



Effects of chrysin in cadmium-induced testicular toxicity in the rat; role of multi-pathway regulation

Sibel Çiğdem Tuncer¹ · Sefa Küçükler² · Cihan Gür² · Serpil Aygörmmez³ · Fatih Mehmet Kandemir¹

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Abstract

Background Cadmium (Cd) is a strong toxic agent and causes serious damage to testicular tissues. Chrysin (CHR) is a natural flavonoid with many effective properties, especially antioxidant, anti-inflammatory and anti-apoptotic properties. The current study describes new evidence for the ameliorative effects of CHR on oxidative stress, apoptosis, autophagy and inflammation pathways in Cd-induced testicular tissue toxicity.

Methods Thirty-five male *Wistar* rats were divided into five groups, control, Cd, CHR, Cd + CHR25, and Cd + CHR50. Cd was administered alone at a dose of 25 mg/kg body weight or in combination with CHR 25 mg/kg and CHR 50 mg/kg for 7 days. Cd and CHR were administered orally. Biochemical, molecular, and histological methods were used to investigate inflammation, apoptosis, autophagy, and oxidant pathways in testicular tissue.

Results Cd increased lipid peroxidation, JAK-2/STAT-3 levels, inflammation-related NF- κ B, TNF- α , IL-1 β , IL-6, COX-2, and iNOS levels, AKT-2, FOXO1, Bax, Apaf-1 and Caspase-3 levels, autophagic Beclin-1, LC3A and LC3B. The Cd also caused a decrease in the activities of antioxidant enzymes and GSH levels, antiapoptotic Bcl-2 levels. CHR, on the other hand, had the opposite effect of all these Cd-induced changes.

Conclusions Overall, the data of this study indicate that testicular damage associated with Cd toxicity could be ameliorated by CHR administration.

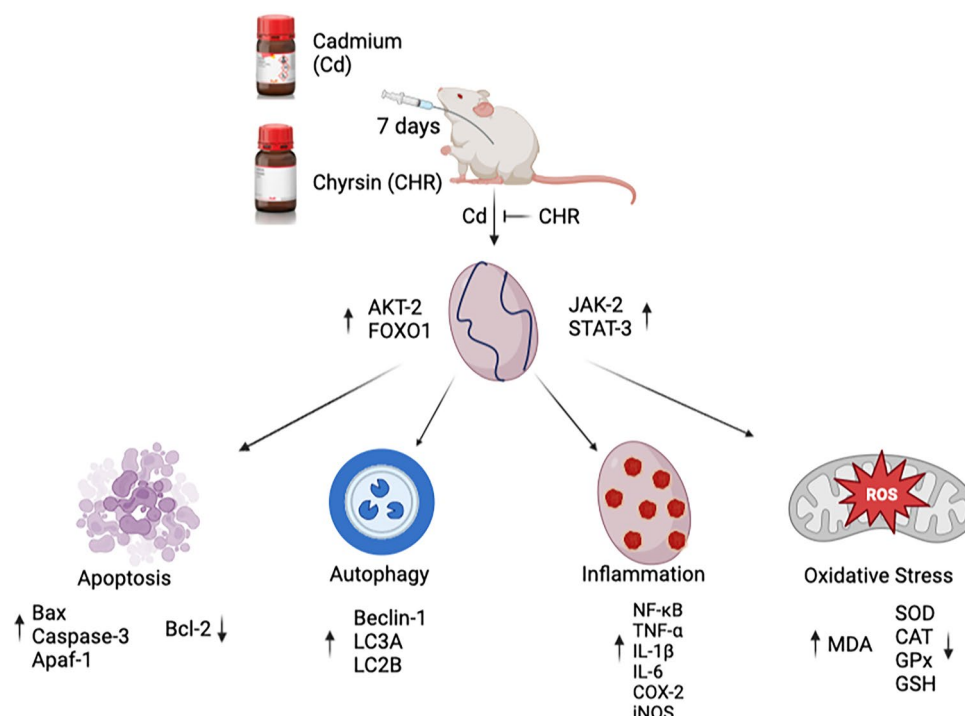
✉ Sibel Çiğdem Tuncer
drozturkc@yahoo.com

¹ Department of Medical Biochemistry, Faculty of Medicine, Aksaray University, Aksaray, Türkiye

² Department of Veterinary Biochemistry, Faculty of Veterinary, Atatürk University, Erzurum, Türkiye

³ Department of Veterinary Biochemistry, Faculty of Veterinary, Kafkas University, Kars, Türkiye

Graphical abstract



Keywords Testicular toxicology · Apoptosis · Inflammation · Cadmium · Oxidative stress · Chrysin

Introduction

The abundance and accessibility of chemical materials, which have been steadily increasing across the world, particularly in the last few decades, have brought about a slew of negative outcomes, like toxic effects. These chemicals, which are uncontrollably released into ecological systems, enter the food chain and are passed to humans indirectly or by direct exposure [1]. Cadmium (Cd) is a toxic heavy metal that is one of the most serious environmental pollutants and has strong toxicity. Cd is ranked eighth among the top 20 most dangerous substances with known toxic effects [2]. Cd is defined as a toxic heavy metal because it has no biological activity and has serious side effects. Since Cd is not biodegradable, its biological half-life extremely long, causing it to accumulate in the environment and is absorbed by the human body in a variety of ways [3]. Exposure to Cd primarily occurs through the ingestion of contaminated food, water, and inhalation. On the other hand, exposure to Cd is higher in some occupational groups including electroplating, plastics and fertilizer production [4]. Since Cd is also found in cigarettes (1.5 µg per cigarette), exposure is even higher in smokers [5].

Reproductive health, one of the global issues, has become a serious concern. This reflected in the reports of the World Health Organization (WHO) shows that 15% of couples are now affected [6]. In a similar vein of the major sources of concern in the field of toxicology is reproductive system dysfunction caused by toxic substances [7]. Cd directly affects the organs such as kidney, liver and lungs, and the testes which are susceptible to toxicity [8]. Men are exposed to higher amounts of Cd than women because of their higher levels of energy consumption and different dietary habits. Hence, the male reproductive system is more vulnerable to Cd toxicity than the female reproductive system [6]. Long-term exposure to Cd has been shown in studies to cause accumulation in the testes. Toxic effects such as sexual dysfunction, especially a decrease in sperm count and quality, fetal growth restriction, and disruption of testicular development occur as a result of accumulation [9].

Oxidative stress is one of the primary mechanisms underlying Cd toxicity in the testis. The disruption of the balance between reactive oxygen species (ROS) and antioxidants in favour of ROS is referred to as oxidative stress. When sperm function becomes impaired due to oxidative stress, cell death occurs [10]. Since ROS has a significant contribution to Cd-induced testicular toxicity, the use of natural antioxidants to mitigate Cd-induced toxic effects has gained attention [11].

