.93** 0.83**
Tail length			1	0.91** 0.93**	0.82** 0.90**	0.85** 0.86**
Tail moment				1	0.83** 0.89**	0.89** 0.86**
Olive moment					1	0.77** 0.78**
% Apoptosis						1

**Correlation is significant at .01 levels (two tailed)

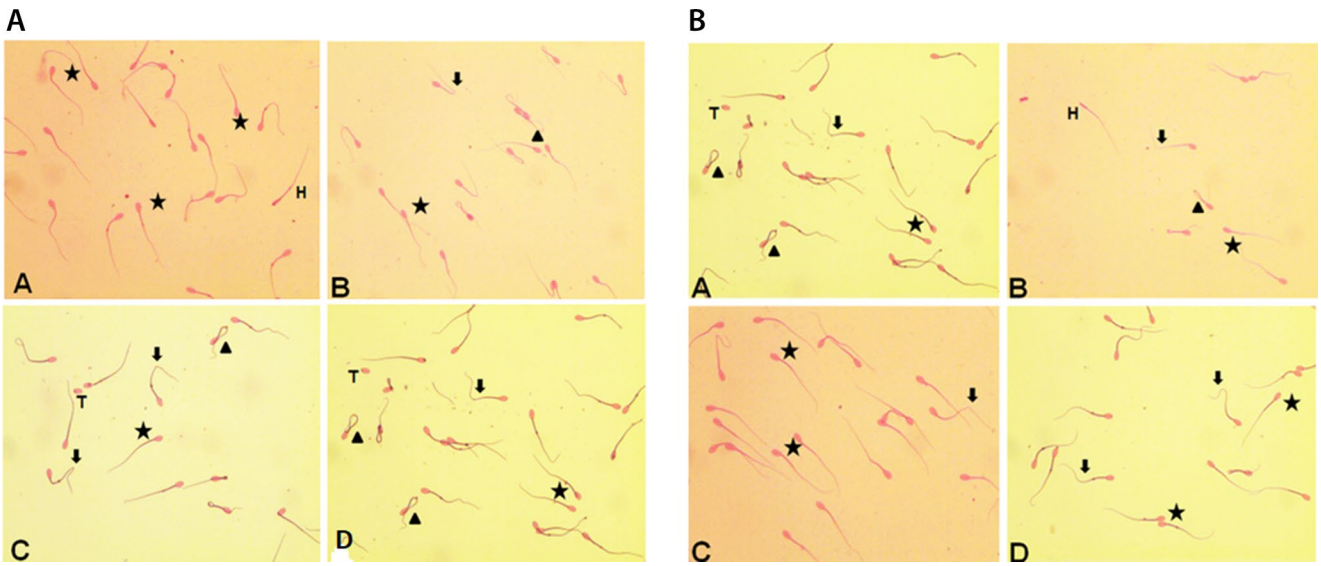

Fig. 5 (**A**) Microphotographs showing various types of sperm deformities in Cd treated group at 10 µM (**B**) 50 µM (**C**) and 100 µM (**D**) in comparison with the control group (**A**) after 4 hours' time duration (**Eosin stain, x400**). **Star** represents healthy sperm with normal morphology; **H**: headless sperm; **T**: tailless sperm; **arrow head**: coiled tail; **arrow**: bent tail. (**B**) Microphotographs showing ameliorative effect of quercetin at 10 µM (**B**) 50 µM (**C**) and 100 µM (**D**) in comparison with the Cd-treated (100 µM) group (**A**) after 4 hours' time duration (**Eosin stain, x400**). **Star** represents healthy sperm with normal morphology; **H**: headless sperm; **T**: tailless; **arrow head**: coiled tail; **arrow**: bent tail

Table 5 Effects of Cd and Cd plus quercetin supplementation on sperm morphology

Sperm deformities	Control	10 µM Cd	50 µM Cd	100µM Cd	100µM Cd + 10 µM Qcn	100µM Cd + 50 µM Qcn	100µM Cd + 100µM Qcn
Headless (%)	1.3	1.3	3.3	2	3.3	1.3	0.6
Tailless (%)	0	0	2.6	4.6	2	2	0
Coiled tail (%)	1.3	2	4.6	3.3	2	2.6	4.6
Bent tail (%)	2	3.3	7.3	11.3	8.6	6	8.6
Total (%)	4.6	6.6	18	21.3	16	12	14

Table 6 Level of activity of steroidogenic enzymes in testicular tissue post 4 h of Cd and Qcn treatment

Steroidogenic enzymes' activity			
Treatment		3β-HSD (nmol NAD reduced/min/mg protein)	17β-HSD (nmol NADPH oxidized/min/mg protein)
10 µM Cd	Control	2.91 ± 0.06^{a}	2.01 ± 0.02^{a}
	Cd	1.38 ± 0.02^{c}	1.23 ± 0.16^{b}
	10 µM Qcn	1.49 ± 0.16^{c}	1.28 ± 0.48^{b}
	50 µM Qcn	1.81 ± 0.15^{b}	1.36 ± 0.07^{b}
	100 µM Qcn	2.73 ± 0.16^{a}	1.68 ± 0.08^{ab}
50 µM Cd	Control	2.91 ± 0.06^{a}	2.01 ± 0.02^{a}
	Cd	1.13 ± 0.06^{d}	0.72 ± 0.18^{d}
	10 µM Qcn	1.20 ± 0.01^{d}	0.92 ± 0.04^{c}
	50 µM Qcn	1.29 ± 0.01^{c}	1.25 ± 0.06^{b}
	100 µM Qcn	1.86 ± 0.06^{b}	1.31 ± 0.13^{b}
100µM Cd	Control	2.91 ± 0.06^{a}	2.01 ± 0.02^{a}
	Cd	0.54 ± 0.06^{c}	0.38 ± 0.06^{d}
	10 µM Qcn	0.60 ± 0.04^{c}	0.33 ± 0.01^{d}
	50 µM Qcn	0.86 ± 0.06^{b}	1.24 ± 0.28^{b}
	100 µM Qcn	0.92 ± 0.08^{b}	0.96 ± 0.08^{c}

Data are presented as mean ± SEM, ANOVA (one-way analysis of variance) between different groups; different small letters in superscripts depicts significant ($p<0.05$) differences post Duncan's test

