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RESEARCH ARTICLE

Etanercept Mitigates Cadmium Chloride-induced Testicular Damage in Rats "An Insight into Autophagy, Apoptosis, Oxidative Stress and Inflammation"

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Abstract

Rationale Cadmium (Cd) is an environmental and occupational toxin that represents a serious health hazard to humans and other animals. One of the negative consequences of cadmium exposure is testicular injury.

Objective This study aimed to investigate the therapeutic effect of etanercept against cadmium chloride-induced testicular damage and the probable underlying mechanisms of its action.

Methods A total of sixty rats were divided into six groups: control, cadmium chloride ($CdCl_2$) (7 mg/ kg i.p.), and $CdCl_2$ treated with etanercept (5,10 and 15 mg/kg s.c.) and etanercept only (15 mg/kg s.c.). $CdCl_2$ was administrated as a single dose, while etanercept was administered every 3 days for 3 weeks.

Results $CdCl_2$ reduced serum testosterone, testicular glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD). However, it elevated the levels of malondialdehyde (MDA) and microtubule-associated protein light chain 3B (LC3B) in the testes. Cadmium caused pathogenic alterations as well as increased levels of inflammatory biomarkers such as tumor necrosis factor-alpha (TNF-α) and nuclear factor-kappa B (NF-κB). Besides, the gene expressions of *caspase-3 and* inducible nitric oxide synthase (*i-NOS*) and Beclin-1 protein increased with $CdCl_2$ exposure. Interestingly, etanercept relieved the previous toxic effects induced by $CdCl_2$ in a dose-dependent manner as evidenced by inhibition of oxidative stress, inflammatory markers, Beclin-1, LC3B, and caspase-3 accompanied by improvement in histopathological changes.

Conclusion Etanercept provides a potential therapeutic approach to treat testicular tissue against the damaging effects of Cd by reducing oxidative stress, inflammation, apoptosis, and autophagy.

Keywords Beclin-1 · Cadmium · Caspase-3 · Etanercept · iNOS · LC3B · Testicular damage · TNF-α

Introduction

Testicular damage is primarily induced by chemical exposure leading to infertility (Ilieva et al. 2020). Heavy metals, such as Cd, lead, and mercury, are environmental toxic contaminants (Fu and Xi, 2020). Cd causes harmful effects at low doses once absorbed by the body (Ciarrocca et al. 2013). $Cdcl_2$ is an extremely poisonous heavy metal that causes cancer in humans and animals (Odewumi et al. 2016). The potential toxicity of Cd to male reproductive organs may cause testicular steroidogenesis inhibition, sperm-cell apoptosis, testes necrosis, and prostate cancer, which finally result in male infertility (Habib et al. 2019a). Moreover, Cd can pass through the blood–testes barrier, causing changes in the hypothalamic–pituitary–testicular axis and DNA damage (Yang et al. 2021).

However, there is a limited and inconsistent research on the mechanism of Cd-induced testicular injury. The key variables involved in cadmium-induced tissue damage include oxidative stress and inflammation (Arafa et al. 2014). Oxidative stress is triggered by reactive oxygen species (ROS) and an imbalance between ROS and antioxidant enzymes

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activities (Antar et al. 2021). As a result, oxidative stress activates the NF-κB signaling pathway, which controls several genes implicated in inflammatory responses such as TNF-α and iNOS (Abdelrazek et al. 2016; Fouad et al. 2013).

The mechanism of interaction between apoptosis and autophagy in testicular damage induced by Cd exposure, still remains unknown. Apoptosis and autophagy are two types of programmed cell death that are important for the development and regulation of male reproductive functions (Bustamante-Marín et al. 2012). Oxidative stress induces Ca2 + channel dysfunction and activation of the intrinsic apoptotic pathway (Knight et al. 2019). In addition, TNF-α acts as a death ligand and activates extrinsic apoptosis (Mukhopadhyay et al. 2014).

Autophagy is activated in response to cellular stress, such as oxidative stress (Singh et al. 2018). Increased generation of ROS can activate mitogen-activated protein kinases (MAPKs) (Son et al. 2011), which induce autophagy by phosphorylating Bcl-2. Phosphorylated Bcl-2 cannot form a complex with Beclin-1 (Filomeni et al. 2015; Kroemer et al. 2010). Hence, Beclin-1 expression may represent the autophagic state of a cell. LC3B is also a marker of autophagic activity that is required for phagophore elongation (Ni et al. 2011; Wang et al. 2015).

Etanercept is a recombinant dimeric fusion protein that binds TNF-α and is composed of the extracellular ligand-binding region of the 75-kDa human TNF receptor coupled to the Fc component of human immunoglobulin G1 (Chadwick et al. 2018). Several inflammatory illnesses, such as rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, are treated with etanercept (Aghdashi et al. 2020). In addition to reducing inflammatory disorders, anti-TNF drugs may improve sperm parameters and hormone levels (Ramonda et al. 2014b). When spermatozoa are exposed to high levels of TNF-α, their genomic and functional integrity may be damaged. Thus, TNF-α may play a role in the pathophysiology of testicular damage (Morsy et al. 2020). Etanercept has shown an effect on testicular function and semen in spondyloarthritis patients (Ramonda et al. 2014b). Moreover, intratesticular expression of mRNAs of both interferon γ and TNF-α is significantly increased in experimental orchitis in mice, indicating that etanercept could be a valuable therapeutic agent in testicular damage (Terayama et al. 2011). Administration of etanercept has been found to promote anti-inflammatory and antioxidant responses in a model of testicular injury (Pascarelli et al. 2017); however, the mechanisms mediating this effect have not been well established and require further research. The above-mentioned possible roles of TNF-antagonists have motivated the desire to explore their potential experimental effects in this regard. Therefore, the current study was conducted to assess the potential therapeutic role of etanercept against chemically induced testicular damage. Several aspects of the activity of etanercept, including its antioxidants, anti-apoptotic, anti-autophagy, and anti-inflammatory potential, have been investigated.

Materials and methods

Drugs and chemicals

Etanercept (Enbrel 50 mg/ 1 ml) prefilled syringe was purchased from Pfizer, Egypt and was diluted with saline. Cadmium chloride was a generous gift from the Analytical Department, Faculty of Pharmacy, Mansoura University, with 96% purity and was dissolved in distilled water.

Animals and experimental design

A total of sixty Wistar male albino rats weighing 190 to 220 g at the start of the experiment were used. The Modern Veterinary Office for Laboratory Animals (Cairo, Egypt) provided for all rats. The rats were allowed to acclimatize under laboratory conditions for two weeks before the experiment. Rats were housed under controlled temperature (25 ºC ± 1) in a 12-h light/dark cycle. Food and water were allowed ad libitum during the study period. The study protocol was approved by the research ethics committee, Faculty of Pharmacy, Suez Canal University (Ismailia, Egypt), according to the Canadian Council on Animal Care Guidelines (License number 201911MA3).

