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Original article

Samarcandin protects against testicular ischemia/reperfusion injury in rats via activation of Nrf2/HO-1-mediated antioxidant responses

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ABSTRACT

The purpose of this study was to evaluate the effectiveness of samarcandin (SMR) in preventing testicular injury caused by ischemia/reperfusion (I/R) in rats. Rats were divided into 4 groups at random: the sham group, the T/D control group (CONT), the T/D group receiving SMR treatment at 10 mg/kg (SMR-10), and the T/D group receiving SMR treatment at 20 mg/kg (SMR-20). When compared to the CONT group, SMR improved the oxidant/antioxidant balance by reducing malondialdehyde (MDA), nitric oxide (NOx), and increasing reduced glutathione (GSH), gluta-thione peroxide (GSH-Px), and superoxide dismutase (SOD). Moreover, SMR increased the levels of the steroid hormones' testosterone (TST), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in the blood as well as controlled the inflammatory mediators; interleukin-6 (IL6), tumor necrosis factor alpha (TNF-α), and nuclear factor κB (NF-κB). Nevertheless, SMR-treated animals showed a considerable downregulation of the apoptotic marker caspase-3. The T/D-induced histopathological changes were reduced and Proliferating Cell Nuclear Antigen (PCNA) protein expression was enhanced by SMR. These effects are associated with upregulation of testicular (Nuclear factor erythroid 2-related factor 2 (Nrf2), Heme oxygenase-1 (HO-1), and downregulation of NF-κB mRNA expression levels. These findings suggest that SMR may be able to prevent T/D-induced testis damage by mainly regulating the expression of Nrf2 and NF-B, which seems to mediate its promising antioxidant, anti-inflammatory and antiapoptotic effects seen in this study.

1. Introduction

Testicular torsion is an urgent urologic condition. This disorder may cause ischemia damage and testis loss if it is not diagnosed in time (Pogorelić et al., 2013). It is widely accepted that viability is not possible after torching of testicles for more than six hours. There is a strong probability of preserving the damaged testicle if treated within 6 h of the onset of injury since 90–100% of testicles can be saved. However, 20–50% of testicles can be saved if treated within 6–12 h; and only 0–10% of testicles can be saved if treated within 12–24 h (Meštrović et al., 2017).

Ischemia-reperfusion (I/R) injury arises after an incident of acute ischemia, when blood flow is restored. The testis appears to be injured by I/R as the primary pathophysiology of testicular torsion/detorsion (T/D). Testicular necrosis during testicular torsion is caused by two different types of injuries: the first is related to ischemia injury during torsion, and the second is owed to reperfusion injury during detorsion. Damage to metabolically active tissues results from the first disruption of the blood flow to the tissue

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in ischemia injury. However, restoring of blood flow to the tissues triggers a paradoxical chain of events that results in further cellular and tissue damage. These processes include the formation of free radicals, intracellular calcium excess, and lipid peroxidation, proinflammatory cytokines and adhesion molecules, apoptosis, anoxia, and changes in microvascular blood flow, resulting in cell damage via which eventually induce testicular shrinkage (Meštrović et al., 2017; Celik et al., 2016; Arena et al., 2017). Moreover, in testicular I/R, active neutrophils have been reported to adhere to microvascular endothelium and subsequently migrate and secrete myeloperoxidase enzyme, which catalyzes the production of ROS (Arena et al., 2017).

Damage from oxidative stress affects connective tissue macromolecules, proteins, nucleic acids, lipids, lipoproteins, and fatty acids (Anwar et al., 2020). ROS overgeneration is thought to play a critical role in I/R injury. Tissues are defended against ROS and oxidative damage by the enzymatic antioxidant defense systems, which include glutathione peroxidase (GPx) and superoxide dismutase (SOD). Malondialdehyde (MDA), a steady byproduct of lipid peroxidation and an indirect biomarker of ROS, is frequently utilized (Lee et al., 2012).

Nrf2 (nuclear factor erythroid 2 related factor 2) and its downstream effector HO-1 (Heme oxygenase-1) are essential for normal spermatogenesis and sperm functions, Since they are the primary regulators of the cellular defense against oxidative stress. The transcription factor nuclear factor-B (NF-B), on the other hand, is involved in the control of inflammatory-immune responses that are linked to the production of cytokines and ROS generation, which furthers testicular dysfunction (Dutta et al., 2021; Rotimi et al, 2022). Therefore, activating Nrf2 and suppressing NF-B may be interesting therapeutic targets for testicular dysfunction.

A number of scientific research concerned with studying the use of natural products in pharmaceutical preparations and their biological activities are quickly growing. The majority of recent research on the biological effects of natural products is focusing on different plant species, especially those exhibiting anticancer, immunostimulant, anti-inflammatory, antioxidant, neuroprotective, and hepatoprotective properties. Using natural products is aiming at decreasing the dose and risk factors of using conventional treatments (Anwar et al., 2020, Ekiert and Szopa, 2020). Sesquiterpene-coumarins are a large group of natural compounds considered as biomarkers in *Ferula* species. Many coumarin derivatives are widely distributed in different plants, have been shown antioxidant and free radical scavenging activities. For instance, a natural coumarin derivative esculetin, revealed neuroprotection on cerebral ischemia/reperfusion injury in mice. On the other hand, umbelliferone; 7-hydroxycoumarin has displayed protective effects against testicular ischemia/reperfusion injury. Likewise, another natural coumarin derivative, osthole reduced oxidative damage, suppressed the inflammatory process, prevented apoptosis, and reduced cell damage in testicular ischemia/reperfusion in rats (Wang et al., 2012; Mahmoud, 2016; Kocaman et al., 2022).

Samarcandin (SMR), a natural sesquiterpene-coumarin, was isolated among other related compounds from *Ferula* species, such as *F. assa-foetida* and *Ferula drudeana* (Abd El-Razek et al., 2001, Ghoran et al., 2016). Plants belonging to the genus *Ferula* are traditionally used to treat various diseases such as asthma, epilepsy, stomachache, flatulence, intestinal parasites, weak digestion and influenza. Moreover, previous studies have shown that the oleo-gum-resin of *Ferula* species has several biological effects such as antioxidant, anticonvulsant, antibacterial, chemopreventive, antidiabetic, antispasmodic, as well as hypotensive (Ghoran et al., 2016). There are numerous studies on chemical compositions and potential of antioxidant activities of Ferula genus essential oils and extracts (Lahazi et al., 2015). *Ferula* is rich in phenylpropanoid ferulic acid, coumarin, sesquiterpene coumarin, and other terpene lactones, most of which have potent antioxidant activities (Zhang et al., 2015). The study conducted by Aydogan, et al. (2020) has established that *F. huber-morathii* possesses potent aphrodisiac activity. Further, the findings of Alqarni et al. (2020) (Alqarni et al, 2020) conducted biologically guided phytochemical study to identify the aphrodisiac compounds in *F. drudeana*. The study provides experimental evidence that the total extract, the chloroform fraction as well as pure isolates including SMR possess promising aphrodisiac activity by enhancing the sexual behavior of male rats. This study also demonstrated that SMR has spaces configuration similar to that of the standard drug sildenafil (Alqarni et al, 2020).