Flavonoids with hydroxyl content have recently become the focus of extensive research into their presence in daily dietary sources and their effects on humans. Hydroxyflavonoids, which have antioxidant, anti-inflammatory, antiallergic, hepatoprotective and neuroprotective properties, are rich in biological activity [12]. Chrysin (CHR-5,7-dihydroxy-2-phenyl-4 H-chromen-4-one; 5,7-dihydroxyflavone) is a natural hydroxyflavone with a 15-carbon skeletal structure [13]. CHR has sparked research interest in recent years due to its protective properties such as antioxidant, anti-inflammatory, antiallergic, hepatoprotective, anticancer, antidiabetic, neuroprotective, reproductive, and cardiovascular health, which are naturally found in many plants, propolis, and honey [14, 15]. Studies have reported that CHR inhibits ROS production, the release of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α), reduces nuclear factor kappa B (NF- κ B), and inducible nitric oxide synthase (iNOS), and shows antioxidant properties by eliminating free radicals thanks to the hydroxyl groups in its structure [14, 16, 17].

There have been very few studies on the effects of CHR against Cd-induced damage in testicular tissues. Therefore, the aim of this study was to ascertain whether CHR could exercise a protective effect against Cd-induced testicular toxicity using biochemical, molecular, and histopathological methods.

Materials and methods

Chemicals

All chemicals (at analytical purities) including Cd (CAS No. 7440-43-9, purity 99%) and CHR (Cas No: 480-40-0, purity 97%) were obtained from Sigma Chemical Co. (St. Louis, US).

Laboratory animals and experimental procedure

Thirty-five *Wistar albino* rats (male, weighing 220–250 g, aged 10–12 weeks) were employed in the study. Rats were acquired from KONÜDAM Experimental Medicine Application and Research Center (Konya/Turkey). And all experiments were carried out here. Rats were housed under standard laboratory conditions (24–25 °C constant temperature, 45–50% humidity, 12 h light-12 h dark). Water and chow were provided *ad libitum*. Rats were randomly divided into five groups with seven animals in each group. Cd and CHR doses were ascertained from previous studies [18, 19].

Control: Saline was administered orally for 7 days.

Cadmium (Cd): it was administered orally for 7 days at a dose of 25 mg/kg.

Chrysin (CHR): It was administered orally for 7 days at a dose of 50 mg/kg.

Cadmium + Chrysin-25 (Cd + CHR25): CHR 25 mg/kg was administered orally half an hour after Cd 25 mg/kg was administered orally for 7 days.

Cadmium + Chrysin-50 (Cd + CHR50): CHR 50 mg/kg was administered orally half an hour after Cd 25 mg/kg was administered orally for 7 days.

Tissue collection

Rats were decapitated under mild isoflurane (IsoFlo; Abbott, Queenborough, UK) anesthesia 24 h after the last administration of CHR (8 day). After the connective and adipose tissues around surrounding the testicular tissues were removed, they were washed with cold physiological saline (0.85% NaCl) and stored in 10% buffered formalin solution, one at –80 °C for molecular and biochemical analyses and the other in 10% buffered formalin solution for histological examinations.

Preparation of tissue homogenates

Testicular tissues were homogenized with a homogenizer after being diluted 1:10 w/v with 1.15% KCl buffer. A portion of the homogenate was centrifuged at 1000 $\times$ g for 15 min at +4 °C to be used in determining the level of malondialdehyde (MDA) and in the analysis of superoxide dismutase (SOD) and catalase (CAT) activities. Another portion was centrifuged at 9000 $\times$ g for 20 min at +4 °C to be used in measurement the activities of glutathione (GSH) and glutathione peroxidase (GPx). Analyses were performed as soon as the supernatant was obtained [19].

Analysis of lipid peroxidation and antioxidant markers

MDA levels, an indicator of lipid peroxidation, and SOD, CAT, GPx activities and GSH levels to determine antioxidant status were analyzed from supernatants obtained from testicular tissues. MDA levels were analyzed by the method of Placer et al. [20]. GSH levels were analyzed by the method of Sedlak and Lindsay [21]; GPx activity by the method of Lawrence and Burk [22]; SOD activity by the method of Sun et al. [23]; CAT activity by the method of Aebi [24]. Protein content was measured by the method of Lowry et al. [25]. Biochemical analyses

were measured by ELISA Plate Reader (Bio-Tek, Winooski, VT, USA).

Total RNA isolation, cDNA synthesis and real time-PCR analysis in testicular tissues

RT-PCR method was used to analysis of the relative mRNA transcript levels of *NF-κB*, *TNF-α*, *Interleukin-1beta* (*IL-1β*), *IL-6*, *Cyclooxygenase* (*COX-2*), *iNOS*, *Janus kinaz-2* (*JAK-2*), *Signal transducer and activator of transcription-3* (*STAT-3*), *Apoptotic protease activating factor-1* (*Apaf-1*), *Cysteine-aspartic acid protease-3* (*Caspase-3*), *Becclin-1*, *Microtubule-associated protein 1 light chain 3 alpha* (*LC3A*) and *Microtubule-associated protein 1 light chain 3 beta* (*LC3B*), *AKT Serine/Threonine Kinase 2* (*AKT-2*), and *Forkhead box protein O1* (*FOXO1*) genes in testicular tissues. The first step for this was to isolate total RNA from tissues using QIAzol Lysis Reagent (79,306; Qiagen). This was done as per the manufacturer's instructions. Afterwards, cDNA synthesis was performed via iScript cDNA Synthesis

Kit (Bio-Rad) from the total RNAs obtained. This was also carried out in accordance with the manufacturer's instructions, and the reaction conditions were designed as follows: Priming time: 5 min at 25 °C, reverse transcription time: 20 min at 46 °C, and RT inactivation time: 1 min at 95 °C. In the final step, cDNAs of all groups were mixed with iTaq Universal SYBR Green Supermix (BIORAD) and primers with the sequences given in Table 1 as per the manufacturer's instructions, and reaction conditions (Polymerase Activation and DNA Denaturation: 95 °C, 30 s, Denaturation: 95 °C, 5 s (40 cycles), Annealing/Extension and Plate Read: 60 °C, 30 s (40 cycles), Melt Curve Analysis: 65–95 °C 0.5 °C increments at 2–5 s/step) were prepared in the Rotor-Gene Q (Qiagen) device as per the manufacturer's instructions as well. CT values were obtained from the device after the cycle was completed. These obtained values have been normalized using β-Actin. The $2^{-\Delta\Delta CT}$ method developed by Livak and Schmittgen [26].