The rats were randomly divided into six experimental groups as follows: in control, rats received saline once daily; in the $CdCl_2$ group, rats received $CdCl_2$ (7 mg/kg; i.p) single dose (Akunna et al. 2017); the third, fourth, and fifth groups received single dose of $CdCl_2$ (7 mg/kg; i.p) and treated with etanercept (5 mg/kg, 10 mg/kg, and 15 mg/kg s.c), respectively, every 3 days for 3 weeks (Totoson et al. 2016); and the sixth group received etanercept only (15 mg/kg/3 days; i.p). Treatment began 8 weeks post-$CdCl_2$ administration.

Killing and biological sample collection

At the end of the experiment, the rats were anesthetized with thiopental sodium (50 mg/kg) and then killed. A dry Eppendorf tube was used to collect blood from a cardiac puncture. The blood samples were allowed to settle for 30 min, followed by centrifugation at 2000 × g for 15 min. The separated serum was stored at -20ºC for further biochemical analyses (Antar et al. 2021). Testes were isolated and collected. One testis was fixed in bouin solution, 5% acetic acid, 9% formaldehyde, and 1.5% picric acid in aqueous solution (Ellenburg et al. 2020) and used in histopathological investigations. Another group of testes was homogenized

by phosphate buffer saline and then centrifuged at 4000 rpm for 15 min; then, the supernatant was separated, collected in clean tubes, and stored at (-20ºC) for ELISA analysis. The remaining tissues were frozen at -80 °C for RT-PCR analysis and Western blot analysis.

Assessment of serum cadmium and testicular cadmium

The serum cadmium level and testicular cadmium content were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. MBS3809624, MyBioSource®, USA) according to the manufacture's instructions. Standard 50 µl was added to the standard well followed by testing sample 10 µl. Then, 100 µl of HRP-conjugate reagent was added to each well and incubated for 60 min at 37 °C. Each well was washed. Chromogen solution A (50 µl) and chromogen solution B (50 µl) were added to each well. They were gently mixed and incubated for 15 min at 37 °C protected from light. The color in the wells changed from blue to yellow. The optical density (O.D.) at 450 nm was read using a microtiter plate reader within 15 min.

Estimation of serum testosterone, testicular TNF-α, and LC3B

The serum testosterone level was determined using an ELISA kit (Cat. No. MBS262661, MyBioSource®, USA) according to the manufacturer's protocol. One hundred microliters of samples or different concentrations of rat testosterone standard samples were added to corresponding wells. The biotinylated rat testosterone antibody liquid was added. Then, 100 µl of color reagent liquid was added to individual well. When color for the high concentration of standard curve became darker and color gradient appeared, 100 µl color reagent C was added. Optical density at (450 nm) was read within 10 min.

The TNF-α content in the testes was determined using a rat TNF-ELISA kit (Cat. No. MBS355371, MyBioSource®, USA) according to the manufacturer's instructions. The testicular content of LC3B was determined using an ELISA kit (Cat. No. MBS938189, MyBioSource®, USA) following the manufacturer's protocol.

Assessment of testicular oxidative stress biomarkers

The MDA levels in the tissues were evaluated using an ELISA kit (MyBioSource®, USA, Cat. No. MBS355371). The levels of SOD in tissues were measured using a rat ELISA kit (Cat. No. MBS036924, MyBioSource®, USA). GSH levels in tissues were measured using an ELISA kit (Cat. No. E02G0367, Shang Hai Blue Gene Biotech CO). Another ELISA kit (Cat. No. MBS) was used to determine the amount of CAT in the tissues. The microtiter plate provided in these kits was pre-coated with an antibody specific to MDA, SOD, GSH, or CAT. Standards or samples were then added to the appropriate wells with a biotin-conjugated polyclonal antibody preparation specific for MDA, SOD, GSH, or CAT, and avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well and incubated. Then, a TMB substrate solution was added to each well. Only those wells that contained MDA, SOD, GSH, or CAT, biotin-conjugated antibody, and enzyme-conjugated avidin exhibited a color change. The enzyme–substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of MDA, SOD, GSH, or CAT in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Assessment of iNOS and caspase-3

iNOS and caspase-3 gene expression was evaluated using reverse transcription-polymerase chain reaction (RT-PCR). In brief, pure RNA was extracted using a total RNA Purification Kit according to the manufacturer's protocol (Thermo Scientific, Fermentas, #K0731). A high-capacity cDNA reverse transcription kit was utilized to convert the total RNA (0.5 to 2 µg) to cDNA. The cDNA samples were then stored at − 20 ◦C. The isolated cDNA was amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). The qRT-PCR assay with the following gene-specific primer sets was optimized with the annealing temperature (*iNOS*; forward primer: ′5 GACCAGAAACTG TCTCACCTG′3, reverse primer: ′5 CGAACATCGAAC GTCTCACA′3, *Caspase3*; forward primer:: ′5 GGTATT GAGACAGACAGTGG′3, reverse primer: ′5 CATGGG ATCTGTTTCTTTGC′3, β-actin; forward primer:: ′5 AAG TCCCTCACCCTCCCAAAAG′3, reverse primer:: ′5 AAG CAATGCTGTCACCTTCCC′3 Real-time PCR amplification and analysis were performed to measure the expression of mRNAs of target genes in the tissue relative to β-actin mRNA expression as an internal reference. The applied biosystems software version 3.1 (StepOneTM, USA) was used to evaluate the expression of mRNAs of target genes in the tissue, with β-actin as an internal reference.

Assessment of NF-κB and Beclin-1 by western blot

Proteins were extracted from testicular tissue using an extraction buffer. Lysates were allowed to remain on ice for 30 min and then centrifuged at 15,000 rpm for 30 min at 4 °C. The soluble lysates were mixed at a 1:4 ratio with 5-ml Laemmle buffer and heated for 4 min at 94 °C. Next,

20 µg of protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), and then blocked with 5% defatted milk in TBS-Tween buffer for 3 h at 4 °C (Daiichikagaku, Tokyo, Japan). The membranes were incubated with specific primary antibodies including anti-Beclin-1 (sc-4834, Santa Cruz, USA) and NF-κB (ab 16,502, Abcam Company). On the next day, β-actin monoclonal antibody (sc- 47,778, Santa Cruz, USA) was added and incubated for 1 h on a roller shaker at 4 °C. To remove unbound primary antibody, the membranes were washed 5 times for 5 min each in TBS-Tween. The membrane incubated with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) diluted at 1:5000 in blocking solution for 1 h at room temperature and then washed three times with 1 × TBS-T. The densitometry analysis of protein bands was performed using Image J. software. The density of each band was normalized with β-actin.

Histopathological examination

The testicular samples collected from all groups were fixed in the bouin solution and then dried using repeated dilutions of alcohol after fixation. Tissues were prepared, embedded in paraffin, sectioned at 5-mm intervals, and stained with hematoxylin and eosin (H&E). For histological investigation under a light electric microscope, 4-µm-thick slices were cut using a microtome and stained with H&E.. The investigation was conducted by a skilled pathologist without previous information about the treatments.