In the present study, we designed an experiment to explore whether SMR could protect against testicular injury induced by torsion/detorsion of testis in rats, and to determine whether this effect is associated with Nrf2, HO-1 and NF-κB.

2. Materials and methods

2.1. Plant material and SMR identification

Plant materials were described earlier. SMR (Fig. 1) has previously been chromatographically separated and spectroscopically identified (Alqarni et al, 2020). Spectral data for SMR are presented in the supporting information.

2.2. Animals

Following approval of the animal experimentation procedure by the Ethical Committee for Medical Research, National Research Center, Egypt, twenty-four adults male Wistar rats weighing 180–200 g were procured from the Animal House Facility of the National Research Centre, Egypt. Animals were kept in standard cages, under pathogen-free conditions, and kept under controlled room temperature and under normal dark –light cycles. Rats were provided with standard food and water ad libitum. Before starting the experimental protocol, animals were given a week to acclimate to these settings. Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC). Handling, use, and euthanasia of rats were complied with the Institutional Animal Care and Use Committee at National research Centre- Egypt (approval No. 12311122022).

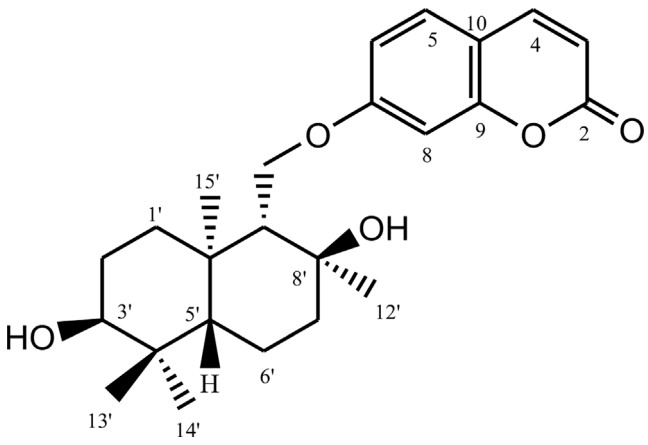

Fig. 1. Chemical structure of SMR.

2.3. Induction of testicular ischemia

Testicular ischemia in rats was induced by the torsion and detorsion (T/D) of the right testis for 2 h, as described earlier (Turner et al., 1997; Ozbal et al, 2012). General anaesthesia was established prior to surgical intervention by intraperitoneal (ip) injection of xylazine hydrochloride (10 mg/kg) and ketamine (50 mg/kg).

The rats were placed on a homeothermic table to maintain core body temperature at 37 °C. All procedures were performed under sterile conditions. After anesthesia, a vertical paramedian incision of the scrotum was made and then the right testis and its spermatic cord were exposed. A unilateral testicular torsion was created by rotating the right testicle with its cord 720° in a clockwise direction. The testicle was fixed to the scrotum's internal surface with 6/0 propylene suture. At 120 min after torsion, reperfusion was achieved by detorsion of the right testis to its previous normal position and the testis was placed back into the scrotum.

2.4. Experimental design

Twenty-four adult male rats were randomly divided into 4 equal groups.

1. Sham group: Rats underwent identical surgical procedure, except for T/D of the right testis and were treated with the vehicle (saline).
2. Control group (CONT): Rats subjected to T/D of the right testis and treated with the vehicle.
3. SMR-10 group: Rats subjected to T/D of the right testis and treated with SMR at 10 mg/kg (Alqarni et al, 2020).
4. SMR-20 group: Rats subjected to T/D of the right testis and treated with SMR at 20 mg/kg.

The vehicle (saline) and SMR were administered intraperitonially, 60 min before the beginning of surgery, and 30 min before testicular detorsion (reperfusion).

After 24 h of reperfusion, blood samples were collected from the *retro*-orbital venous plexus. Sera were separated by centrifugation for 1,538 g for 10 min. Then, rats were euthanized by decapitation; the right testicles were separated and divided into two sections. One section was stored at −80 °C for further biochemical analysis and the other section for histopathological and immunohistochemical examinations.

2.5. Estimation of sex hormones in serum

ELISA kits (BioVision, Inc., MA, USA), were used for the quantitative protein detection of testosterone (TST, Catalog# K7621-100), follicle-stimulating hormone (FSH, Catalog# K7425-100), and luteinizing Hormone (LH, Catalog# K7426-100), following the directions provided by the manufacturer.

2.6. Preparation of tissue homogenates

The right testes were homogenized (MPW-120; Medical Instruments) in 10% (w/v) ice-cold phosphate buffer. Then, the homogenate was centrifuged using a cooling centrifuge (2 k15; Sigma/Laborzentrifugen) for 5 min. The resulting supernatants were collected and stored at −80 °C till analysis.

2.6.1. Assessment of oxidative stress markers in testicular homogenate

The levels of malondialdehyde (MDA) (Ruiz-Larrea et al., 1994), reduced glutathione (GSH) (Ellman, 1959), glutathione peroxide (GSH-Px) (Mohandas et al., 1984), superoxide dismutase (SOD) (Sun and Zigman, 1978), and nitric oxide (NOx) (Miranda et al., 2001) were determined by utilizing the assay kits acquired from Cayman Chemical Company (Michigan, USA) in accordance with the procedures outlined in guidelines provided by the manufacturer.

2.6.2. Estimation of inflammatory biomarkers in testicular homogenate

Inflammatory cytokines (IL6, NF-κB and TNF-α) were determined in the right testis homogenate using commercially available ELISA kits according to the manufacturer's instructions (Immuno-Biological Laboratories, Inc., MN, USA).

2.7. Gene expression analyses

The mRNA expression levels of Nrf2, HO-1, and NF-κB1 genes were monitored by the quantitative real-time PCR (qRT-PCR). Briefly, the total testicular RNA was isolated and purified from the entire right testes using TRIzol reagent (Invitrogen, CA, USA) following the instructions provided. The first strand DNA synthesis was performed by using RevertAid™ First Strand cDNA Synthesis kit (Thermo Scientific). Real-time PCR was done using SYBR green mix kit with the primers sets specific for target genes and ß. actin as an internal reference gene for data normalization (Table 1). Primers were based on our previously published sequences (El Badawy et al., 2021; Alsharif et al., 2022; Ogaly et al., 2021; Ogaly et al., 2022). These primers were designed using Primer-BLAST (NCBI) for amplification of the full coding sequence of Nrf2, HO-1, NF-κB1, and ß. actin using the predicted rat mRNA sequences in NCBI (GenBank accession: XM_006234398.3, NM_012580.2, XM_006233360.3, and NM_031144.3, respectively). PCR thermal program was run with 40 cycles of 95 °C for 30 sec, followed by 58 °C for 30 sec and 72 °C for 25 sec. Using the comparative Ct method, the expression of each gene was calculated and plotted relative to negative control which was set at a value of 1.