Table 1 Primer sequences

Gene	Sequences (5'–3')	Length (bp)	Accession No.
NF-κB	F: AGTCCCGCCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC	106	NM_001276711.1
IL-1β	F: ATGGCAACTGTCCCTGAACT R: AGTGACACTGCCTTCCTGAA	197	NM_031512.2
IL-6	F: AGCGATGATGCACTGTCAGA R: GGAACTCCAGAAGACCAGAGC	127	NM_012589.2
TNF-α	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCACTT	139	NM_012675.3
COX-2	F: AGGTTCTTCTGAGGAGAGAG R: CTCCACCGATGACCTGATAT	240	NM_017232.3
iNOS	F: AGATCAATGCAGCTGTGCTC R: GGCTCGATCTGGTAGTAGTAGA	235	NM_012611.3
JAK-2	F: TAGGTACGGAGTATCTCGTG R: TGGAGTTATAGACAGCCAGG	215	NM_031514.1
STAT-3	F: TACCTGGAGCAGCTTCATCA R: GATCTCGCCCAAGAGGTTAT	153	NM_012747.2
Apaf-1	F: ACCTGAGGTGTCAGGACC R: CCGTTCGAGCATGAGCCAA	192	NM_023979.2
Caspase-3	F: ACTGGAATGTCAGCTCGCAA R: GCAGTAGTCGCCTCTGAAGA	270	NM_012922.2
Becclin-1	F: TCTCGTCAAGGCGTCACTTC R: CCATTCTTTAGGCCCCGACG	198	NM_053739.2
LC3A	F: GACCATGTAAACATGAGCGA R: CCTGTTTCATAGATGTCAGCG	139	NM_199500.2
LC3B	F: GAGCTTCGAACAAAGAGTGG R: CGCTCATATTCACGTGATCA	152	NM_022867.2
AKT-2	F: GAGTACTTGCACTCGACGGA R: CCATGAGGATGAGCTCGAAG	304	NM_017093.1
FOXO1	F: CAGCCAGGCACCTCATAACA R: TCAAGCGGTTTCATGGCAGAT	143	NM_001191846.3
β-Actin	F: CAGCCTTCCTTCTTGGGTATG R: AGCTCAGTAACAGTCCGCCT	360	NM_031144.3

Western blot analysis

Western blot analyses for testicular tissues were performed in our laboratory similar to the previous study of Kandemir et al. [27]. Testicular tissues powdered with liquid nitrogen were weighed and homogenized in RIPA buffer. After homogenizing, they were centrifuged at $16,000\times g$ for 20 min. The obtained supernatants were reconstituted with Laemmli sample buffer and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 30 g protein per well. Proteins were transferred to PVDF membranes after being separated by molecular size. After the proteins were transferred to the membranes, they were blocked for 1.5 h with 4% bovine serum albumin (BSA) dissolved in phosphate buffered saline with 0.1% tween (PBS-T). The membranes were washed 5 times for 5 min each with PBS-T after blocking. Primary antibodies (Caspase-3, Bax, and Bcl-2) were then added and incubated overnight. After incubation, the membranes were washed with PBS-T 5 times for 5 min each and incubated for 1.5 h with goat anti-mouse IgG secondary antibody conjugated to HRP (1:2000 dilution). After incubation with secondary antibodies, the membranes were washed 5 times for 5 min each time with PBS-T, and then the bands were visualized using BioRad Clarity Max ECL substrate (Bio-Rad, Hercules, USA). Densitometric analysis of blots was performed using ImageLab program (Bio-Rad, Hercules, USA). At least 3 replicate measurements were taken for each sample.

Histopathological analysis

Testicular tissues obtained from rats were fixed in 10% formaldehyde for 24 h. It was then washed overnight in tap water to perform the routine histological follow-up procedure. Afterwards, the blocks were prepared by passing through graded alcohol series, after the dehydration process, clearing with xylol and infiltration with paraffin. Sections of 5 μ m thickness were removed from the blocks by microtome and stained with Hematoxylin-Eosin (H&E). Stained sections were examined and photographed using a Binocular Olympus CX 43 microscope.

Statistical analysis

IBM SPSS was used to perform statistical evaluation of the findings obtained from testicular tissue analysis (version 20.0; IBM Co, North Castle, NY). To determine whether there were statistical differences between the experimental groups, One-way ANOVA and Tukey's post hoc tests were used. Each sample was run in triplicate in RT-PCR analyses,

and the results are presented as mean $\pm$ SD. The values of $p < 0.05$, $p < 0.01$ ve $p < 0.001$ were considered statistically significant.

Results

Effects of Cd and CHR administrations on MDA, GSH, SOD, CAT and GPx

MDA level in testicular tissues were measured as an indicator of lipid peroxidation. As antioxidant indicators, GPx, SOD, and CAT activities, and GSH levels, were analyzed. When compared to the control group, an increase in MDA ($p < 0.001$, 105%) and a decrease in SOD (58%), GPx (64%), and CAT (64%) activities and GSH (55%) were detected ($p < 0.001$). When compared to the Cd group, a decrease in MDA level ($p < 0.001$, 28%), an increase in SOD ($p < 0.001$, 142%), GPx ($p < 0.001$, 46%), and CAT ($p < 0.01$, 53%) activities, and GSH ($p < 0.001$, 42%) levels were found in Cd + CHR25 group. All of these changes were more pronounced in the Cd + CHR50 group ($p < 0.001$, MDA:43%, GPx:102%, SOD:83%, CAT:137%, GSH:100%) (Fig. 1).

Effects of Cd and CHR administrations on NF- κ B, TNF- α , IL-1 β , IL-6, COX-2 and iNOS

The RT-PCR method was used to analyze the parameters of NF- κ B (127%), TNF- α (119%), IL-1 β (129%), IL-6 (96%), COX-2 (93%) ve iNOS (67%) as inflammation indicators in testicular tissues (Fig. 2). According to RT-PCR results, all of these parameters increased in the Cd group compared to the control group ($p < 0.001$). In comparison to the Cd group, it was found that NF- κ B (11%), IL-1 β (18%), IL-6 (16%), COX-2 (11%) and iNOS (18%) were decreased in Cd + CHR25 group and all parameters were decreased in Cd + CHR50 group ($p < 0.001$, NF- κ B: 43%, TNF- α : 18%, IL-1 β : 22%, IL-6: 43%, COX-2: 19% ve iNOS: 27%).

Effects of Cd and CHR administration on JAK-2 and STAT-3

The findings from RT-PCR analysis revealed that JAK-2 (43%) and STAT-3 (81%) mRNA transcript levels were found to be significantly up-regulated in the Cd group compared to the control group ($p < 0.001$). JAK-2 (25 mg/kg: 20%, 50 mg/kg: 25%) and STAT-3 (25 mg/kg: 13%, 50 mg/