Statistical analysis

All data are presented as a as mean ± SD. One-way analysis of variance (ANOVA) was used to determine statistical significance, followed by Tukey's multiple comparison analysis. The level of significance was fixed at a p-value of < 0.05.

Results

Effect of etanercept on testes/body weight index

In the $CdCl_2$ control group, $CdCl_2$ significantly decreased testes/body weight index by approximately 0.61-fold compared with that in the control group. In a dose-dependent way, treatment with etanercept (5 mg/kg and 10 mg/kg) significantly increased the testes/body weight index by 21.85% and 36.02%, respectively, compared with the $CdCl_2$ group. Treatment with 15 mg/kg etanercept induced a further significant increase in testes/body weight index by 46.83% in comparison with the $CdCl_2$ group (Table 1).

Table 1 Effect of etanercept (5,10, 15 mg/kg) on the testes/body weight index in experimental groups

Study groups	Testis Wt/ body Wt index (mg/g)
Control	$7.702 \times 10^{-3} \pm 2.657 \times 10^{-4}$
$CdCl_2$ group	$4.708 \times 10^{-3} \pm 2.765 \times 10^{-4}$ *
$CdCl_2$ + Etanercept (5 mg/kg)	$5.737 \times 10^{-3} \pm 2.883 \times 10^{-4}$ *&
$CdCl_2$ + Etanercept (10 mg/kg)	$6.404 \times 10^{-3} \pm 2.888 \times 10^{-4}$ *&
$CdCl_2$ + Etanercept (15 mg/kg)	$6.913 \times 10^{-3} \pm 2.639 \times 10^{-4}$ *&#
Etanercept (15 mg/kg)	$7.417 \times 10^{-3} \pm 2.101 \times 10^{-4}$ &#$

Table 2 Effect of etanercept (5 mg/kg, 10 mg/kg, 15 mg/kg) on serum testosterone level in experimental groups

Study groups	Testosterone level (ng/dL)
Control	3.800 ± 0.100
$CdCl_2$ group	1.467 ± 0.1155*
$CdCl_2$ + Etanercept (5 mg/kg)	1.833 ± 0.0577*&
$CdCl_2$ + Etanercept (10 mg/kg)	2.167 ± 0.1155*&
$CdCl_2$ + Etanercept (15 mg/kg)	2.800 ± 0.100 *&#
Etanercept (15 mg/kg)	3.700 ± 0.100&#$

Table 1 Effect of etanercept (5,10, 15 mg/kg) on the testes/body weight index in experimental groups.

Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P<0.05$. * Significantly different from control, & significantly different from $CdCl_2$ group, # significantly different from 5 mg/kg etanercept-treated group, $ significantly different from 10 mg/kg etanercept-treated group.

Effect of etanercept on testosterone level

The testosterone level in the $CdCl_2$ control group was significantly decreased by threefold compared to the control group. In a dose-dependent way, treatment with etanercept (5, 10, and 15 mg/kg) significantly increased the serum testosterone by 24. 94%, 47.71%, and 90.86%, respectively, compared with the $CdCl_2$ group. In comparison with the $CdCl_2$ group, treatment with etanercept alone (15 mg/kg) resulted in a significant increase in serum testosterone level by 152.21%. These findings indicate favorable effect of etanercept on the testicular steroidogenic changes following Cd exposure (Table 2).

Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P<0.05$. * Significantly different from

control [&] significantly different from $CdCl_2$ group, [#] significantly different from 5 mg/kg etanercept-treated group, [$] significantly different from 10 mg/kg etanercept-treated group.

Effect of etanercept on serum cadmium and testicular cadmium levels

In comparison with the control group, the serum cadmium level was significantly increased by threefold in the $CdCl_2$ group. Treatment with etanercept (5, 10, and 15 mg/kg) significantly reduced the serum cadmium level by 36.27%, 39.72%, and 58.87%, respectively, compared with the $CdCl_2$ group.

The testicular cadmium content was also markedly increased by 9.19-fold in the $CdCl_2$ group compared with the control group. Treatment with etanercept (5 mg/kg) reduced testicular cadmium content by 70.64% compared with the $CdCl_2$ group, and treatment with etanercept (10 mg/kg) significantly reduced the testicular cadmium content by 75.56%, compared with the $CdCl_2$ group. Optimum enhancement was observed with etanercept (15 mg/kg) treatment with a significant decrease in testicular cadmium content by 87.48% compared with the $CdCl_2$ group (Fig. 1).

Effect of etanercept on $CdCl_2$-induced changes in testes oxidative and anti-oxidative stress markers

Exposure to cadmium chloride raised the testicular MDA content by fourfold and reduced the testicular SOD, CAT, and GSH contents by approximately (7), (7), and (5.6) folds, respectively, in the $CdCl_2$ group compared with the control group. Treatment with etanercept (5, 10, and 15 mg/kg) significantly ameliorated $CdCl_2$-induced damage to different ranges. Etanercept at (5 mg/kg) decreased the testicular MDA content and increased the SOD, GSH, and CAT contents by 32.21%, 53.58%, 37.75%, and 56.25%, respectively, compared with those in the $CdCl_2$ group, and etanercept at (10 mg/kg) significantly decreased testicular MDA content and restored SOD, GSH, and CAT contents by 102.61%, 72.02, 68.53%, and 71.27%, respectively, compared with those in the $CdCl_2$ group. The dose of (15 mg/kg) etanercept provided the best results, with a considerable decrease in the testicular MDA content and an increase in the SOD, GSH, and CAT levels by 188.16%, 80.37%, 76.98%, and 78.77%, respectively, compared with those in the $CdCl_2$ group as shown in Fig. 2

Effect of etanercept on testicular inflammatory markers (TNF-α, iNOS, and NF-κB)

The content of TNF-α was markedly elevated by 6.45-fold. Likewise, *iNOS* gene expression was significantly increased by approximately 5.9-fold along with a significant rise in NF-κB protein expression by approximately 2.43-fold, in the $CdCl_2$ group compared with those in the control group.