Table 7 Level of activity of steroidogenic enzymes in testicular tissue post 8 h of Cd and Qcn treatment

Steroidogenic enzymes' activity			
Treatment		3β-HSD (nmol NAD reduced/min/mg protein)	17β-HSD (nmol NADPH oxidized/min/mg protein)
10 µM Cd	Control	2.17 ± 0.01^{a}	1.71 ± 0.04^{a}
	Cd	1.12 ± 0.08^{d}	0.59 ± 0.02^{e}
	10 µM Qcn	1.32 ± 0.04^{c}	0.78 ± 0.02^{d}
	50 µM Qcn	1.66 ± 0.10^{b}	0.98 ± 0.03^{c}
	100µM Qcn	1.79 ± 0.09^{b}	1.33 ± 0.16^{b}
50 µM Cd	Control	2.17 ± 0.01^{a}	1.71 ± 0.04^{a}
	Cd	0.43 ± 0.13^{d}	0.38 ± 0.01^{c}
	10 µM Qcn	0.48 ± 0.16^{d}	0.40 ± 0.08^{c}
	50 µM Qcn	0.88 ± 0.04^{c}	0.41 ± 0.01^{c}
	100µM Qcn	1.50 ± 0.01^{b}	0.58 ± 0.18^{b}
100µM Cd	Control	2.17 ± 0.01^{a}	1.71 ± 0.04^{a}
	Cd	0.22 ± 0.02^{d}	0.10 ± 0.00^{c}
	10 µM Qcn	0.27 ± 0.01^{cd}	0.11 ± 0.00^{c}
	50 µM Qcn	0.32 ± 0.01^{c}	0.22 ± 0.03^{b}
	100µM Qcn	0.78 ± 0.09^{b}	0.24 ± 0.00^{b}

Data are presented as mean ± SEM, ANOVA (one-way analysis of variance) between different groups; different small letters in superscripts depicts significant ($p<0.05$) differences post Duncan's test

Furthermore, treatment with Cd led to a significant reduction in levels of testosterone in testicular tissue after 8 h (Fig. 6), justifying its toxicity effect in testicular steroidogenic process. Simultaneously Qcn supplementation at 50 and 100 µM concentrations increased the Cd-mediated decrease in testosterone hormone level to a significant extent (Fig. 6). However, statistically non-significant increase was observed at 10 µM dose of Qcn at 50 and 100 µM concentrations of Cd treatment.

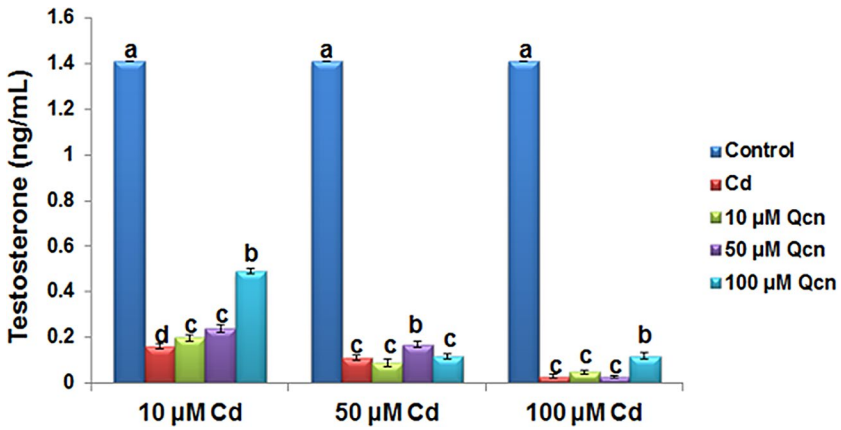

Fig. 6 Graphs depicting changes in testosterone level in testicular tissue by Cd treatment and simultaneous quercetin supplementation after 8 h. Data are presented as mean ± SEM, ANOVA (one-way analysis of variance) between different groups; different small letters on bars depicts significant ($p < 0.05$) differences whereas similar small letters denote non-significant ($p > 0.05$) difference post Duncan's test. Experiment was performed in triplicates to ensure statistical validity

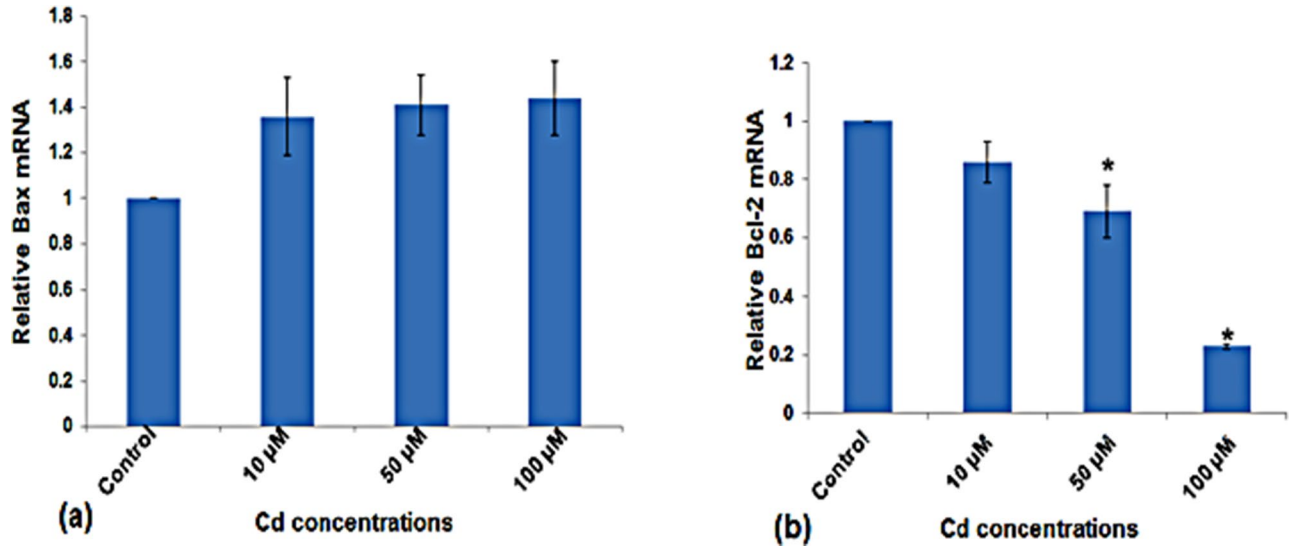

Fig. 7 Graphs depicting gene expression profile of Bax (**a**) and Bcl-2 (**b**) in testicular cells after Cd treatment at different doses and 8 h exposure duration. Relative gene expressions are shown as mean ± SEM followed by t-test to compare the significant differences (*$p < 0.05$) between the means of experimental and control groups