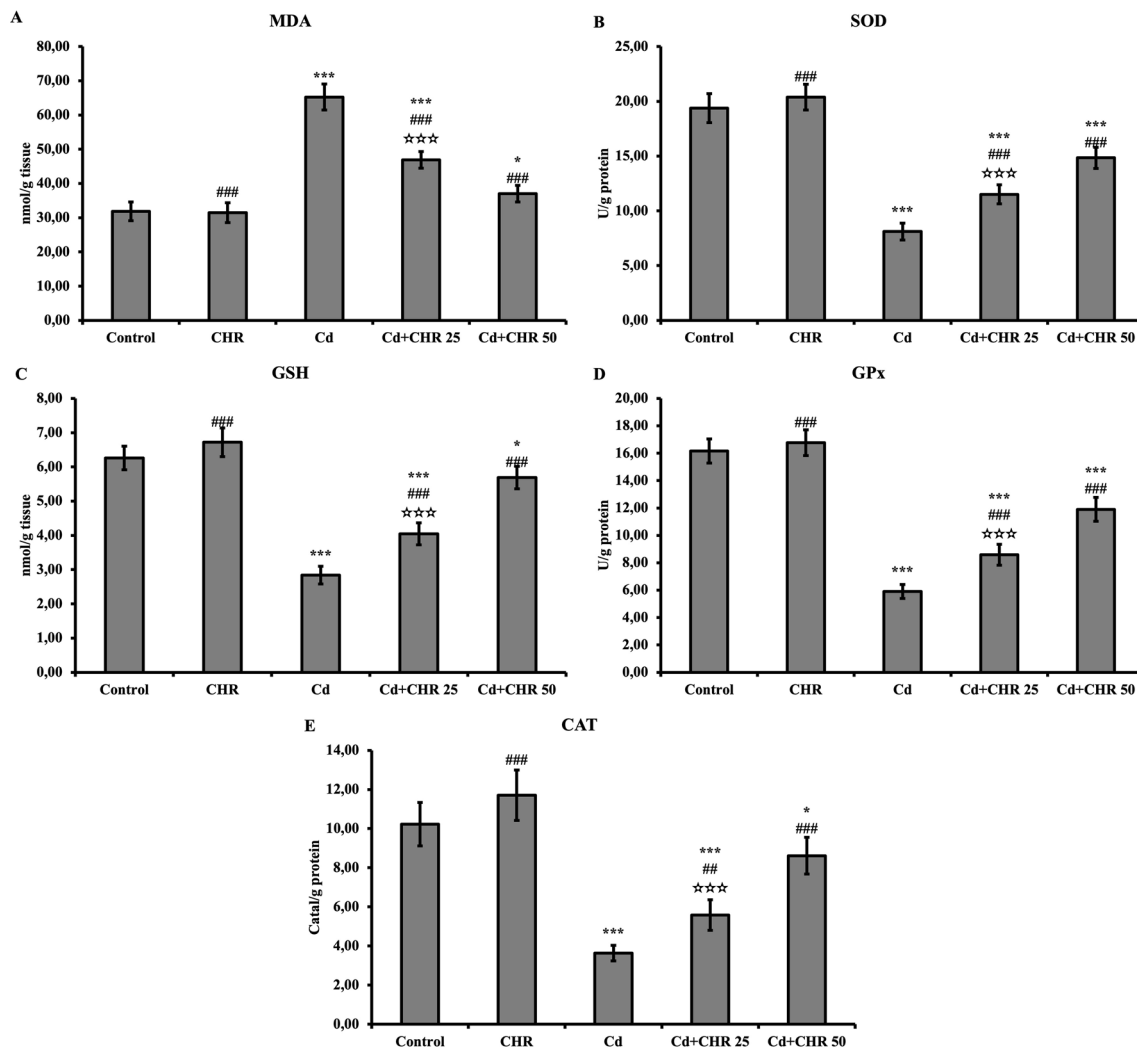


Fig. 1 The effects of Cd and CHR on MDA, SOD, GSH, GPx and CAT in the testicular tissues of rats. All data were expressed as mean $\pm$ SD. Statistical significance (Control vs. others: * p < 0.05, *** p < 0.001, Cd vs. others: # p < 0.05, ## p < 0.01, ### p < 0.001, Cd + CHR25 vs. Cd + CHR50: * p < 0.05, ** p < 0.01, *** p < 0.001) was analyzed using One Way ANOVA

** p < 0.01, *** p < 0.001, Cd vs. others: # p < 0.05, ## p < 0.01, ### p < 0.001, Cd + CHR25 vs. Cd + CHR50: * p < 0.05, ** p < 0.01, *** p < 0.001) was analyzed using One Way ANOVA

kg: 21%) mRNA transcript levels were suppressed after CHR administration (p < 0.001 at both doses) (Fig. 3).

Effects of Cd and CHR administrations on Apaf-1 and Caspase-3

Caspase-3 and Apaf-1 mRNA transcript levels were analyzed for apoptosis level in testicular tissues. Caspase-3 (p < 0.001, %162) and Apaf-1 (p < 0.001, %112) mRNA transcript levels were increased in the Cd group compared to the control group. Caspase-3 (25 mg/kg: 27%, 50 mg/kg: 39%) and Apaf-1 (25 mg/kg: 17%, 50 mg/kg: 40%) mRNA

transcripts were suppressed with CHR administration at both doses (p < 0.001). CHR was more effective at 50 mg/kg dose compared to 25 mg/kg dose (p < 0.001) (Fig. 4).

Effects of Cd and CHR administrations on AKT-2 and FOXO1

When the results of the RT-PCR analysis were evaluated, a decrease in AKT-2 (43%) and FOXO1 (45%) mRNA transcript levels was detected in the Cd group compared to the control group (p < 0.001). It was found that the mRNA transcript levels of AKT-2 and FOXO1 were increased in Cd + CHR25 (AKT2: 26%, FOXO1: 73%) and