2.8. Histopathological examination of the testis

Right testis was prepared and stained for light microscopy by fixing in 10% neutral-buffered formalin for 24 h, then washed with tap water. Serial alcohol dilutions were used to dehydrate the samples, and then they were cleared in xylene and embedded in paraffin wax in a hot air oven for 6 h at 56 °C. Paraffin wax tissue blocks were sectioned by using microtome at 4–6 μm thickness. Then, sections were collected on glass slides and deparaffinized. They were stained for routine histological examination using Hematoxylin and Eosin stain (Suvarna et al., 2018).

2.9. Immunohistochemical examination of caspase-3 and proliferating cell nuclear antigen (PCNA)

Testicular specimens of all groups underwent deparaffinization and then rehydrated. The antigen retrieval was carried out using 10 mM citrate buffer, pH 6.0 in line with methods described by Rabbit polyclonal anti-Caspase-3 Antibody (Elabscience, Cat# E-AB-63602, Dil.1:50), and Rabbit polyclonal anti-PCNA antibody (CYANAGEN, Cat. No. ABP141, Dil 1:50), was applied to tissue samples at 1:50 and incubated overnight in a humid environment. Furthermore, for the slides used as negative controls, the primary antisera were eliminated along with 1 mg/mL BSA (Sigma). To get rid of unconjugated antibodies, the tissue slices were extensively washed in Tris buffered saline. The slices were then treated with a goat anti-rabbit and mouse antibody that had been biotinylated (using Vectastain ABC-HRP kit, Vector laboratories) for 10 min then washed thoroughly with TBS and DAB (3,3di-aminobenzidine) (produced by Sigma) was added to the sections.

Table 1
Primer sequences and accession number of the target genes.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Reference
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC	El Badawy et al, 2021
HO-1	ACAGGGTGACAGAAGAGGCTAA	CTGTGAGGGACTCTGGTCTTTG	Alsharif et al., 2022
NF-κB1	CTGGCAGCTCTTCTCAAAGC	CCAGGTCATAGAGAGGCTCAA	Ogaly et al, 2021
ß. actin	ATGGTGGGTATGGGTCAG	CAATGCCGTGTTCAATGG	Ogaly et al, 2022

The sections were then mounted with DPX and counterstained with Mayer's hematoxylin. ImageJ was used to analyze the captured images. In 10 microscopic fields/group, the proportion of immune-staining cells that were positive was determined.

2.10. Statistical analysis

All the attained data were shown as mean ± SEM. Utilizing Graph Prism®, statistical analysis was conducted via one-way analysis of variance (one-way ANOVA), proceeded by Tukey's test to ascertain intergroup variability. A probability threshold of less than 0.05 was regarded as acceptable for statistical significance.

3. Results

3.1. Serum sex hormones levels

Serum levels of testosterone (TST), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) significantly decreased in CONT group compared to sham control ($p \leq 0.05$). Serum testosterone TST, FSH, and LH levels significantly increased in rats treated with SMR-10 and SMR-20 as compared to the CONT rats (see Fig. 2).

3.2. Testicular antioxidant markers

The oxidative stress markers; MDA and NOx exhibited significant elevation in the testicular tissues of CONT rats, as compared to sham rats. While, significant reduction in GSH contents and the activities of GSH-Px and SOD was detected in the testicular tissues of CONT rats (Fig. 3). Further, SMR-10 and SMR-20 significantly improved the oxidative status in testicular tissues of rats. GSH contents and the activities of GSH-Px and SOD were significantly increased in SMR-10 and SMR-20 groups as compared to CONT group. Moreover, MDA and NOx were significantly reduced in SMR treated rats as compared to CONT rats.

3.3. Testicular inflammatory markers

As depicted in Fig. 4, the testicular inflammatory biomarkers, TNF-α, NF-κB, and IL-6, were shown to be markedly elevated in CONT rats versus the sham group. However, SMR therapy at both dose levels markedly reduced the levels of the pro-inflammatory markers.

3.4. Effect on Nrf2, HO-1, and NF-κB1 gene expression

As shown in Fig. 5, the mRNA levels of Nrf2, HO-1, and NF-κB were analyzed by qRT-PCR. The mRNA expression levels of Nrf2 and its downstream antioxidant genes were significantly increased in SMR-10 and SMR-20 groups compared with CONT group ($p \leq 0.05$). In addition, higher mRNA expression levels of NF-κB were determined in CONT group compared to sham group ($p \leq 0.05$); Nevertheless, NF-κB expression levels were significantly decreased in SMR-10 and SMR-20 treated groups, compared to CONT group ($p \leq 0.05$).

3.5. Histopathological examination of testis

Testicular T/D resulted in histological changes in terms of disruptions of spermatogonia across the lumen of seminiferous tubules due to edema that led to increase spaces between seminiferous tubules, presence of necrobiotic changes in spermatocytes and pyknosis in spermatogonia nuclei (Fig. 6B). However, treatment with SMR-10, and SMR-20 resulted in improved the seminiferous tubules' structural and cellular architecture as well as the interstitial spaces (Fig. 6C-D). A modified Cosentino scoring was

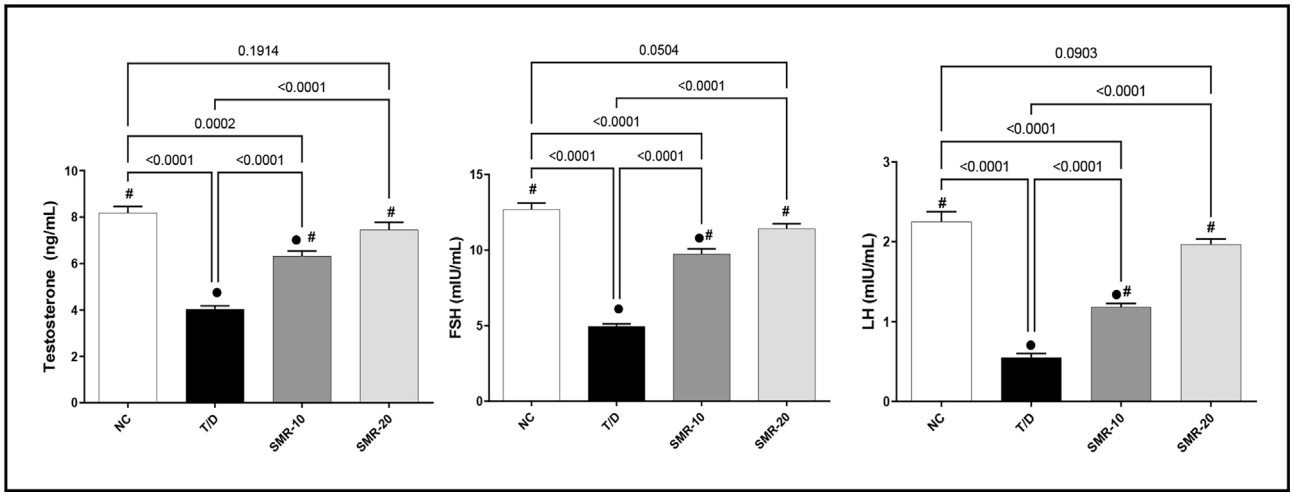

Fig. 2. Effect on serum male hormones. Values are presented as the Mean SEM of six animals from each group. •Significantly different from the values of the negative control rats at $p \leq 0.05$. #Significantly different from the values of CONT rats at $p \leq 0.05$. TST: testosterone; FSH: follicle stimulating hormone; LH: luteinizing hormone.