Treatment with etanercept (5, 10, and 15 mg/kg) lowered testicular TNF-α content by 31.08%, 50.09%, and 64.13%, respectively, compared with the $CdCl_2$ group. The testicular *iNOS* expressions were also reduced by 35.97%, 46.64%, and 62.65%, respectively, compared with that in the $CdCl_2$ control group. Similarly, treatment with etanercept (5, 10, and 15 mg/kg) reduced the testicular NF-κB protein expression by 33.88%, 37.77%, and

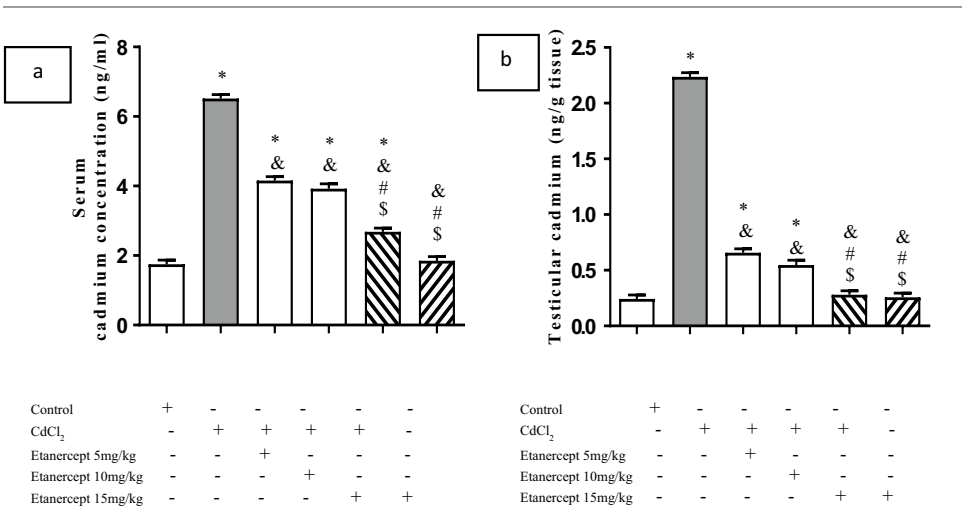

Fig. 1 Effect of etanercept (5 mg/kg, 10 mg/kg, 15 mg/kg) on: (a) serum cadmium level, (b) content of testicular cadmium in experimental groups. Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$. [*]Significantly different from control, [&]significantly different from $CdCl_2$ group, [#]Significantly different from 5 mg/kg etanercept-treated group, [$]Significantly different from 10 mg/kg etanercept-treated group (+: present, -: absent)

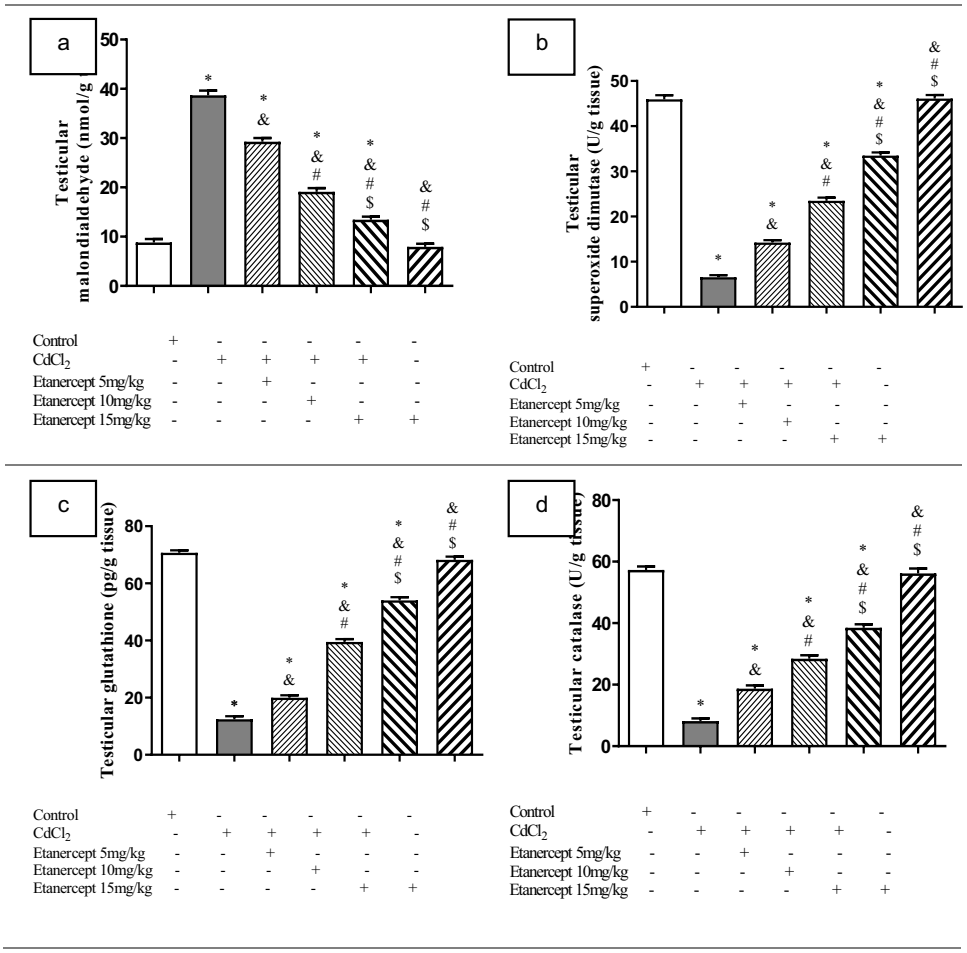

Fig. 2 Effect of etanercept (5 mg/kg, 10 mg/kg, 15 mg/kg) on oxidative stress biomarkers in experimental groups. (a) Testicular content of malondialdehyde. (b) Testicular content of superoxide dismutase enzyme. (c) Testicular content of glutathione. (d) Testicular content of catalase enzyme. Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$. *Significantly different from control, &significantly different from $CdCl_2$ group, #Significantly different from 5 mg/kg etanercept-treated group, $Significantly different from 10 mg/kg etanercept-treated group(+: present, -: absent).

56.80%, respectively, compared with the $CdCl_2$ control group (Fig. 3).

Effect of etanercept on caspase-3

Exposure to cadmium chloride increased the testicular *caspase-3* gene expression by threefold compared with that in the control group. Treatment with etanercept (5 and 10 mg/kg) reduced the testicular *caspase-3* gene expression by 29.48% and 41.34%, sequentially compared with the $CdCl_2$ group. Treatment with (15 mg/kg) etanercept provided the best results, with a significant reduction in the testicular *caspase-3* gene expression by 53.52% (Fig. 4).

Effect of etanercept on autophagy biomarkers

Beclin-1 protein expression was significantly elevated by approximately 3.45-fold. Similarly, the LC3B content was significantly increased by approximately 5.62-fold in the $CdCl_2$ group compared with the control group. Treatment with etanercept (5, 10 and 15 mg/kg) reduced the testicular Beclin-1 protein expression by 17.98%, 34.52%, and 52.05%, respectively, compared with the $CdCl_2$ group. Similarly, the testicular LC3B content was, respectively, reduced by 43.08%, 47.38%, and 71.07% compared with that in the $CdCl_2$ group (Fig. 5).