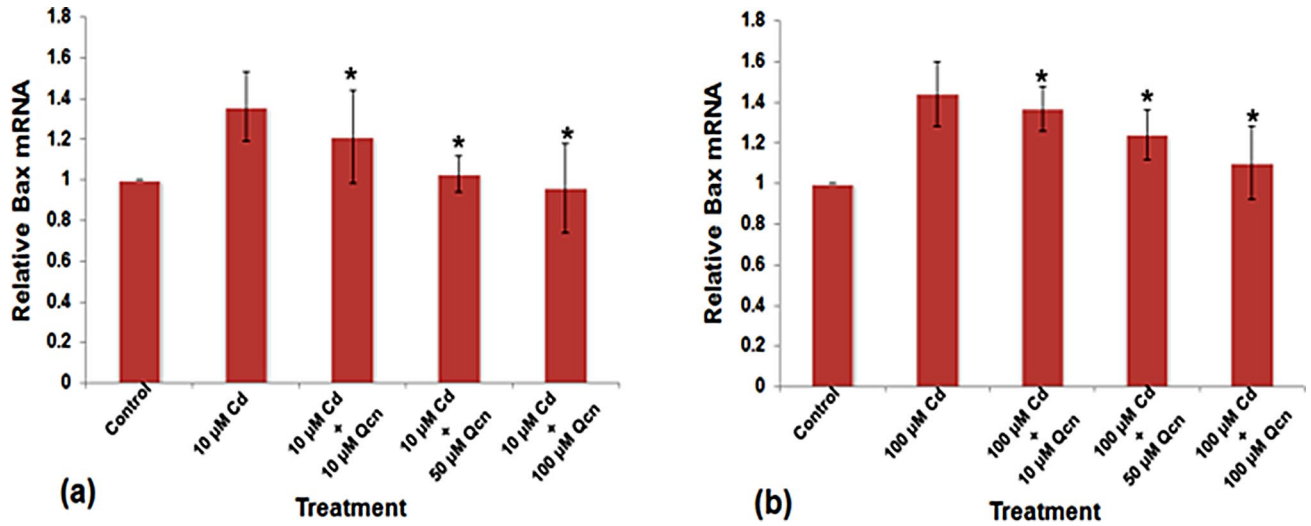

Fig. 8 Graphs depicting gene expression profile of Bax in testicular cells post Cd treatment at 10 µM (a) and 100 µM (b) with simultaneous supplementation of quercetin (Qcn) at different concentrations after 8 h exposure duration. Relative gene expressions are shown as mean ± SEM followed by t-test to compare the significant differences (*$p < 0.05$) between the means of experimental and control groups

Cadmium-Induced Apoptosis

The gene expression profile of pro-apoptotic Bax at 10, 50 and 100 µM Cd concentrations was upregulated but statistically insignificant (Fig. 7a). Conversely, the expression level of anti-apoptotic Bcl-2 at 50 and 100 µM Cd concentrations was significantly downregulated in comparison to the untreated control group ($p < 0.05$); however, at 10 µM concentration, Cd did not significantly downregulate the level of Bcl-2 expression (Fig. 7b). Furthermore, when compared to the control and only Cd treated groups, simultaneous Qcn supplementation significantly downregulated the Cd-mediated upregulation in Bax expression (Fig. 8), while upregulated the Bcl-2 expression (Fig. 9), which is downregulated by Cd treatment. On the other hand, mRNA levels of Bcl-2 were significantly up regulated in all the Qcn supplemented groups in comparison to the groups treated with Cd only (Fig. 9). However, non-significant results for Bcl-2 were obtained at 10 µM Qcn supplementation when compared to both control and Cd-treated (10 µM) groups.

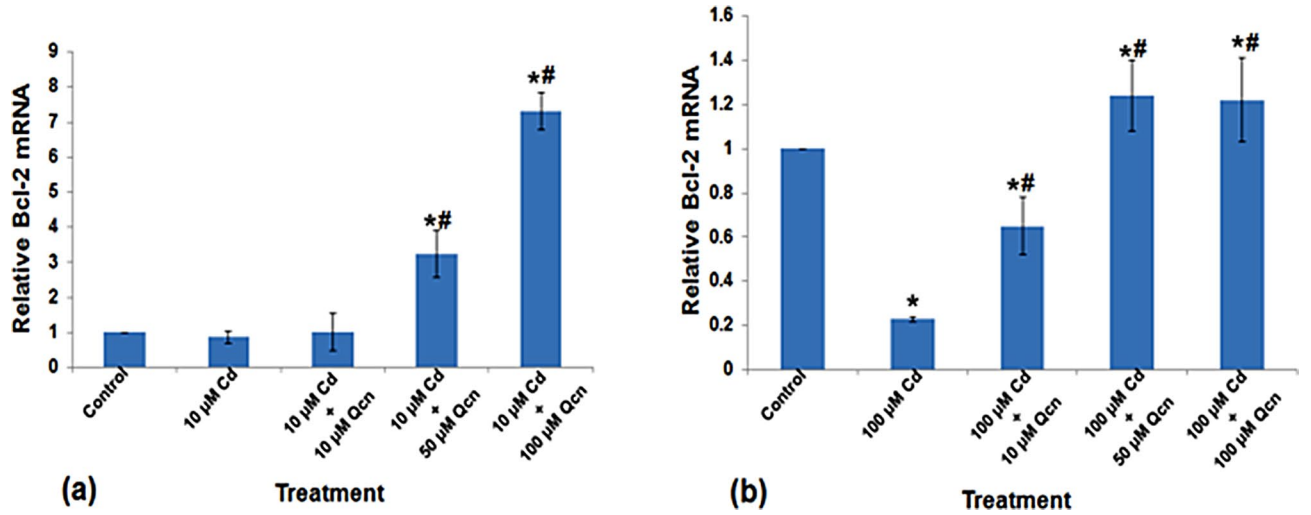

Fig. 9 Graphs depicting gene expression profile of Bcl-2 in testicular cells post Cd treatment at 10 µM (a) and 100 µM (b) with simultaneous supplementation of quercetin (Qcn) at different concentrations after 8 h exposure duration. Relative gene expressions are shown as mean ± SEM followed by t-test to compare the significant differences (*$p < 0.05$) between the means of experimental and control groups (*$p < 0.05$ from control; #$p < 0.05$ from only Cd treated)