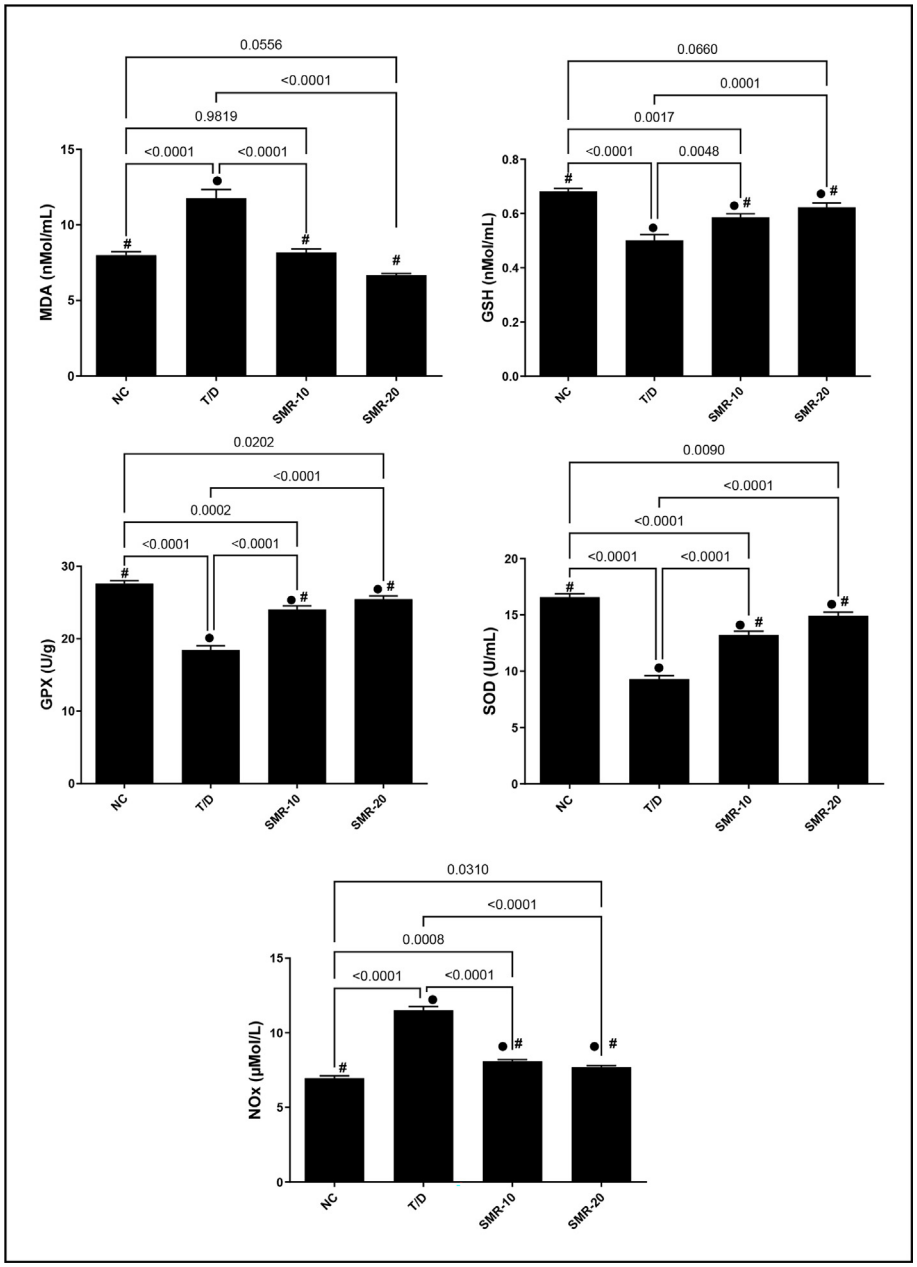

Fig. 3. Effect on testicular antioxidant markers. Values are presented as the Mean SEM of six animals from each group. ● Significantly different from the values of the sham rats at $p \leq 0.05$. # Significantly different from the values of CONT rats at $p \leq 0.05$. MDA: Malondialdehyde; GSH: Reduced glutathione; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; NOx: Nitric oxide.

done to the testicular tissues according to Ufuk et al., (2016), as shown in Fig. 6E.

3.6. Immunehistochemical examination of testis

Decreased expression of Caspase-3 was observed in the testis of sham rats (Fig. 7), while diffuse expression of PCNA was shown as dark brown cytoplasmic and nuclear color in testicular sections of the sham group (Fig. 8). Tissue sections of CONT group revealed increased expression of Caspase-3 immunopositive cells with a marked decrease in PCNA immunopositive cells (Fig. 7-B and Fig. 8-B). A noteworthy deceased expression of Caspase-3 and increased expression of PCNA were observed in SMR -treated groups as shown in Figs. 7 and 8.

4. Discussion

Testicular dysfunction associated with extensive male infertility is one of the serious side effects of testicular torsion that affects all age groups. testicular torsion leads to impairment of testicular perfusion by arterial obstruction, venous congestion, and subsequently ischemic and necrotic alterations (Ozbal et al, 2012). Management of such urological emergency needs immediate detorsion of the twisted testis. Detorsion results in reperfusion of the ischemic tissues, which triggers further biochemical and morphological alterations such as excessive free radical production, lipid peroxidation (LPO), increased intracellular Calcium, ATP depletion, and inflammatory response in the testicular tissues (Akhigbe et al, 2021; Xu et al., 2022).