Histopathological examination

The H&E-stained testicular sections of the control group exhibited normal seminiferous tubules lined with spermatogenic cell layers (arrows) with the presence of free sperms within their lumen (arrowheads). The $CdCl_2$ group revealed severe testicular degeneration within the spermatogenic cell layers (arrows), hyperplasia of Leydig cells within edematous (arrowheads) and atypia within some spermatogenic cells (tailed-arrow). $CdCl_2$ with etanercept (5 mg/kg) treatment led to a decrease in testicular degeneration within the spermatogenic cell layers (arrows) with a core of desquamative spermatogenic cells (arrowheads). $CdCl_2$ with etanercept (10 mg/kg) treatment resulted in a marked decrease in testicular degeneration

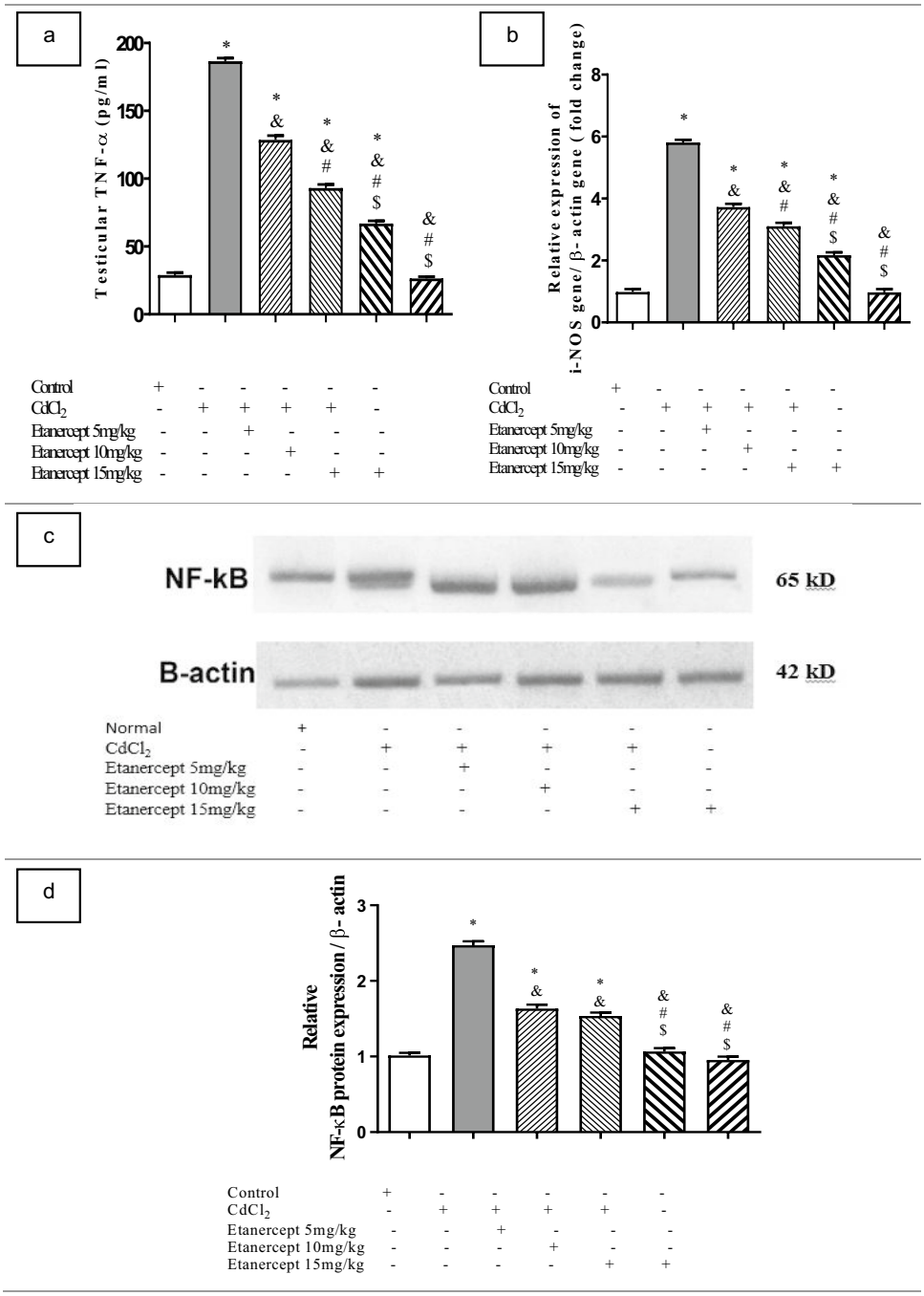

Fig. 3 Effect of etanercept (5 mg/kg, 10 mg/kg, 15 mg/kg) on inflammatory biomarkers in experimental groups. (a) Testicular content of TNF-α. (b) Testicular gene expression of *i-NOS*. (c) Western blot analysis showing protein expression of NF-κB and β-actin. (d) Testicular protein expression of NF-κB. Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$. *Significantly different from control, &significantly different from $CdCl_2$ group, #Significantly different from 5 mg/kg etanercept-treated group, $Significantly different from 10 mg/kg etanercept-treated group(+: present, -: absent).

within the spermatogenic cell layers (arrows) with the appearance of free sperms within the lumen (arrowheads). Finally, $CdCl_2$ with etanercept (15 mg/kg) treatment induced a remarkable decrease in the spermatogenic cell layer damage (arrows) and an increase in spermatogenesis (arrowheads indicate free sperms). Furthermore, testes sections of the group treated with etanercept (15 mg/kg)-treated group only revealed a normal seminiferous tubule lined with spermatogenic cell layers (arrows) with the presence of free sperms within their lumen (arrowheads) as shown in Fig. 6.

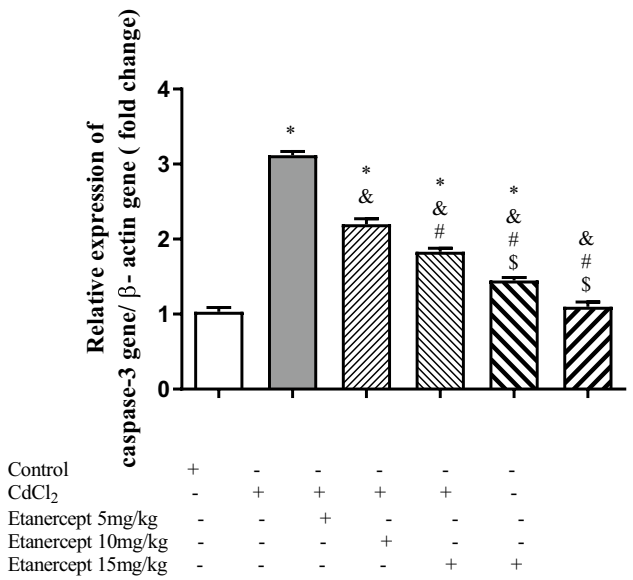

Fig. 4 Effect of etanercept (5 mg/kg, 10 mg/kg, 15 mg/kg) on testicular *caspase-3* gene expression in experimental groups. Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$. *Significantly different from control, &significantly different from $CdCl_2$ group, #Significantly different from 5 mg/kg etanercept-treated group, $Significantly different from 10 mg/kg etanercept-treated group(+: present, -: absent)

Discussion

Human exposure to environmental pollutants that negatively affect the male reproductive function has become the most pressing public health issue (Ma et al. 2019). In the present study, it was observed that $CdCl_2$ exposure led to a remarkable reduction in the testes/body weight index. These results were in agreement with the other studies, which have shown that $CdCl_2$ reduced the weight of sex organs (Bashandy et al. 2016; Erboga et al. 2016; Nna et al. 2017). $CdCl_2$ was found to cause necrotic degeneration of testicular tissues as a result of oxidative stress and inflammation. Interestingly, the testicular weight primarily depends on the mass of undifferentiated spermatogenic cells. Hence, $CdCl_2$ may have a detrimental impact on the number of germ cells and elongated spermatids, resulting in a reduction of testicular weight (Ola-Mudathir et al. 2008). Moreover, $CdCl_2$ has the potential to degrade the functional and structural integrity of testicular tissues (CHEN et al. 2013).