Discussion

The most critical public health issue now involves both human and animal exposure to environmental contaminants that have a deleterious impact on male reproductive function [30]. According to certain reports on semen quality testing, chronic heavy metals' exposure in some work fields greatly increases the likelihood of male infertility [31]. The present study was performed to assess the protective effects of quercetin (Qcn), a potent antioxidant, against Cd-induced genotoxicity-mediated apoptotic profile, sperm characteristics, and steroidogenesis in goat testicular tissue under in-vitro culture conditions. Cd induced damage to the DNA of testicular cells that subsequently causes apoptosis and thus indicating the genotoxicity-mediated apoptotic cell death in a dose- and time-dependent manner. Cd treatment interferes with the biosynthetic pathway of steroid hormone (testosterone) synthesis by significantly reducing the activity of key steroidogenic enzymes (3β-HSD and 17β-HSD) and thus leads to impaired spermatogenic process. Consequently, increased DNA damage and reduced spermatogenesis may result in the alteration of apoptotic (Bax) and anti-apoptotic (Bcl-2) genes' expression leading to activation of apoptotic pathway in testicular cells. Being a potent natural antioxidant, Qcn significantly attenuated the Cd-elicited DNA damage and impaired steroidogenesis in testicular cells in a concentration- and time-dependent manner. Furthermore, quercetin significantly causes reduction in frequency of apoptosis in testicular cells, suggesting its anti-apoptotic property.

Results indicated a significant decline in cell viability percentage after the acute exposure of testicular cells' culture to varied concentration of Cd in comparison to the control group. Exposure of Cd at different concentrations has significantly lowered the viability of Leydig cells when assessed by the MTT assay and trypan blue method in rat [32]. MTT assay revealed a significant increment in cell death via apoptosis or necrosis after Cd treatment in cultured kidney proximal tubule cells, thus decreasing the percentage of viable cells [33]. Another study, showed the half-maximal inhibitory concentration (56.08 µM) for Cd in mice Sertoli cells [34]. Over the last decade, the single cell gel electrophoresis (SCGE) or alkaline comet assay have been considered the most commonly utilized techniques for determining the damage to DNA with applications in genotoxicity testing, ecogenotoxicology, and fundamental DNA damage and repair research [35]. Results of present investigation demonstrated the occurrence of different types of comets (Type 1-Type 4), which signifies the different degree of DNA damage caused by Cd at varied concentrations and time of exposure in testicular spermatogenic cells. Tail DNA percentage is considered to be the best descriptor for frequencies of DNA breaks, or comets, and the degree of harm may be simply envisaged [36]. The quantitative study revealed that tail DNA percent, indicating more DNA damage, was more in groups treated with Cd as those of control groups. Moreover, a positive type of correlation has also been observed between apoptosis percentage and percent tail DNA in testicular cells, therefore suggesting the genotoxicity-mediated apoptosis in testicular cells. Cd causes DNA damage in a variety of cells, including Sertoli cells [37] and liver cells [38]. Normal cells may repair damage to the DNA and prevents mutations from occurring; however, exposure to Cd can impair the functions of genes that repair