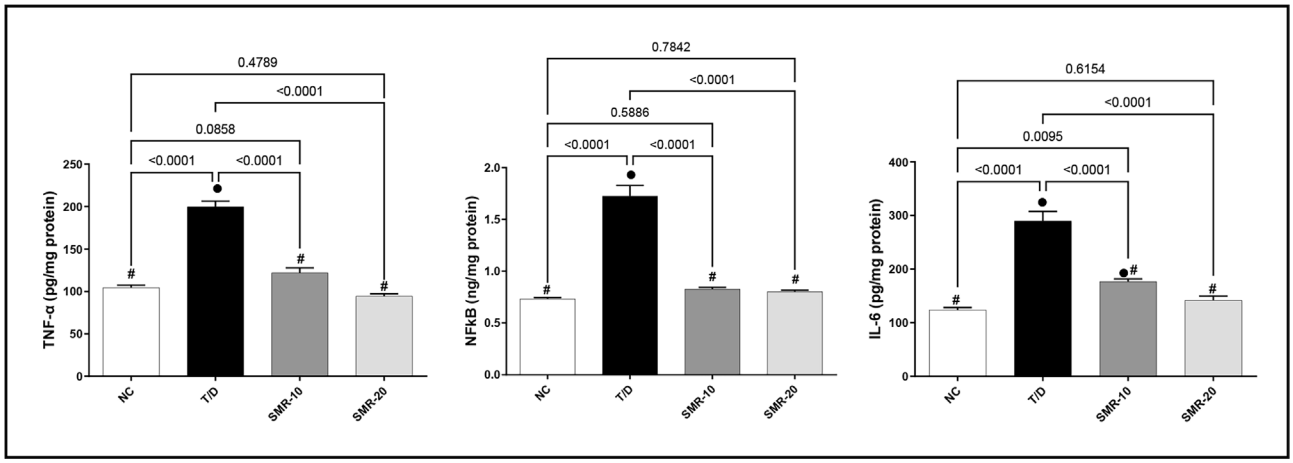

Fig. 4. Effect on testicular inflammatory markers. Values are presented as the Mean SEM of six animals from each group. ● Significantly different from the values of the sham rats at $p \leq 0.05$. # Significantly different from the values of CONT rats at $p \leq 0.05$. TNF-α: Tumor necrosis factor-alpha; NF-κB: Nuclear factor-kappa B; IL-6: Interleukin-6.

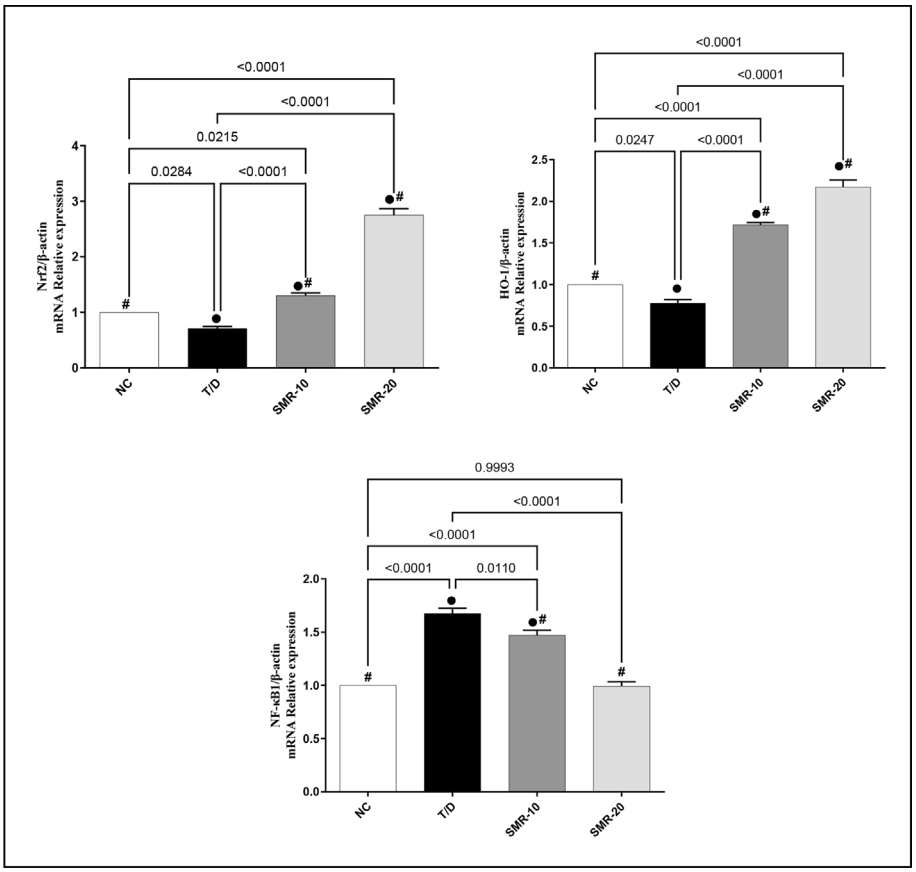

Fig. 5. Effects on testicular Nrf2, HO-1, and NF-κB1 mRNA expression by qRT-PCR. Values are presented as fold changes of normal control value ± SEM after normalization by β-actin. ● Significantly different from the values of NC rats at $p \leq 0.05$. # Significantly different from the values of T/D rats at $p \leq 0.05$.

Pathogenesis of I/R injury is a multifactorial process in which different contributing factors are implicated. However, oxido-inflammatory response has been reported to be a significant factor for T/D testicular damage (Bei et al., 2019). Ischemia brought on by the torsion results in the primary pathophysiological deuteriation of testicular tissues, and the reperfusion-induce release of reactive oxygen species (ROS) including nitric oxide (NO.), superoxide anions (O2.), hydrogen peroxide (H_2O_2.), and hydroxyl radicals (OH.) (Wilhelm Filho et al., 2004). In conjunction, excessive ROS trigger cytokine storm, DNA and cellular apoptotic damage (Afolabi et al. 2022).

Different parts of Ferula have been reputed in the treatment of numerous ailments for instance neurological disorders, inflammatory conditions, digestive disorders, diabetes arthritis, headache, and dizziness. Additionally, Ferula was believed to had aphrodisiac characteristics and enhancement of sexual desire. Moreover, many Ferula-isolated compounds were reported to target several factors of the signal transduction cascade giving rise to antitumor and