There are different therapeutic agents used in management of cd-induced testicular damage such as natural products and monoclonal antibodies. Previous studies showed that kolaviron and quercetin have protective effects on cadmium-induced testicular damage and endocrine pathology in rats by their antioxidant activity (Farombi et al. 2012). Moreover, fenugreek seed powder mitigates cadmium-induced testicular damage and hepatotoxicity in male rats by its antioxidant and anti-inflammatory activities (Arafa et al. 2014). On the other hand, infliximab, anti-TNF-α, abrogates cadmium-induced testicular damage and spermiotoxicity via enhancement of steroidogenesis and suppression of inflammation and apoptosis mediators (Habib et al. 2019b). In the current study, etanercept, anti-TNF-α, showed therapeutic effect against cadmium-induced testicular damage by suppressing oxidative stress, inflammation, apoptosis, and autophagy. Indeed, anti-TNF-α drugs can improve testicular function (Ramonda et al. 2014a).

The most common side effects linked to etanercept use in clinical trials are non-upper respiratory tract infections followed by upper respiratory infections (Scheinfeld, 2004). Etanercept is associated with serious bacterial infections (Elwood et al. 2003). It can also promote the reactivation of tuberculous, though to a lesser extent of infliximab. Infliximab has been related to viral and bacterial infection. As foreign substances, TNF-α blockers are immunogenic and can result in the development of neutralizing antibodies. Treatment with infliximab can be associated with the development of neutralizing antibodies to infliximab in approximately 10% of patients. On the other hand, 5% of patients treated with etanercept for rheumatoid and psoriatic arthritis develop antibodies to etanercept. However, these antibodies are not related to the side effects or efficacy of etanercept (Scheinfeld, 2004).

Treatment with etanercept restored the testes weight in a dose-dependent manner. Consistent with this result, treatment with infliximab (anti-TNF-α) was found to significantly alleviate Cd-induced weight loss in the reproductive organs. High levels of TNF-α "the most important inflammatory mediator" played an essential role in reducing the quantity of germ cells and elongated spermatids (Bashandy et al. 2016). Therefore, TNF-α inhibition by etanercept could be responsible for its effect on the testicular weight increase in the present study.

Treatment with etanercept decreased Cd concentration. Administration of Cd to rats causes acute tubular necrosis, which results in decreasing its elimination from the body and increasing its serum levels (Aoyagi et al. 2003). Previous studies showed that etanercept is a nephroprotective drug by its antioxidant properties, decreasing serum creatinine, and proteinuria (Kim et al. 2013). It has been reported that simultaneous administration of $CdCl_2$ and quercetin decreased Cd accumulation in serum, testis, and epididymis (Nna et al. 2017). Quercetin improved renal function in cadmium-exposed rats by lowering oxidative stress in the kidneys, with increase in creatinine, urea, and cadmium clearance (Morales et al. 2006; Renugadevi et al. 2010). By the same mechanism of decreasing oxidative stress, etanercept could improve renal function, decrease proteinuria, and increase