DNA and subsequently enhance instability in the genome, causing development of cancer [39]. Yang and coworkers (2003) have demonstrated that Cd induced single strand break in Leydig cells in vitro. Cd exposure correlates with the extent of DNA damage in Leydig cells. These findings confirmed that 10 μM Cd concentrations are directly toxic to primary culture of Leydig cells and associated with DNA damage [40]. Another in vitro study has also analyzed the Cd-induced DNA damage in piglet Sertoli cells. Results revealed that 10 μM Cd can cause DNA trailing, indicating more DNA damage in piglet Sertoli cells in comparison to control group [37]. Recently, a study has revealed the increment in tail DNA percentage in groups treated with Cd in comparison to the control group in rat testicular cells [15]. Another study has indicated the Cd-induced damage to the DNA of sperm in mice as assessed by comet assay [41]. Similarly, several other studies have reported the Cd-induced DNA damage in different organs and cells such as rat proximal tubular cell line [42], hepatocytes [43] and in amphibians' testis [44]. Thus, our findings support other previous findings regarding the Cd-induced direct damage to genetic material of cell and subsequently its death. Androgenesis in testis is regulated by two steroidogenic enzymes i.e., 3β- and 17β-HSDs, which are essential and considered as rate limiting factors in synthesis of testicular testosterone [45]. Testosterone is essential for the attachment of germ cells of different generations within the seminiferous tubules, and thus low intratesticular testosterone levels may cause germ cells detachment from the epithelium of seminiferous tubules and start apoptosis of germ cells [46]. During the present study, the activities of these enzymes (3β- and 17β-HSDs) were found to be considerably reduced in groups treated with Cd in comparison to the control group. Due to this reduction in the activity levels of these steroidogenic enzymes, the level of testosterone was also found to be lower in Cd-treated groups. The current findings are consistent with the other previous studies [46, 47] regarding the steroidoenic toxicity induced by Cd in testicular tissue. Similarly, massive apoptosis of testicular germ cells is thought to be caused by Cd exposure or changes in hormonal support provided by Leydig cells [48]. In another study, significant reduction in expression of StAR, P450scc, and SF1 mRNA and levels of P450scc, StAR, 3β- and 17β-HSDs protein-expression were induced by Cd in rat in comparison to the control group [49]. Apoptosis is a process initiated by various signals through mitochondria- or death receptor-mediated pathways. In mitochondrial-mediated pathway, Bcl-2 proteins act as apoptotic inhibitors [50] and Bax proteins stimulates apoptosis [51]. In the present study, the Bax mRNA expression were increased, whereas expression of Bcl-2 mRNA was reduced markedly in testicular tissue treated with Cd in comparison to the untreated control groups. Likewise, Bax and caspase-3 expression were shown to be induced by Cd while Bcl-2 expression was inhibited by Cd in mice testis [52]. Another study has also shown that rates of Bax and Bcl-2 protein expression were significantly higher and lower, respectively, in mice spermatogenic cells exposed to Cd than that of normal untreated group [53]. In most of the cases, apoptosis induced by Cd is mediated through mitochondrial pathway [54]. The current findings intensely advocate that Cd-induced apoptosis in goat testicular cells might be mediated via the mitochondrial pathway through alteration of Bax/Bcl-2 expression ratio. In accordance with the results of present study, previous findings showed that inflammatory responses induced by Cd stimulate Bax production in the mitochondria-mediated apoptotic pathway. Bax enhanced the permeability of mitochondrial membrane and thus facilitate release of cytochrome c into the cytosol that subsequently causes caspase activation and eventually apoptosis via caspase-3 [55]. Some researchers have discovered that exposure to Cd enhances mitochondrial fission [56], resulting in structural damage to mitochondria, cytochrome c release and mitochondrial fragmentation [57], which causes caspase-cascade activation and finally apoptosis [58]. Results of another recent study showed increased Bax and caspase-3 levels and reduced Bcl-2 levels in Cd intoxicated rats, and therefore further demonstrating the critical role of mitochondria in Cd-mediated apoptosis [59]. Studies in the literature have demonstrated the antioxidant and cyto-protective nature of Qcn in averting the toxicity induced by Cd in different organs under both in vitro and in vivo conditions [27, 60]. Qcn is one of the most effective antioxidants among flavonoids and is recommended for use in dietary supplements to protect against toxicity of Cd [61]. According to findings of a recent study, combined Qcn and Cd treatment reduced the level of Cd in serum and testis [62]. One possible mechanism for this is the ability of Qcn to chelate metals [63, 64]. In our previous study, simultaneous Qcn supplementation has significantly decreased the Cd-elevated oxidative stress and enhanced the cellular antioxidant defense in testicular tissue, preventing the apoptosis of testicular cells [24]. Therefore, it stands to reason that Qcn's antioxidant property is responsible for its anti-apoptotic effects. However, it is well recognised that oxidative stress produced by mitochondrial dysfunctions causes apoptosis under both physiological and pathological conditions [65]. Moreover, mitochondrial pathway of apoptosis plays an important role in initiation of Cd-induced apoptosis in testicular germ cells [66]. Qcn has been shown to modulate various mitochondria associated pathways [67]. In the current investigation, supplementation of Qcn significantly reversed the Cd-mediated alteration in the ratio of apoptotic (Bax) and anti-apoptotic (Bcl-2) genes, and thus preventing the apoptosis of

testicular cells via modulating mitochondrial pathway of apoptosis. The findings of a study clearly revealed that oral administration of Cd markedly reduced sperm motility and count while increasing the sperm abnormality percentage in comparison to the control. Additionally, a severe decline in levels of total serum LH, FSH, and testosterone were also detected in Cd-treated group [62]. However, it has been noticed that Qcn administration counteracted the adverse influence of Cd on quality of sperm and production of sex hormones [62, 68]. This is in accordance with the findings of our work, where Qcn supplementation to in-vitro culture of sperm cell significantly reduced the Cd-elicited increase in defects of sperm morphology and increased the level of decreased testosterone in Cd-treated groups. Studies have shown that antioxidant therapy utilizing Qcn has the capacity to maintain redox balance, stimulate androgen production, and subsequently inhibit apoptosis of germ cells that leads to restoration of testis function in estrogenized rats [69]. Moreover, Qcn showed high anti-genotoxic ability by decreasing damage to DNA in sperm after exposure to food mutagens in vitro [70].

However, it is important to note here that this study was performed under in-vitro conditions, and thus unable to adequately convey the innate complexity of the body's interior environments and organ systems. Therefore, more comprehensive long term in-vivo studies are warranted in future to reflect the more realistic scenario of cadmium toxicity mechanism and ameliorative power of quercetin in testicular tissue.

Consequently, the current study hypothesized the ameliorative potential of a potent natural antioxidant, Qcn, against Cd-induced male gonadal toxicity in one of the most prolific domestic animal goats. Qcn showed its protective influence by inhibiting the Cd-induced apoptosis of testicular cells via mitochondrial pathway, which may be induced by Cd-mediated increase in DNA damage and decreased androgen level in testicular tissue in a concentration- and duration-dependent manner. Therefore, these findings will further enrich the study of gonadal toxicity induced by Cd and its molecular mechanism in males. Moreover, the study would also pave the way towards the development of new therapeutic agents to protect from the Cd-elicited damage in testicular tissue.

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Author Contributions Jitender Kumar Bhardwaj has conceptualized, designed and reviewed the manuscript. Harish Panchal has performed the literature search, experimental work, data analysis, compilation, and writing of the draft.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate However, the goat testes for the present study were obtained as a by-product of routine castration from the government slaughterhouse and did not cause any harm to the animal. Therefore, the study did not require any ethical approval.

Competing Interests The authors declare no competing interests.