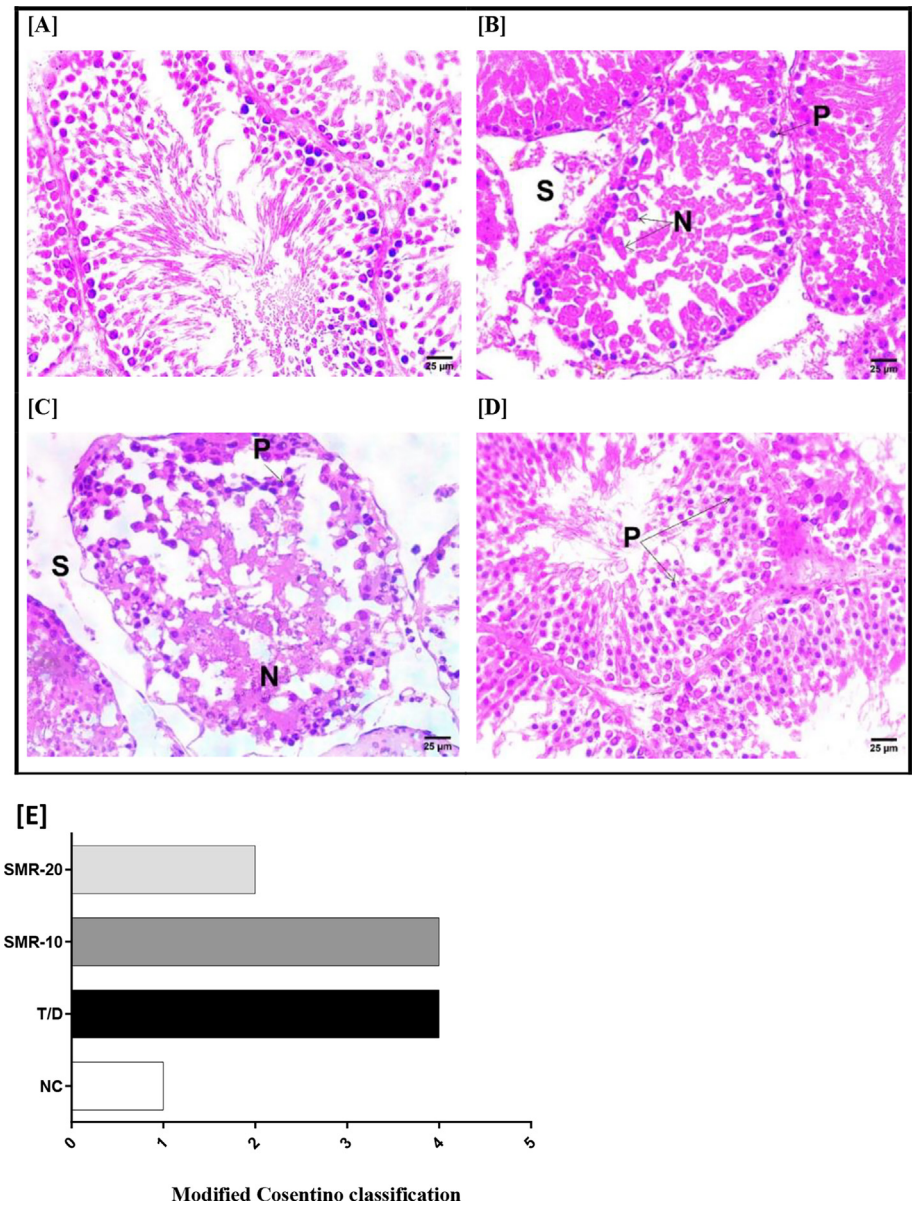

Fig. 6. Effect of SMR on testicular ischemic damage (H& E). Sham group (A) showing normal histological picture of testis; CONT group (B) showing edema led to increase spaces between seminiferous tubules (S), presence of necrobiotic changes in spermatocytes (N) and pyknosis (P) in spermatogonia nuclei; SMR-10 group (C) showing pyknotic nuclei (P) of some spermatocytes while others showing necrobiotic changes (N) with edema leading to increase spacing between seminiferous tubules (S); and SMR-20 group (D): showing pyknotic nuclei of spermatocytes (P). (E): Modified Cosentino scoring.

anti-inflammatory activities (Nazari and Iranshahi, 2011; Yusufoglu et al., 2015).

SMR is a natural sesquiterpene coumarins first isolated from *F. samarcandica* in 1968 (Kir'yalov and Movchan, 1968; Bagirov et al., 1970). In 2016, SMR was isolated from *F. assa-foetida*'s oleo-gum-resin and its structure was confirmed by X-ray crystallography. The cytotoxicity of the compound was explored against AGS and WEHI-164 cancerous cell lines by the MTT assay (Ghoran et al., 2016). Recently, SMR was estimated by HPTLC ecofriendly method in five *Ferula* species: *F. drudeana, F. tenuissima, F. Huber-morathii, F. duranii*, and *F. assa-foetida* (Abdel-Kader et al., 2022). The highest amount of SMR were estimated in *F. drudeana* followed by *F. Huber-morathii*. The structure of SMR was identified via various spectroscopic methods (Supplementary materials) and its potential aphrodisiac activity was demonstrated in male rats supporting the traditional use of genus *Ferula* members (Alqarni et al, 2020).

To our knowledge, the present study is the first to evaluate the therapeutic efficacy of SMR on spermatogenic function after testicular T/D in the rat model. In addition, the involvement of Nrf2 and NF-kB were explored as suggested mechanistic targets.

In the present study, SMR administration exhibited a significant enhancement in the quality of spermatogenesis and boosted the hormonal activities against the damage caused by testicular ischemia reperfusion. Serum TST, FSH, and LH showed increased levels in rats treated with SMR-10 and SMR-20 as compared to the CONT rats. TSH and FSH are the main regulators of spermatogenesis and fertility in males (Zhao et al., 2020).

The main ROS-scavenging enzymes in the male rat reproductive system are GPx and SOD. GPx, is one of the antioxidant enzymes, serves as the first line of defense against oxidative stress in injured testis are by ischemia/reperfusion. Additionally, some experimen-