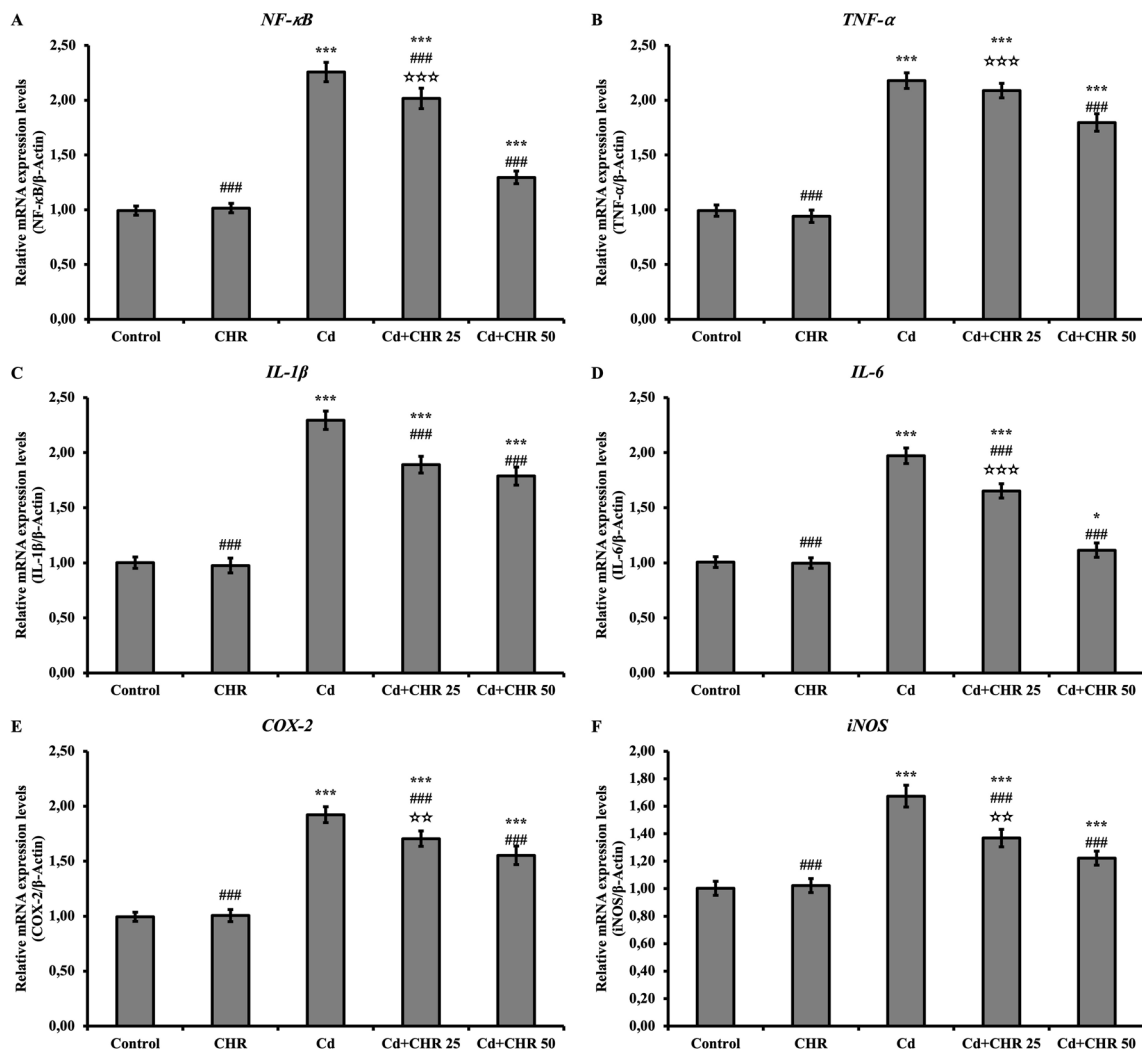


Fig. 2 The mRNA transcript level of NF-κB, TNF-α, IL-1β, IL-6, COX-2 and iNOS in the testicular tissues of rats. All data were expressed as mean ± SD. Statistical significance (Control vs. others: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd+CHR25 vs. Cd+CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

ers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd+CHR25 vs. Cd+CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

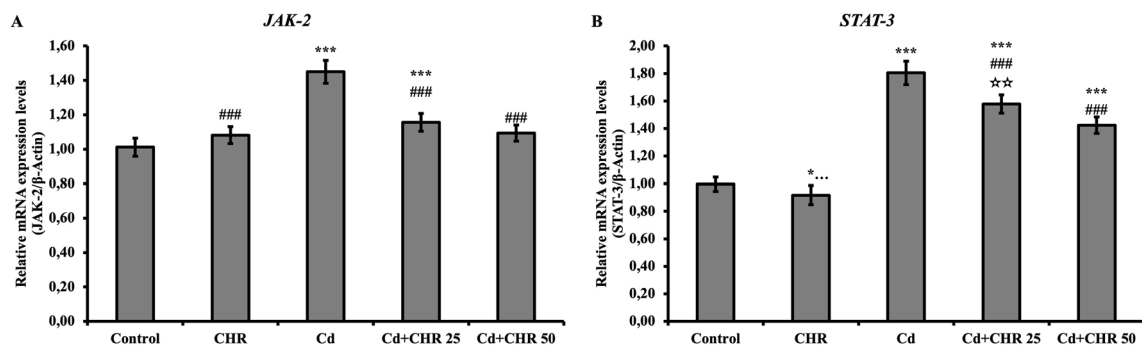


Fig. 3 The mRNA transcript level of JAK-2 and STAT-3 in the testicular tissues of rats. All data were expressed as mean ± SD. Statistical significance (Control vs. others: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd+CHR25 vs. Cd+CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

*** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd+CHR25 vs. Cd+CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

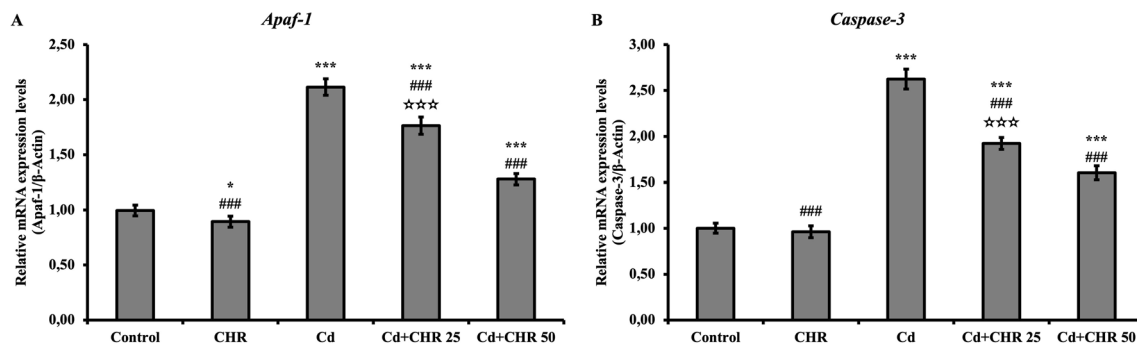


Fig. 4 The mRNA transcript level Apaf-1 and Caspase-3 in the testicular tissues of rats. All data were expressed as mean $\pm$ SD. Statistical significance (Control vs. others: * $p < 0.05$, ** $p < 0.01$,

*** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd + CHR25 vs. Cd + CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

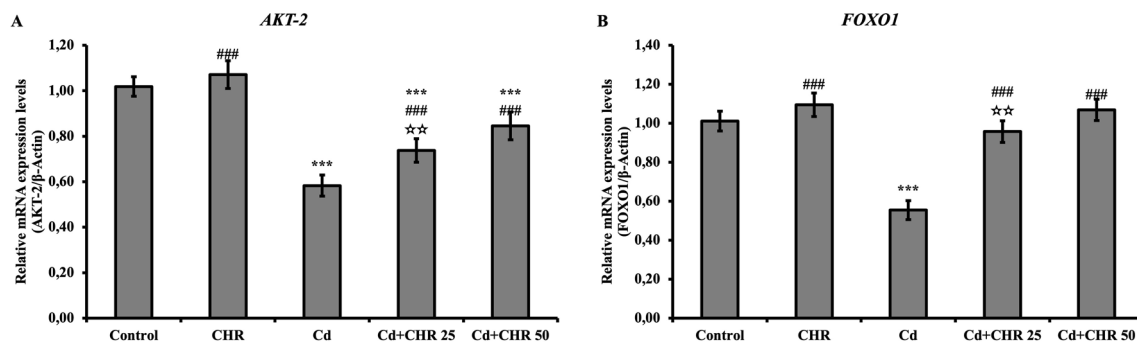


Fig. 5 The mRNA transcript level of AKT-2 and FOXO1 in the testicular tissues of rats. All data were expressed as mean $\pm$ SD. Statistical significance (Control vs. others: * $p < 0.05$, ** $p < 0.01$,

*** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd + CHR25 vs. Cd + CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

Cd + CHR50 (AKT2: 45%, FOXO1: 93%) groups compared to Cd group ($p < 0.001$). When CHR doses were compared, 50 mg/kg dose was more effective than 25 mg/kg dose ($p < 0.01$) (Fig. 5).