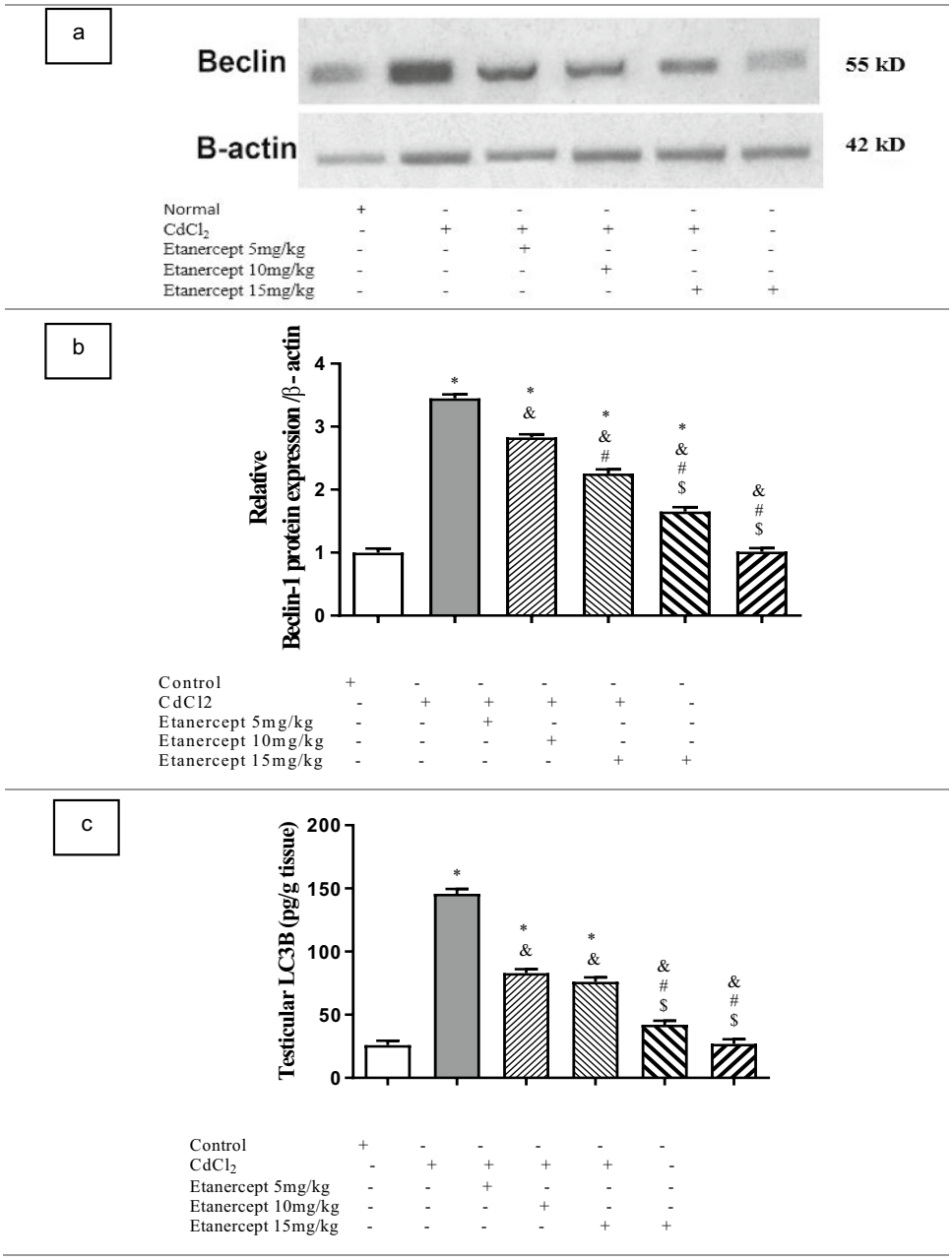

Fig. 5 Effect of etanercept (5 mg/kg, 10 mg/kg, 15 mg/kg) on autophagy biomarkers in experimental groups (a) Western blot analysis showing protein expression of Beclin-1 and β-actin. (b) Testicular protein expression of Beclin-1. (c) Testicular content of LC3-B. Results are expressed as mean ± SD. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$. *Significantly different from control, &significantly different from $CdCl_2$ group, #Significantly different from 5 mg/kg etanercept-treated group, $Significantly different from 10 mg/kg etanercept-treated group(+: present, -: absent).

Cd clearance from the body. In addition, it has been reported that Cd triggers disturbances in the lipid composition so that the macrophages will release TNF-α and increase oxidative stress in organs. In addition, there is a linear relationship between Cd concentration and TNF-α protein levels (Rumahlatu et al. 2019). Thus, it may be considered that the inhibition of TNF-α by etanercept is responsible for the decreased serum cadmium concentration.

Oxidative stress is considered as the main mechanism by which $CdCl_2$ mediates testicular injury. It was observed that $CdCl_2$ increased the MDA content and decreased the GSH, SOD, and CAT contents. These results came in line with previous research, which revealed that Cd can initiate oxidative stress and cause an indirect increase in ROS by binding to the sulfhydryl (-SH) groups of the ROS scavenging proteins and non-protein molecules like GSH (Djuric et al. 2015; Erboga et al. 2016; Nna et al. 2017). Lipid peroxidation is also evidence of oxidative stress induced by Cd exposure (Al Olayan et al. 2020). In addition, $CdCl_2$ interacts with the structure of enzyme protein, inhibiting catalytic activity, and replaces the other divalent cation required for antioxidant enzyme activity (Almeer et al. 2018). Treatment with etanercept reduced the testicular oxidative stress, as evidenced by lower MDA levels and increased GSH, SOD, and CAT activities. A previous study showed that etanercept exerts a protective effect against myocardial ischemia/

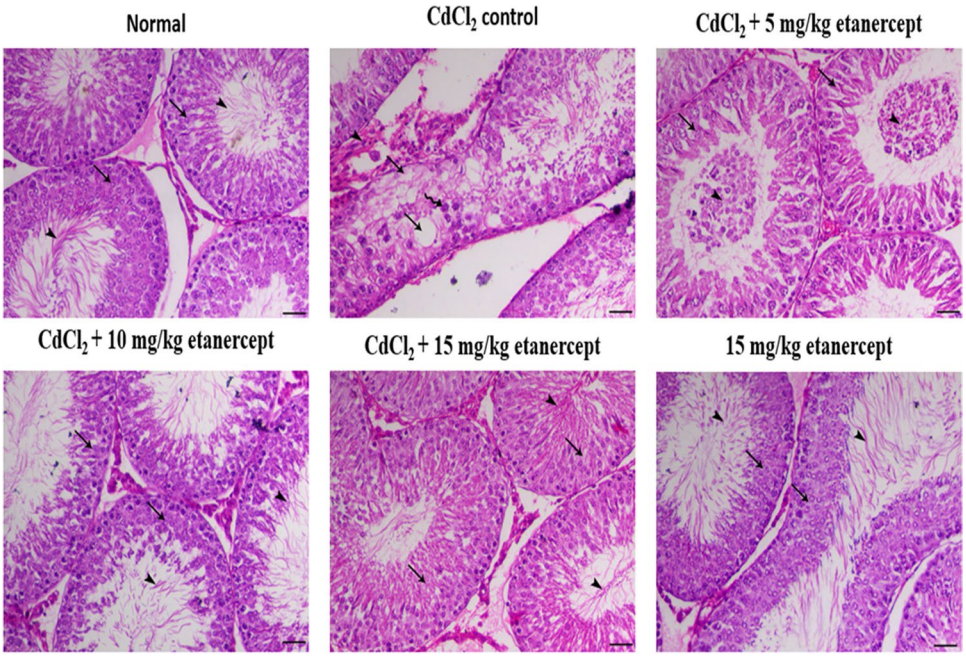

Fig. 6 Effect of etanercept (5,10, 15 mg/kg) assessed by histopathological examination of different experimental groups. All images were captured with magnification of 400 x

reperfusion injury in rats, which could be due to its ability to reduce lipid peroxidation and increase antioxidant enzyme activity (Yang et al. 2014).

Inflammation is a crucial process in Cd-induced testicular damage. $CdCl_2$ increased the testicular TNF-α content and induced the expression of NF-κB and iNOS leading to excessive production of nitric oxide (NO). These results matched with previous experimental studies, which showed that $CdCl_2$ induced the NF-κB signaling pathway by dissociation from its inhibitory IκBα (inhibitory kappa B) as a result of oxidative stress. This signaling promotes the production of proinflammatory cytokines such as TNF-α (Ansari et al. 2017; Habib et al. 2019b; Jiaxin et al. 2020). $CdCl_2$ also triggers cytotoxicity by increasing the expression of iNOS. The activation of macrophages and other leukocytes by a high amount of NO intensifies inflammation, thus contributing to testicular disease (Elmallah et al. 2017). However, treatment with etanercept dose-dependently decreased the testicular TNF-α content together with inhibiting the expression of NF-κB and iNOS. This result can be explained by the following sequence: etanercept is a competitive inhibitor of TNF-α, which regulates other inflammatory mediators such as NF-κB and iNOS (Goffe and Cather 2003; Yang et al. 2014). So, the inhibition of TNF-α by etanercept is followed by inhibition of NF-κB and iNOS.

Testosterone is required for normal spermatogenesis and the maintenance of normal seminiferous tubule structure (Sadik 2008). Moreover, male sex organ mass loss is considered as the main sign of androgenic status alterations. $CdCl_2$ administration decreased the levels of serum testosterone. It has been demonstrated that the effects of $CdCl_2$ on Leydig cells and the hypothalamic–pituitary–testicular axis probably cause endocrine disruption (Siu et al. 2009). $CdCl_2$ accumulates in the hypothalamus and pituitary gland, stimulating oxidative stress, and adversely affects the hormonal secretions of these organs (Nna et al. 2017). Moreover, inflammatory stimuli, in particular TNF-α, enhance the iNOS expression, which catalyzes the synthesis of a large quantity of NO. Then, NO interacts with superoxide anion to form peroxynitrite radicals, which cause cell damage and steroidogenesis suppression in Leydig cells and the adrenal cortex (Cameron and Hinson, 1993; Sokanovic et al. 2013).