References

1. McConnell JR, Edwards R (2008) Coal burning leaves toxic heavy metal legacy in the Arctic. Proc Natl Acad Sci U S A 105(34):12140–12144. https://doi.org/10.1073/pnas.0803564105
2. Kapusta P, Sobczyk L (2015) Effects of heavy metal pollution from mining and smelting on enchytraeid communities under different land management and soil conditions. Sci Tot Environ 536:517–526. https://doi.org/10.1016/j.scitotenv.2015.07.086
3. Centres for Disease Control and Prevention (CDC) (2013) Infertility FAQs
4. Sharpe RM (2010) Environmental/lifestyle effects on spermatogenesis. Philos Trans R Soc Lond Series B Biol Sci 365:1697–1712. https://doi.org/10.1098/rstb.2009.0206
5. Selvaraju V, Baskaran S, Agarwal A, Henkel R (2021) Environmental contaminants and male infertility: effects and mechanisms. Andrologia 53(1):e13646. https://doi.org/10.1111/and.13646
6. Manouchehri A, Shokri S, Abbaszadeh S (2022) The effects of toxic heavy metals lead, cadmium and copper on the epidemiology of male and female infertility. JBRA Assist Reprod 26(4):627–630. https://doi.org/10.5935/1518-0557.20220013
7. Arab HH, Gad AM, Reda E, Yahia R, Eid AH (2021) Activation of autophagy by sitagliptin attenuates cadmium-induced testicular impairment in rats: Targeting AMPK/mTOR and Nrf2/HO-1 pathways. Life Sci 269:119031. https://doi.org/10.1016/j.lfs.2021.119031
8. Mead MN (2010) Cadmium confusion: do consumers need protection? Environ Health Perspect 118(12):A528–A534. https://doi.org/10.1289/ehp.118-a528
9. Ma Y, He X, Qi K, Wang T, Qi Y, Cui L et al (2019) Effects of environmental contaminants on fertility and reproductive health. J Environ Sci 77:210–217. https://doi.org/10.1016/j.jes.2018.07.015
10. Harlev A, Agarwal A, Ozgur GS, Shetty A, du Plessis SS (2015) Smoking and male infertility: an evidence-based review. World J Men's Health 33:143–160. https://doi.org/10.5534/wjmh.2015.33.3.143
11. Bernard A (2008) Cadmium & its adverse effects on human health. Indian J Med Res 128(4):557
12. Zhang C, Lin J, Ge J, Wang LL, Li N, Sun XT, Cao HB, Li JL (2017) Selenium triggers Nrf2-mediated protection against cadmium-induced chicken hepatocyte autophagy and apoptosis. Toxicol in Vitro 44:349–356. https://doi.org/10.1016/j.tiv.2017.07.027
13. Huang M, Liu Z (2018) Cadmium accumulation and distribution in *Xenopus laevis* after Long-term