Effects of Cd and CHR administration on Beclin-1, LC3A and LC3B

mRNA transcript levels of Beclin-1 (77%), LC3A (107%), and LC3B (75%) were found to be increased in the Cd group compared to the control group ($p < 0.001$). When compared to the Cd group, the Cd + CHR25 (Beclin-1: 20%, LC3A: 17%, LC3B: 10%) and Cd + CHR50 (Beclin-1: 29%, LC3A: 23%, LC3B: 19%) groups showed a decrease ($p < 0.001$). The 50 mg/kg dose was more effective at CHR doses (Beclin 1: $p < 0.01$; LC3A: $p < 0.05$; LC3B: $p < 0.001$) (Fig. 6).

Effects of Cd and CHR administrations on Caspase-3, bax and Bcl-2 protein levels

Protein levels of Caspase-3, Bax and Bcl-2 parameters related to apoptosis were also analyzed by Western blot. Caspase-3 (120%) and Bax (300%) protein levels increased in the Cd group compared to the control group ($p < 0.001$), while antiapoptotic factor Bcl-2 (35%) protein level decreased ($p < 0.001$). Compared to Cd group, Bax protein level decreased ($p < 0.001$, 160%) in Cd + CHR25 group; Caspase-3 (36%) and Bax (57%) protein levels decreased ($p < 0.001$), and Bcl-2 protein level increased ($p < 0.05$, 31%) in Cd + CHR50 group (Fig. 7).

Effect of CHR on Cd-induced testicular histological changes

When the testicular tissue morphology of the control group was examined, it was seen that the seminiferous

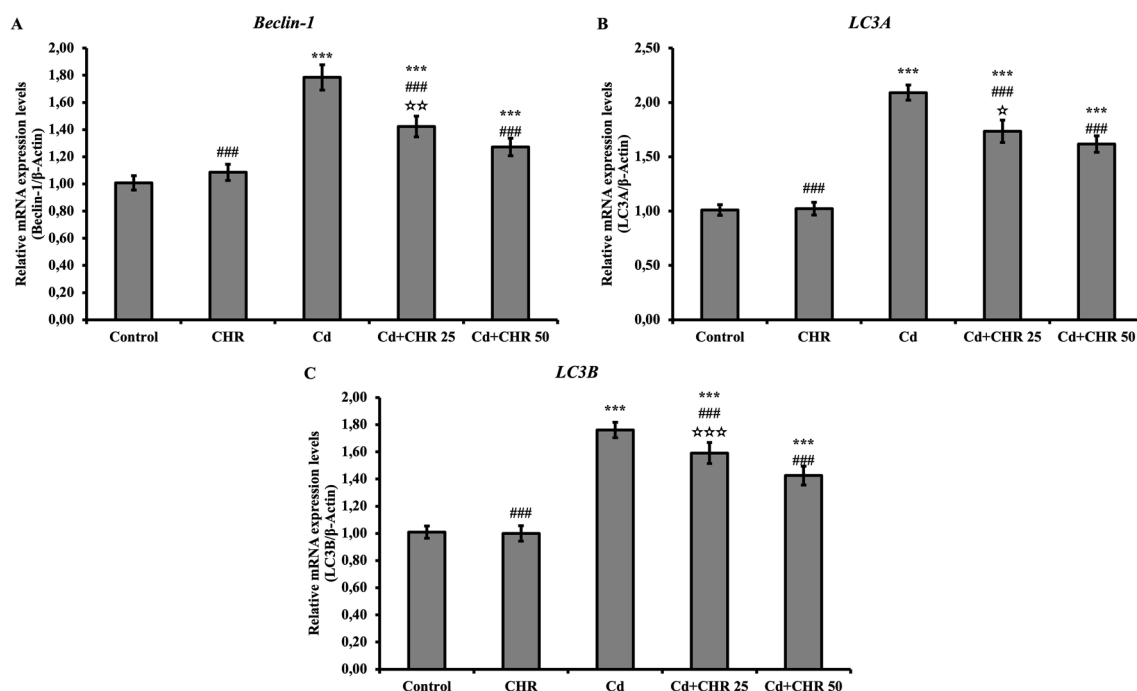


Fig. 6 The mRNA transcript level of Beclin-1, LC3A and LC3B in the testicular tissues of rats. All data were expressed as mean $\pm$ SD. Statistical significance (Control vs. others: * $p < 0.05$, ** $p < 0.01$,

*** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd+CHR25 vs. Cd+CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

tubules and interstitial connective tissue area were normal. The germinal epithelium and basement membrane were in a smooth structure and the epithelium consisted of sertoli, spermatogonia, spermatocyte, spermatid and spermatozoa cells. Especially spermatozoa cells were abundant in the tubule lumen (Fig. 8A, A1). When the sections in the CHR group were evaluated, it was seen that they were similar to the control group and the seminiferous tubules were normal and the seminiferous tubule epithelium had a typical cell distribution (Fig. 8B, B1). There were amorphous seminiferous tubules in the testis tissues of the Cd applied group. It was observed that spermatozoa in the lumen were less than in the control group. Additionally, desquamation of spermatogenic cells forming the germinal epithelium and a decrease in germ cell layers were observed. There was increased vacuolization in germinal epithelial cells in this group. In addition, congestion occurred in some of the vessels in the connective tissue (Fig. 8C, C1). In the testicular tissues in the Cd + CHR25 and Cd + CHR50 groups, the seminiferous tubules were more arranged and spermatogenic cells were more abundant. The spermatozoa in the lumen started to increase and the severity of vacuolization decreased. Particularly in the images in the group treated with high-dose CHR, seminiferous tubules characterized by the presence of all

different stages of germ cells were observed in the tubule epithelium (Fig. 8D, D1 and E, E1).

Discussion

Heavy toxic metals are among the leading causes of environmental pollution. Heavy metal exposure to human is gradually increasing as a result of their increasing accumulation, which leads to a rise in pathophysiology of diseases [28]. One of these heavy toxic metals is Cd, which is a persistent environmental and occupational pollutant with toxic effects on humans and other living organisms [18]. Cd damages tissues by inducing oxidative stress, epigenetic changes in DNA expression, and inhibition of transportation pathways or up-regulation. Flavonoids have protective effect against these damages by activating various cellular signaling pathways [2]. Many studies have been conducted to investigate the protective effects of natural antioxidants such as CHR against heavy metal toxicity, and researchers have found promising results [18]. CHR's protective effects in Cd-induced testicular toxicity were investigated using biochemical, molecular, and histological methods in the current study.