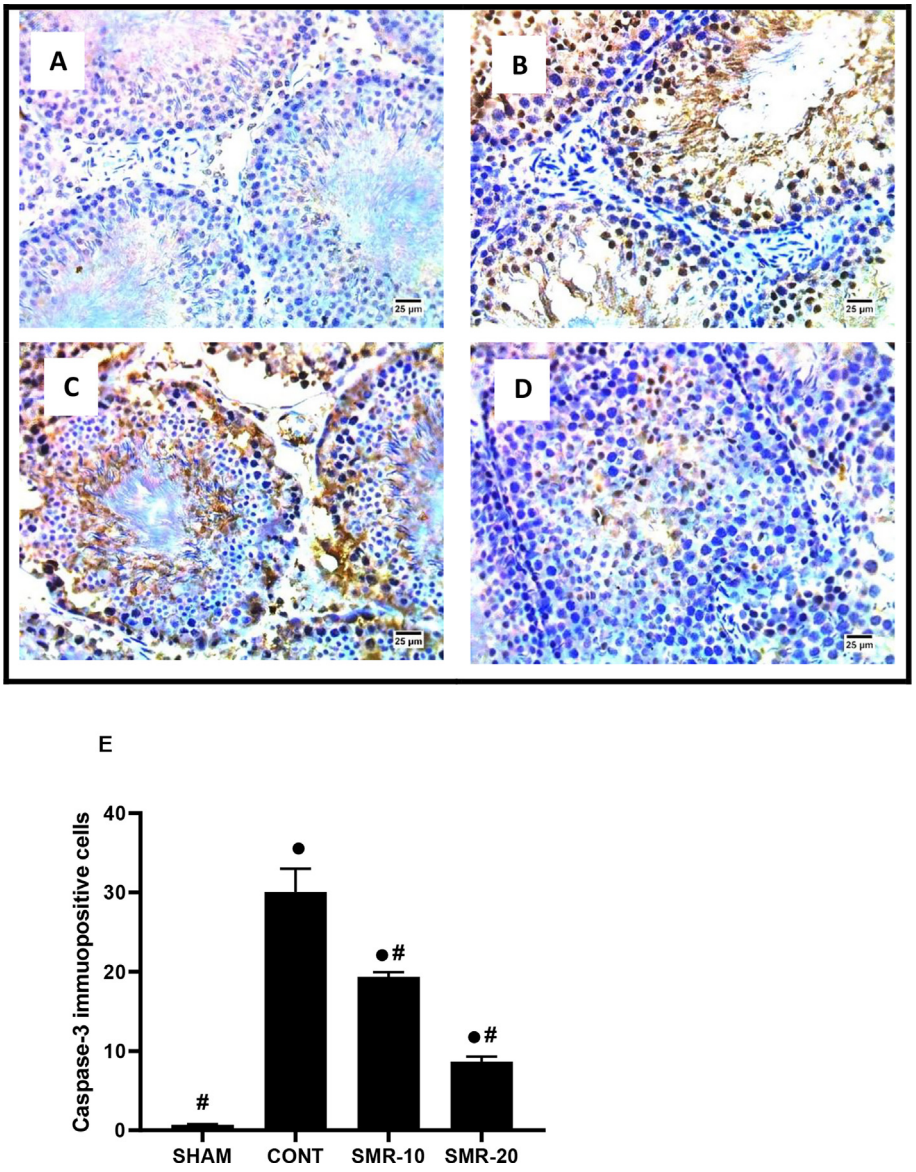

Fig. 7. Caspase-3 immunopositive cells are distinguished by their dark brown cytoplasm and nuclei. Sham group (A), CONT group (B), SMR-10 group (C), SMR-20 group (D), The bar chart (E) designates the percentage of Caspase-3 immunopositive cell in testicular section. Each value is represented as mean ± SEM (n = 6). $^{\bullet,\#}$ denotes significant difference from Sham and CONT groups, respectively at $p \leq 0.05$.

tal investigations have demonstrated that using SOD or SOD analogues significantly reduced I/R injury (Sangodele et al., 2021).

ROS-induced chain reactions of lipid peroxidation in the cell membranes culminate in the production of MDA, the primary lipid peroxidation product (Ayala et al., 2014). Furthermore, it was reported that NO derived from iNOS exalted testicular germ cell necrosis in the delayed phase following experimental testicular torsion in rats (Zhang et al., 2013). The oxidative stress markers; MDA and NOx displayed a substantial increase in the testicular tissues of CONT rats, as compared to sham animals. While CONT rats' testicular tissues showed a substantial decline in GSH levels and GPX and SOD activity, denoting declined free radical scavenging ability and increased tissue apoptosis. Through a variety of mechanisms, give rise to excessive ROS generation that can harm sperm cells including motility and morphology. Additionally, the duration and degree of testicular torsion directly correlate with the severity of ischemic tissue damage (Moghimian et al., 2016). Moreover, SMR-10 and SMR-20 significantly increased GSH contents and the activities of GPX and SOD, as compared to CONT rats. Additionally, MDA and NOx levels in SMR-treated rats were considerably lower, as compared to CONT rats. Based on these findings, SMR showed antioxidant properties and protected testicular tissues from the damaging effects of free radicals.

Spermatogenic cell apoptosis constitutes an important factor in regulation testicular germ cell population. The pathological consequences of testicular T/D involve a propagated rate of apoptotic cell death, which may be a result of the amplified generation of ROS. This provokes further testicular dysfunction and male infertility (Xu et al., 2022; Kostakis et al., 2017). This study showed that SMR did not only maintain endogenous antioxidant balance, it also regulated spermatogenic and testicular cell apoptosis by the abated caspase 3 activation; thus, maintaining sperm and testicular cell integrity and improve spermatogenesis. On the other hand, PCNA is a nuclear protein expressed in association with cell proliferation and DNA replication during the cell cycle (Celis et al. 1987). Our findings showed a reduced number of PCNA-positive cells in

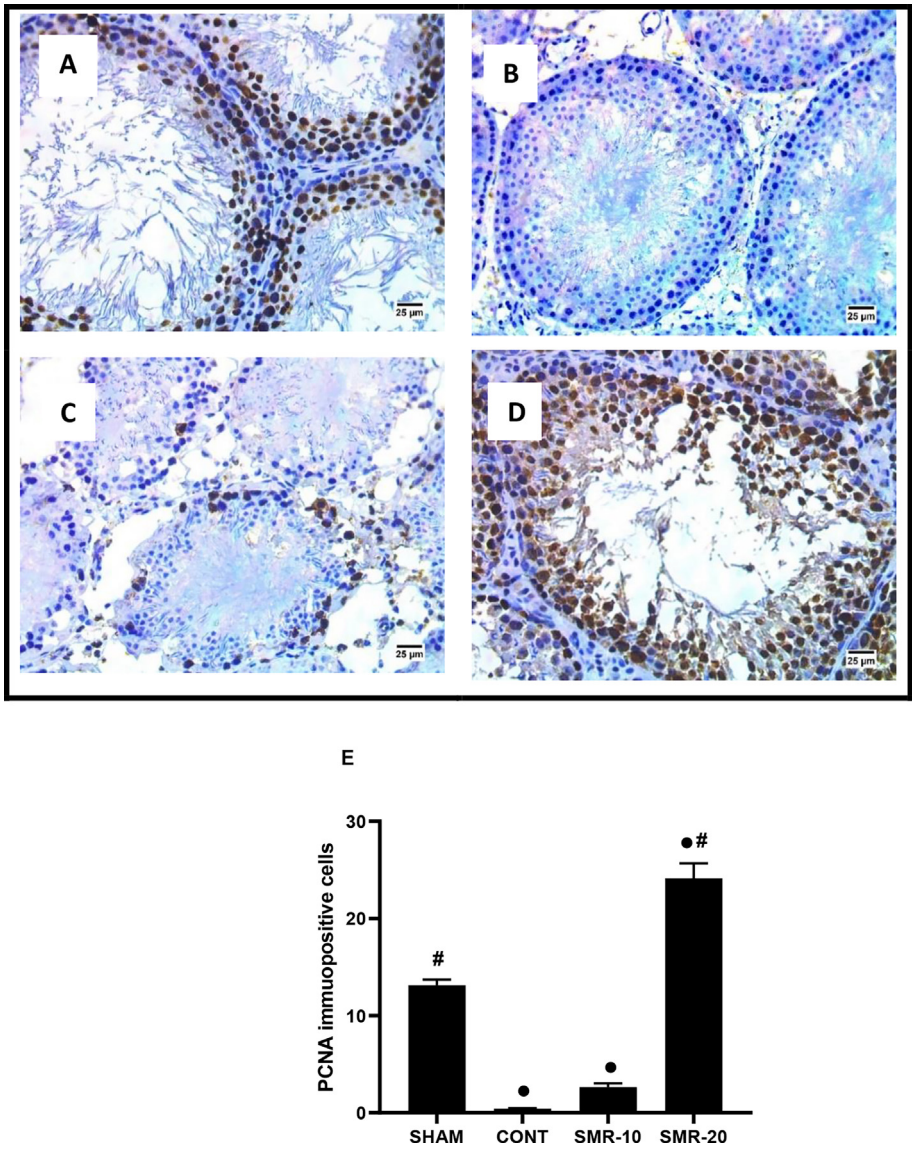

Fig. 8. Cells that are PCNA immunopositive display dark brown cytoplasm and nuclei. Sham group (A), CONT group (B), SMR-10 group (C), SMRR-20 group (D), The bar chart (E) designates the percentage of PCNA immunopositive cell in testicular section. Each value is represented as mean ± SEM (n = 6). $^{\bullet,\#}$ denotes significant difference from Sham and CONT groups, respectively at $p \leq 0.05$.

the CONT group. SMR effectively upregulated PCNA expression in testicular tissues as compared with CONT group indicating the improved spermatogenic and proliferative activities.