cadmium treatment. In: IOP Conference Series: Materials Science and Engineering 452(2):022159, IOP Publishing. https://doi.org/10.1088/1757-899X/452/2/022159
14. Habib R, Wahdan SA, Gad AM, Azab SS (2019) Infliximab abrogates cadmium-induced testicular damage and spermiotoxicity via enhancement of steroidogenesis and suppression of inflammation and apoptosis mediators. Ecotoxicol Environ Saf 182:109398. https://doi.org/10.1016/j.ecoenv.2019.109398
15. Bashir N, Shagirtha K, Manoharan V, Miltonprabu S (2019) The molecular and biochemical insight view of grape seed proanthocyanidins in ameliorating cadmium-induced testes-toxicity in rat model: implication of PI3K/Akt/Nrf-2 signaling. Biosci Rep 39(1):BSR20180515. https://doi.org/10.1042/BSR20180515
16. Etemadi T, Momeni HR, Ghafarizadeh AA (2020) Impact of silymarin on cadmium-induced apoptosis in human spermatozoa. Andrologia 52(11):e13795. https://doi.org/10.1111/and.13795
17. Bhardwaj JK, Panchal H, Saraf P (2021a) Cadmium as a testicular toxicant: a review. J Appl Toxicol 41(1):105–117. https://doi.org/10.1002/jat.4055
18. Al-Azemi M, Omu FE, Kehinde EO, Anim JT, Oriowo MA, Omu AE (2010) Lithium protects against toxic effects of cadmium in the rat testes. J Assist Reprod Genet 27(8):469–476. https://doi.org/10.1007/s10815-010-9426-3
19. Kelly GS (2011) Quercetin. Altern Med Rev 16(2):172–195
20. Abdou HM, Abd Elkader HT (2022) The potential therapeutic effects of Trifolium alexandrinum extract, hesperetin and quercetin against diabetic Nephropathy via attenuation of oxidative stress, inflammation, GSK-3β and apoptosis in male rats. Chem Biol Interact 352:109781. https://doi.org/10.1016/j.cbi.2021.109781
21. Zhao X, Wang J, Deng Y, Liao L, Zhou M, Peng C, Li Y (2021) Quercetin as a protective agent for Liver Diseases: a comprehensive descriptive review of the molecular mechanism. Phytother Res 35(9):4727–4747. https://doi.org/10.1002/ptr.7104
22. Mehany A, Belal A, Santali EY, Shaaban S, Abourehab MA, El-Feky OA, Diab M, Abou Galala F, Elkaeed EB, Abdelhamid G (2022) Biological effect of quercetin in repairing brain damage and cerebral changes in rats: molecular docking and in vivo studies. Biomed Res Int 8962149. https://doi.org/10.1155/2022/8962149
23. Youl E, Bardy G, Magous R, Cros G, Sejalon F, Virsolvy A, Richard S, Quignard JF, Gross R, Petit P (2010) Quercetin potentiates insulin secretion and protects INS-1 pancreatic β-cells against oxidative damage via the ERK1/2 pathway. Br J Pharmacol 161(4):799–814. https://doi.org/10.1111/j.1476-5381.2010.00910x
24. Bhardwaj JK, Panchal H (2021) Quercetin mediated attenuation of cadmium-induced oxidative toxicity and apoptosis of spermatogenic cells in caprine testes in vitro. Environ Mol Mutagen 62(6):374–384. https://doi.org/10.1002/em.22450
25. Panchal H, Sachdeva SN, Bhardwaj JK (2022) Ultrastructural analysis of cadmium-induced toxicity and its alleviation by antioxidant quercetin in caprine testicular germ cells in vitro. Ultrastruct Pathol 46(3):259–267. https://doi.org/10.1080/01913123.2022.2060396
26. Khanna S, Lakhera PC, Khandelwal S (2011) Interplay of early biochemical manifestations by cadmium insult in sertoli–germ coculture: an in vitro study. Toxicology 287(1–3):46–53. https://doi.org/10.1016/j.tox.2011.05.013
27. Wang J, Qian X, Gao Q, Lv C, Xu J, Jin H, Zhu H (2018) Quercetin increases the antioxidant capacity of the ovary in menopausal rats and in ovarian granulosa cell culture in vitro. J Ovarian Res 11(1):1–11. https://doi.org/10.1186/s13048-018-0421-0
28. Ahuja YR, Saran R (1999) Alkaline single cell gel electrophoresis assay protocol. J Cytol Genet 34:57–62
29. Bergmeyer HU (1974) β-hydroxy steroid dehydrogenase. In: Methods of Enzymatic analysis. Ist Edition, Academic Press, New York, 447–489
30. Antar SA, El-Gammal MA, Hazem RM, Moustafa YM (2022) Etanercept mitigates Cadmium Chloride-induced testicular damage in rats an insight into Autophagy, apoptosis, oxidative stress and inflammation. Environ Sci Pollut Res Int 19:28194–28207. https://doi.org/10.1007/s11356-021-18401-6
31. Ho HY, Wei HJ (2013) The relationship between heavy metal exposure and risk of infertility in Taiwan. Fertil Steril 100(3):S12
32. Qing LIU, Hong GJ, Yan YUAN, Zhong LX, Jun WY, Dong WH, Ping LZ (2013) Effect of cadmium on rat leydig cell testosterone production and DNA integrity in vitro. Biomed Environ Sci 26(9):769–773. https://doi.org/10.3967/0895-3988.2013.09.009
33. Lee WK, Abouhamed M, Thevenod F (2006) Caspase-dependent and-independent pathways for cadmium-induced apoptosis in cultured kidney proximal tubule cells. Am J Physiol Renal Physiol 291(4):F823–F832. https://doi.org/10.1152/ajprenal.00276.2005
34. Yang SH, Yu LH, Li L, Guo Y, Zhang Y, Long M, Li P, He JB (2018) Protective mechanism of sulforaphane on cadmium-induced sertoli cell injury in mice testis via Nrf2/ARE signaling pathway. Molecules 23(7):774. https://doi.org/10.3390/molecules23071774
35. Neri M, Milazzo D, Ugolini D, Milic M, Campolongo A, Pasqualetti P, Bonassi S (2015) Worldwide interest in the comet assay: a bibliometric study. Mutagenesis 30(1):155–163. https://doi.org/10.1093/mutage/geu061
36. Langie SA, Azqueta A, Collins AR (2015) The comet assay: past, present, and future. Front Genet 6:266. https://doi.org/10.3389/fgene.2015.00266
37. Zhang M, He Z, Wen L, Wu J, Yuan L, Lu Guo C, Zhu L, Deng S, Yuan H (2010) Cadmium suppresses the proliferation of piglet sertoli cells and causes their DNA damage, cell apoptosis and aberrant ultrastructure. Reprod Biol Endocrinol 8(1):1–12. https://doi.org/10.1186/1477-7827-8-97
38. Jia X, Zhang H, Liu X (2011) Low levels of cadmium exposure induce DNA damage and oxidative stress in the liver of Oujiang colored common carp Cyprinus carpio var. Color. Fish Physiol Biochem 37(1):97–103. https://doi.org/10.1007/s10695-010-9416-5
39. Al-Brakati A, Albarakati AJA, Lokman MS, Theyab A, Algahtani M, Menshawi S, AlAmri OD, Essawy EA, Kassab RB, Abdel Moneim AE (2021) Possible role of kaempferol in reversing oxidative damage, inflammation, and apoptosis-mediated cortical injury following cadmium exposure. Neurotox Res 39(2):198–209. https://doi.org/10.1007/s12640-020-00300-2
40. Yang JM, Arnush M, Chen QY, Wu XD, Pang B, Jiang XZ (2003) Cadmium-induced damage to primary cultures of rat leydig cells. Reprod Toxicol 17(5):553–560. https://doi.org/10.1016/s0890-6238(03)00100-x
41. Chatterjee M, Sadhukhan GC, Kundu JK (2017) Indian gooseberry and Lycopodium 200c can effectively reduce cadmium induced testicular damage in 40 days exposed mice. Int J Zool Stud 2(6):31–37
42. Chen S, Yu Q, Dong W, Zhang H, Zou H (2020) Isoorientin plays an important role in alleviating Cadmium-induced DNA damage and G0/G1 cell cycle arrest. Ecotoxicol Environ Saf 187:109851. https://doi.org/10.1016/j.ecoenv.2019.109851
43. Jama AM, Mitic-Culafic D, Kolarevic S, Durasevic SF, Knezevic-Vukcevic J (2012) Protective effect of probiotic bacteria against cadmium-induced genotoxicity in rat hepatocytes in vivo and in vitro. Arch Biol Sci 64(3):197–1206. https://doi.org/10.2298/ABS1203197?
44. Zhang H, Cai C, Shi C, Cao H, Han Z, Jia X (2012) Cadmium-induced oxidative stress and apoptosis in the testes of frog *Rana*