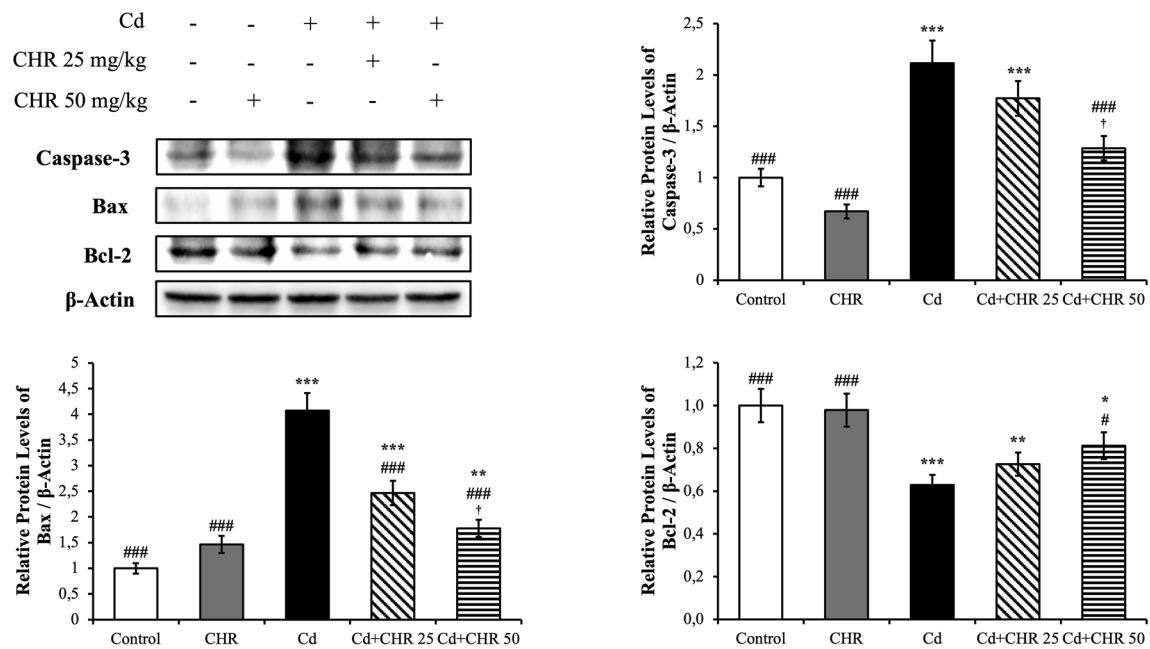


Fig. 7 Effects of Cd and CHR administrations Caspase-3, Bax and Bcl-2 protein levels in testicular tissues of rats. All data were expressed as mean $\pm$ SD. Statistical significance (Control vs. oth-

ers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Cd vs. others: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Cd+CHR25 vs. Cd+CHR50: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was analyzed using One Way ANOVA

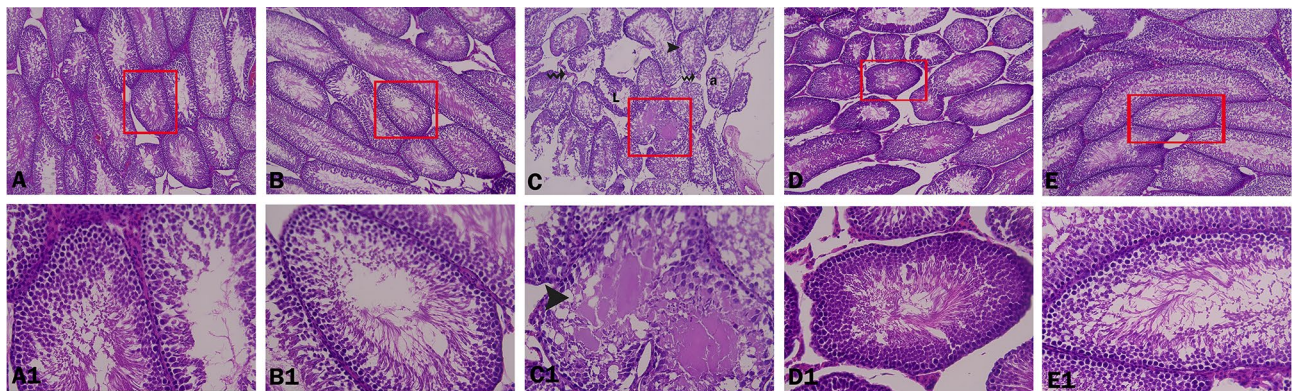


Fig. 8 Representative photomicrographs of rat testis tissues treated with cadmium and chrisin. (H&E staining, original magnification; top row 100 $\times$; bottom row 400 $\times$). Control group with normal spermatogenic cells and normal interstitial space and normal seminifer-

ous tubules (A, A1), CHR group (B, B1), Cd group (D, D1); arrow-head: severe vacuolization, a: amorphous seminiferous tubule, curved arrow: congestion, L: seminiferous tubule lumen without spermatozoa, Cd+CHR 25 group (D, D1), Cd+CHR 50 group (E, E1)

The oxidant/antioxidant balance in the body is disrupted in favor of oxidants due to excessive ROS production, resulting in oxidative stress. When this balance cannot be restored, serious damage may occur in tissues. An increase in ROS has a negative impact on spermatologic parameters, the reproductive system, and thus male fertility due to the fact that testicular tissue is particularly sensitive. Increased lipid peroxidation is one of the primary causes of increased ROS [29]. The increase in MDA level is one of the best indicators of lipid peroxidation [30]. SOD, CAT, and GPx enzymes are

the first line of defense against oxidative stress. GSH is the second line of defense [31]. CHR has antioxidant capacity due to the presence of both 5- and 7- hydroxyl groups [19]. It was ascertained in a study conducted by Ilertürk et al. that CHR caused an increase in GSH levels and a decrease in MDA levels, as well as antioxidant enzyme activities as a result of this property [19]. Cd was found to cause oxidative stress in testicular tissue by increasing MDA levels, decreasing SOD, CAT, and GPx enzyme activity, and increasing GSH levels in our current study. With CHR administration,

these effects shifted in the opposite direction, and it was found that its antioxidant properties protected against tissue damage.

Evidence on the link between oxidative stress and inflammatory response suggests that oxidative stress triggers the inflammatory process [17]. ROS, which contribute in the oxidative process, contribute as well in inflammation by activating the NF- κ B pathway. NF- κ B acts on proinflammatory cytokines during inflammation [17]. After being stimulated, NF- κ B becomes activated by dissociating from I κ B in the cytoplasm. It then travels to the nucleus and triggers the production of cytokines such as TNF- α and IL-1 β , which significantly contribute to the inflammatory process [27, 32]. NF- κ B also regulates the expression of the proteins iNOS and COX-2. As a result, inhibiting NF- κ B is crucial for therapeutic purposes [17]. Oxidative stress is also linked to increased amount of iNOS [33]. Previous studies have reported that exposure to Cd causes inflammation by altering the expression of these parameters in tissues [2]. CHR, on the other hand, exerts anti-inflammatory effects by inhibiting the expression of cytokines like TNF- α and inactivating proinflammatory mediators like iNOS [2]. In the study by Mantawy et al., CHR was also found to significantly inhibit NF- κ B expression. CHR inhibited the inflammatory pathway with a decrease in iNOS and TNF- α , providing satisfactory anti-inflammatory effects, according to the same study [34]. CHR was found to significantly reduce Cd-induced increases in NF- κ B, TNF- α , IL-1 β , IL-6, COX-2, and iNOS expression in testicular tissue in the current study.