In the current study, etanercept treatment increased the serum testosterone levels in a dose-dependent manner. This effect could be due to its antioxidant properties, which protect Leydig cells against $CdCl_2$-induced oxidative stress and improve testicular steroidogenesis (Arafa et al. 2014). These findings are in parallel with studies showing that etanercept may have a potential impact on the management of TNF-α-induced infertility (Pascarelli et al. 2017). TNF-α has been shown to impair gonadal activities, specifically gene expressions of steroidogenic enzymes and steroidogenesis in Leydig cells (Hong et al. 2004; Sadasivam et al. 2015). In addition, etanercept inhibits the effect of the aromatase enzyme "the enzyme that converts testosterone to estrogen" because proinflammatory cytokines, such as TNF-α, activate the aromatase enzyme (Atzeni et al. 2008; Cutolo et al. 2006).

Cadmium exposure induces DNA damage of testicular cells. Apoptosis is a process in which cells with substantial DNA damage die (Han et al. 2020). Excessive apoptosis of testicular germ cells is caused by direct alterations of

hormonal support from Leydig cells (Fouad et al. 2009). Low intratesticular testosterone levels in response to Cd-induced toxicity may cause germ cell detachment from the seminiferous epithelium and apoptosis of germ cells because, in seminiferous tubules, testosterone is required for the attachment of distinct generations of germ cells (Sadik 2008). This coincides with the result obtained in the present study showing the upregulation of caspase-3 expression by $CdCl_2$.

Oxidative stress stimulates the mitochondrial pathway of apoptosis by Cd exposure. ROS generation and accumulation cause Ca^{+2} channel dysregulation, which alters mitochondrial membrane permeability and cytochrome c release into the cytoplasm. In the presence of adenosine triphosphate (ATP), cytochrome c can activate apoptotic protease activating factor-1 (Apaf-1) that results in the activation of caspase-9. Activated caspase-9 cleaves caspase-3 zymogen that produces activated caspase-3, which causes DNA fragmentation (Kassab et al. 2020; Knight et al. 2019; Ye et al. 2007). Inflammation also triggers apoptosis by stimulating death receptor signaling pathways. TNF-α binds to TNFR1, the death receptor (DR), on the plasma membrane. Activation of DR can recruit and activate caspase-8. Caspase-8 recruitment stimulates caspase-3. $CdCl_2$ was found to cause an upregulation of DR expression (Jiaxin et al. 2020). Furthermore, NF-κB activation induces the expression of apoptotic markers (Behl et al. 2008; Ye et al. 2019).

Etanercept administration downregulated the expression of caspase-3 in the testicular tissue. TNF-α was found to change the expression of vascular adhesion molecules, allowing lymphocytes and macrophages to reach the target site, activate the inflammation, and trigger apoptosis by the release of cytotoxic substances. A study demonstrated that etanercept could preserve the retina of diabetic rats by reducing the leakage of retina and apoptosis of retinal cells (Ye et al. 2019). The anti-apoptotic effect of etanercept may be due to its antioxidant and anti-inflammatory properties (Yildirim et al. 2016).

Simultaneously, strong autophagy induces cell death and apoptosis, whereas weak autophagy prevents apoptosis and maintains cells alive (Gump and Thorburn 2011). The link between autophagy and apoptosis is still unclear because the 2 processes occur independently and may either induce or oppose one other (Mi et al. 2016). Autophagy is involved in a variety of physiological and pathological processes, including cell survival, cell death, cell metabolism, and immunity (Chuang et al. 2014; Circu and Aw 2012). Under stress conditions, autophagy plays a vital role in preventing cell death by removing harmful particles and protein aggregates. However, the protective effects of autophagy on the body are limited. When the dosage and exposure period of cadmium surpass the safety threshold, it can induce irreversible cell damage by causing autophagic death (Wang et al. 2017b).

In the present study, $CdCl_2$ exposure increased the Beclin-1 protein expression and testicular content of LC3B. These results were supported by previous experimental research (Wang et al. 2017a). Cadmium prevents the synthesis of functional metallothionein, which primarily reduces inflammatory responses. Therefore, increasing levels of inflammatory cytokines, such as TNF-α, caused by cadmium absorption lead to an increase in the number of cells undergoing autophagy (Inoue, 2013; Lee et al. 2015). Moreover, cadmium stimulates the endoplasmic reticulum, causing calcium to leak out and cause autophagic cell death (Kalogeris et al. 2012). Cd also harms cells by prohibiting cell communication via gap junctions. This impact can be amplified by autophagy, resulting in significant harm (Zou et al. 2015). Etanercept treatment, especially at a high dose, reduced the expression of Beclin-1 and LC3B. These results may be attributed to the effect of TNF-α in autophagy induction (Yuan et al. 2018). TNF-α promotes hepatocyte apoptosis and autophagy in humans, according to an earlier study (Ezquerro et al. 2019). Therefore, etanercept could inhibit the overactivity of autophagy induced by cadmium exposure.

Histopathological examination showed that $Cdcl_2$ led to severe testicular degeneration within the spermatogenic cell layers, hyperplasia of Leydig cells within edematous as well as atypia in some spermatogenic cells. The presence of atypical germ cells is the characteristic finding of testicular damage (Michalova et al. 2020). In contrast, etanercept treatment dose-dependently led to a remarkable decrease in the degenerative changes within the spermatogenic cell layers and interstitial cell proliferation and an increase in spermatogenesis. This result supports that etanercept may have a potential impact on the management of TNF-α induced infertility (Pascarelli et al. 2017).

Conclusion

Current data demonstrated a new mechanistic pathway by which etanercept alleviated Cd-induced testicular damage. The observed improvement in testicular function and physiology was achieved through a variety of mechanisms including reducing oxidative stress, improving the antioxidant activity, inhibition of pro-inflammatory cytokines (TNF-α and i NOS), suppression of autophagy markers (Beclin-1 and LC3B) and testicular damage-induced apoptosis. It is worth noting that anti-inflammatory, antioxidant, and anti-apoptotic properties of etanercept are dose-dependent. More investigations are needed for explaining the molecular mechanisms of etanercept protective effect.

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Declarations

Ethics approval and consent to participate The study protocol has been approved by the research ethics committee, Faculty of Pharmacy, Suez Canal University (Ismailia, Egypt) following the Canadian Council on Animal Care Guidelines (License number 201911MA3).

Availability of data and materials All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declaration of competing interest No conflict of interest to be declared.

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