limnocharis. Aquat Toxicol 122:67–74. https://doi.org/10.1016/j.aquatox.2012.05.014
45. Miller WL (2007) Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. Biochim Biophys Acta 1771:663–676. https://doi.org/10.1016/j.bbalip.2007.02.012
46. Yao Y, Wan Y, Shi X, Guo L, Jiang H, Zhang X, Xu B, Hua J (2022) Letrozole protects against cadmium-induced inhibition of spermatogenesis via LHCGR and Hsd3b6 to activate testosterone synthesis in mice. Reprod Biol Endocrinol 20(1). https://doi.org/10.1186/s12958-022-00915-4
47. Bhattacharya S (2020) The role of probiotics in the amelioration of cadmium toxicity. Biol Trace Elem Res 197(2):440–444. https://doi.org/10.1007/s12011-020-02025-x
48. Yu W, Xu Z, Gao Q, Xu Y, Wang B, Dai Y (2020) Protective role of wogonin against cadmium induced testicular toxicity: involvement of antioxidant, anti-inflammatory and anti-apoptotic pathways. Life Sci 258:118192. https://doi.org/10.1016/j.lfs.2020.118192
49. Shi X, Fu L (2019) Piceatannol inhibits oxidative stress through modification of Nrf2-signaling pathway in testes and attenuates spermatogenesis and steroidogenesis in rats exposed to cadmium during adulthood. Drug Des Devel Ther 13:2811. https://doi.org/10.2147/DDDT.S198444
50. Lin ML, Lu YC, Chung JG, Li YC, Wang SG, Sue-Hwee NG, Wu CY, Su HL, Chen SS (2010) Aloe-Emodin induces apoptosis of human nasopharyngeal carcinoma cells via caspase-8-mediated activation of the mitochondrial death pathway. Cancer Lett 291(1):46–58. https://doi.org/10.1016/j.canlet.2009.09.016
51. Cory S, Adams JM (2005) Killing cancer cells by flipping the Bcl-2/Bax switch. Cancer Cell 8:5–6. https://doi.org/10.1016/j.ccr.2005.06.012
52. Wang XK, Cai YF, Zhang K, Rui F, Li JG, Wu GL, Wang GL (2008) Apoptosis induced by cadmium and the expression of Caspase-3, Bcl-2 and Bax mRNA in testis of mouse. Acta Laser Biology Sinica 17:569–576
53. Jin LJ, Fang ZX, Zhang C, Lou ZF, Dong JY, Chen XW (2005) DNA damage, Bcl-2, Bax expression and ultrastructure change in spermatogenic cell of mice exposed to cadmium. Chin J Indust Hygiene Occup Dis 23:271–273
54. Rana SV (2008) Metals and apoptosis: recent developments. J Trace Elem Med Biol 22(4):262–284. https://doi.org/10.1016/j.jtemb.2008
55. Yang Z, He Y, Wang H, Zhang Q (2021) Protective effect of melatonin against chronic cadmium-induced hepatotoxicity by suppressing oxidative stress, inflammation, and apoptosis in mice. Ecotoxicol Environ Saf 228:112947. https://doi.org/10.1016/j.ecoenv.2021.112947
56. Wan N, Xu Z, Liu T, Min Y, Li S (2018) Ameliorative effects of selenium on cadmium-induced injury in the chicken ovary: mechanisms of oxidative stress and endoplasmic reticulum stress in cadmium-induced apoptosis. Biol Trace Elem Res 184(2):463–473. https://doi.org/10.1007/s12011-017-1193-x
57. Bhardwaj JK, Kumar V, Saraf P, Panchal H, Rathee V, Sachdeva SN (2021e) Efficacy of N-acetyl-L-cysteine against glyphosate induced oxidative stress and apoptosis in testicular germ cells preventing infertility. J Biochem Mol Toxicol 36:e22979. https://doi.org/10.1002/jbt.22979
58. Zhang P, Guan P, Ye X, Lu Y, Hang Y, Su Y, Hu W (2022) SOCS6 promotes mitochondrial fission and cardiomyocyte apoptosis and is negatively regulated by quaking-mediated miR-19b. Oxid Med Cell Longev 1121323. https://doi.org/10.1155/2022/1121323
59. Kassab RB, Lokman MS, Daabo HMA, Gaber DA, Habotta OA, Hafez MM, Zhery AS, Moneim AEA, Fouda MS (2020) Ferulic acid influences Nrf2 activation to restore testicular tissue from cadmium-induced oxidative challenge, inflammation, and apoptosis in rats. J Food Biochem 44(12):e13505. https://doi.org/10.1111/jfbc.13505
60. Yuan Y, Ma S, Qi Y, Wei X, Cai H, Dong L, Lu Y, Zhang Y, Guo Q (2016) Quercetin inhibited cadmium-induced autophagy in the mouse kidney via inhibition of oxidative stress. J Toxicol Pathol 29(4):247–252. https://doi.org/10.1293/tox.2016-0026
61. Ozgen S, Kilinc OK, Selamoglu Z (2016) Antioxidant activity of quercetin: a mechanistic review. Turkish J Agricul Food Sci Technol 4(12):1134–1138
62. Badr GM, Elsawy H, Sedky A, Eid R, Ali A, Abdallah BM, Alzahrani AM, Abdel-Moneim AM (2019) Protective effects of quercetin supplementation against short-term toxicity of cadmium-induced hematological impairment, hypothyroidism, and testicular disturbances in albino rats. Environ Sci Pollut Res 26(8):8202–8211. https://doi.org/10.1007/s11356-019-04276-1
63. Ravichandran R, Rajendran M, Devapiriam D (2014) Antioxidant study of quercetin and their metal complex and determination of stability constant by spectrophotometry method. Food Chem 146:472–478. https://doi.org/10.1016/j.foodchem.2013.09.080
64. Bhardwaj JK, Panchal H, Saraf P (2021b) Ameliorating effects of natural antioxidant compounds on female infertility: a review. Reprod Sci 28(5):1227–1256. https://doi.org/10.1007/s43032-020-00312-5
65. Liu G, Wang ZK, Wang ZY, Yang DB, Liu ZP, Wang L (2016) Mitochondrial permeability transition and its regulatory components are implicated in apoptosis of primary cultures of rat proximal tubular cells exposed to lead. Arch Toxicol 90(5):1193–1209. https://doi.org/10.1007/s00204-015-1547-0
66. Amanpour P, Khodarahmi P, Salehipour M (2020) Protective effects of vitamin E on cadmium-induced apoptosis in rat testes. Naunyn-Schmiedeberg's Arch Pharmacol 393(3):349–358. https://doi.org/10.1007/s00210-019-01736-w
67. de Oliveira MR, Nabavi SM, Braidy N, Setzer WN, Ahmed T, Nabavi SF (2016) Quercetin and the mitochondria: a mechanistic view. Biotechnol Adv 34(5):532–549. https://doi.org/10.1016/j.biotechadv.2015.12.014
68. Rotimi DE, Olaolu TD, Adeyemi OS (2022) Pharmacological action of quercetin against testicular dysfunction: a mini review. J Int Med 20(5):396–401. https://doi.org/10.1016/j.joim.2022.07.001
69. Bharti S, Misro MM, Rai U (2014) Quercetin supplementation restores testicular function and augments germ cell survival in the estrogenized rats. Mol Cell Endocrinol 383(1–2):10–20. https://doi.org/10.1016/j.mce.2013
70. Ruf AA, Webb J, Anderson D (2003) Modulation by flavonoids of the effects of a food mutagen in different thalassaemia genotypes in the Comet assay. Teratog Carcinog Mutagen 23(S2):93–102. https://doi.org/10.1002/tcm.10083