Studies on IL-6 have shown that it plays an important role in the regulation of pathological pain. IL-6 activates the JAK/STAT signaling pathway via its specific receptor (Gp80) [35]. The JAK2/STAT3 signaling pathway plays a prominent role in proliferation, apoptosis, immunity and inflammation, as well as underlies various diseases [31]. JAKs are primarily responsible for STAT3 activation [31]. Information in the literature about the effects of JAK2/STAT3 pathway in Cd toxicity is insufficient. In this study, it was ascertained that Cd increased the mRNA transcription levels of JAK2 and STAT3, whereas CHR administration decreased the transcription levels of these genes significantly. As a result, it is believed that CHR has protective properties against inflammation via this pathway as well.

Oxidative stress increases inflammation on the one hand and stimulates apoptosis on the other. While apoptosis is effective in destroying damaged or dangerous cells in the body, as its severity increases, it may begin to act on healthy cells, resulting in the occurrence of pathologic conditions [36]. The effect of increased ROS on apoptosis is achieved by stimulating pro-apoptotic genes [37]. Members of the caspase family are prominent in all stages of the apoptosis pathway [38]. Caspase-3 is one of these members that

determines the boundary between life and death of cell life [39]. The intrinsic pathway, which involves mitochondrial factors, is another pathway involved in apoptosis. When the Bax/Bcl-2 balance is disrupted, cytochrome c (CytC) is released from the mitochondria into the cytoplasm via this pathway [40]. After CytC enters cytoplasm, it binds to apoptotic protease activating factor-1 (Apaf-1) and activates it in the presence of adenosine triphosphate. Activated Apaf-1 activates Caspase-9. Caspase-9 then activates Caspase-3, resulting in apoptosis. Apaf-1 is the key caspase regulator in this pathway [2]. While Bax exerts an apoptotic effect by destroying growth factors, Bcl-2 exerts an anti-apoptotic effect by inhibiting the activation of proapoptotic proteins [31]. Many studies have reported that Cd administration causes apoptosis by increasing the levels of Bax, Apaf-1, and caspase-3 levels while decreasing Bcl-2 levels [18, 28]. In this study, Cd was found to direct cells to apoptosis in testicular tissues by increasing Apaf-1 and Caspase-3 expression. CHR administration exhibited anti-apoptotic effect in testicular tissues by suppressing Apaf-1 and Caspase-3 expressions. However, when protein levels were examined, Cd induced apoptosis in testicular tissue by increasing the amounts of Caspase-3 and Bax while decreasing the amount of Bcl-2. These changes were reversed by CHR administration, and the tissue attempted to recover from apoptosis. Given the role of ROS in apoptosis, CHR can be said to have protective properties against Cd-induced apoptosis.

To counteract apoptosis, cells activate various pathways [41]. Akt is a serine/threonine kinase that has three isoforms: AKT1, AKT2, and AKT3. It is involved in many cellular processes [42]. Phosphorylated Akt controls cell survival and proliferation by activating target genes [43]. The anti-apoptotic effect of Akt is based on its ability to regulate the Bcl-2 family and affect the Caspase-3 cascade [44, 45]. FOXO1 is also involved in a variety of biological functions such as DNA repair, apoptosis, cell cycle arrest, and oxidative stress resistance [46]. According to RT-PCR data obtained in this study, mRNA transcription levels of AKT-2 and FOXO1 were significantly decreased in the testicular tissue of Cd-induced rats. CHR (25 and 50 mg/kg) administration reversed this situation and increased the mRNA transcription levels of AKT-2 and FOXO1.

The increase in oxidative stress also triggers autophagy [41]. Autophagy is basically an effective mechanism for controlling cellular homeostasis. In the presence of damaged organelles or misfolded protein, autophagy is activated and some of the cytoplasmic components are transferred to the lysosomes and they are removed [31]. However, overstimulation of autophagy damages cells and causes functional and structural disorders in tissues [41]. The main active proteins in the autophagy pathway are Beclin-1, LC3A, and LC3B [47]. Beclin-1 is an indispensable protein involved in many important biological processes such as immunity, development,

and tumor suppression [48]. LC3, another specific marker of autophagy, provides the formation of autophagosomes. Upon activation of autophagy, LC3A is converted to the LC3B form by conjugation with phosphatidylethanolamine [31]. LC3B is the most common autophagic marker that is activated by oxidative damage and contributes to the autophagosome [49]. Increased levels of Cd-mediated inflammatory cytokines in Cd-exposed rat tissues lead to increased autophagy [2]. Our results showed that there was an increase in Beclin 1, LC3A and LC3B levels with Cd exposure in rat testicular tissues, reversing these effects due to morin treatment. It was shown in our study that CHR plays an important anti-autophagic role in Cd-induced testicular toxicity.

Cd toxicity causes structural changes in testicular tissues, damage to germ and Sertoli cells, degeneration by causing rupture of the blood-testis barrier, and testicular necrosis [8]. Li et al. in their study, they reported that Cd exposure induced edema, necrosis, degeneration of seminiferous tubules, and apoptosis in the spermiogenic region of the seminiferous epithelium in rat testicles [50]. In the present study, both structural and functional disorders were detected in testicular tissues exposed to Cd. The most striking changes are the amorphous seminiferous tubules. With this, a decrease in sperm count in the lumen, desquamation of spermatogenic cells forming the germinal epithelium and a decrease in germ cell layers were detected. Another detected structural defect was increased vacuolization in germinal epithelial cells. In addition, some blood vessels in the connective tissue were blocked. With the CHR application, all these structural and functional damages were alleviated. Especially when CHR was administered at a dose of 50 mg/kg, this alleviation was much more pronounced. As a matter of fact, the observation of seminiferous tubules characterized by the presence of all different stages of germ cells in the tubule epithelium in this group has been important proof of this situation.

Conclusions

In conclusion, CHR was found to inhibit Cd-induced toxicity in rat testicular tissue. It exhibited this effect by lowering oxidative stress, inflammation, apoptotic and autophagic cell death, and increasing antioxidant capacity. As a result, our present study supports the hypothesis that CHR may have a beneficial (protective) effect against Cd-induced testicular toxicity.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SÇT, SK, CG, SA, and FMK. The first draft of the manuscript was written by SÇT and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available on request.

Declarations

Conflict of interest Sibel Çiğdem Tuncer, Sefa Küçükler, Cihan Gür, Serpil Aygörmöz, Fatih Mehmet Kandemir declares that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Ethics committee approval was received for this study from the ethics committee of Necmettin Erbakan University KONDAM Experimental Medicine Research and Study Center's (No: 2022/65, Date: 30.12.2022). This article does not contain any studies with human participants performed by any of the authors.

Consent to participate The authors gave their explicit consent to publish the manuscript. Sibel Çiğdem Tuncer is free to contact any of the people involved in the research to seek further clarification and information.

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