Since inflammation is a substantial contributor to testicular I/R injury, T/D has been reported to produce an abundance of pro-inflammatory cytokines and cell adhesion molecules such as IL-1β and TNF-α, as well as NF-kB, which mediate I/R-induced testicular damage (Schindler, 2019). Moreover, these cytokines induce a chemotactic effect on neutrophils in the testis, resulting in ROS overproduction and trigger cytokine storm (Bo et al., 2020). Our findings imply that testicular NF-κB, TNF-α, and IL-6, levels were markedly elevated in CONT rats versus the sham group. Nonetheless, SMR treatment at both dose levels (SMR-10 and SMR-20) markedly reduced NF-κB mRNA and protein levels with decreased levels of TNF-α and IL-6.

Added to that, the transcription factor nuclear factor-B (NF-κB) is a pleiotropic factor involved in the regulation of inflammatory/immune cellular responses that link to cytokine production, inflammation, apoptosis, as well as cell growth and survival. NF-κB is held in the cytoplasm in a "resting" state, associated with inhibitor of κB (IκB) protein. Activating stimuli such as cytokines and excessive ROS trigger signaling cascade that ultimately activates NF-κB through phosphorylation of IκB, which sets a series of ubiquitination and proteasomal degradation. Subsequently, active NF-κB undergoes nuclear translocation where the inflammatory cytokine TNF-α, IL-1β, and IL-6, are upregulated (Qi et al., 2017). Thus, we investigated the possibility that SMR downregulates NF-κB.

The mechanism of NF-κB inhibition of various anti-inflammatory agents may be due to interfering with phosphorylation of IκBα, proteasomal IκB degradation, NF-κB DNA binding activity, and/or NF-κB transactivation. Indirect NF-κB inhibition may be occur through downregulation of NF-κB–targeted genes such as NOS and gelatinase B via COX-2 and PPARγ-dependent mechanisms (Biswas and Bagchi, 2016; Kunnumakkara et al., 2020).

Another transcription factors, such as Nrf2, is a master regulator for the cellular homeostasis in response to oxidative stress and

toxic insults including T/D (Qi et al., 2017). The role of Nrf2 in cellular protection from oxidative stress is to boost the expression of antioxidant and ROS detoxifying gene (Qu et al, 2020). SMR treatment significantly enhanced the expression of Nrf2 and HO-1 expression, compared to CONT group ($p \leq 0.05$), suggesting its protective effects against testicular T/D injury. SMR significantly upregulated Nrf2 expression, and such was correlated with a significant upregulation of its downstream gene HO-1 in testicular issues. Under physiological state, Nrf2 is anchored in the cytosolic portion due to its tight binding with Keap1, resulting in to subsequent Nrf2 ubiquitination and degradation (Ding et al., 2015). Electrophilic antioxidants dissociate Nrf2-Keap1 cytoplasmic complex, allowing the nuclear translocation of Nrf2 where it activates the expression of ARE-driven antioxidant and detoxifying genes (Duan et al., 2017; Zhang et al., 2018). In accordance with these previous studies, our findings indicate that SMR significantly activates Nrf2 expression and subsequently promotes HO-1 expression. Those results demonstrated that SMR mediated its protective potential against oxidative insults through enhancing the Nrf2/HO-1 pathway.

The potential for crosstalk between the two redox-regulated transcription factors Nrf2 and NF-κB has been noted. Impaired Nrf2 signaling is associated with upregulation of NF-κB with the subsequent increase in the inflammatory mediator production. Besides, upregulation of Nrf2 reduces ROS production, interferes with IkB proteasomal degradation and NF-kB nuclear translocation. Furthermore, Nrf2 stimulates HO-1 and phase II enzymes which subsequently block IkB-α degradation of (Peng et al., 2020). On the other hand, activation of NF-κB adversely affects Nrf2 transcription and activity and subsequently dysregulates its downstream antioxidant genes (Ekiner et al., 2022; Gao et al., 2022).

This study has demonstrated that SMR protected the testes of rat against T/D injury by activating Nrf2 and its downstream effector HO-1 as well as downregulating NF-κB, which is crucial for the anti-inflammatory effect against T/D testicular injury. The precise mechanism by which SMR could inhibit NF-κB were not identified in this study. Therefore, further studies to explore interior mechanisms between SMR and NF-κB pathway following are highly encouraged and may be considered in future studies.

As well, the obtained results showed that SMR clearly ameliorated the histopathological damage caused by T/D in the testes. T/D rat group showed disruptions of spermatogonia across the lumen of seminiferous tubules, edema, necrobiotic changes in spermatocytes and pyknosis (Fig. 6B). However, treatment with SMR-10 and SMR-20 enhanced the structural and cellular architecture of the seminiferous tubules as well as their interstitial spaces (Fig. 6C-D). These protective effects SMR may be attributed to its strong antioxidant activity, promoting the production of testosterone by the interstitial Leydig cells, and reducing the apoptosis rate of spermatogenic cells.

5. Conclusions

Summing up, the current study is novel to demonstrate that SMR administration could effectively protect against testicular injury associated with torsion/detorsion in rats. The beneficial effects of SMR might be attributable to blockade of oxidative stress, defeating inflammation, and maintaining testicular integrity and function. The therapeutic potential of SMR against testicular T/D was associated with upregulation of Nrf2/antioxidant redox pathway, and downregulation of NF-κB and caspase 3 apoptotic pathway. The present findings support promising value of SMR in the management of Testicular T/D and other ischemic conditions.

CRediT authorship contribution statement

Maged S. Abdel-Kader: Funding acquisition, Project administration, Resources. **Rehab F. Abdel-Rahman:** Writing – original draft, Visualization, Data curation. **Hassan N. Althurwi:** Visualization, Writing – original draft, Data curation. **Gamal A. Soliman:** Resources, Project administration, Funding acquisition. **Hanan A. Ogaly:** Writing – original draft, Visualization, Data curation. **Faisal F. Albaqami:** Writing – original draft, Visualization, Data curation.

Declaration of Competing Interest

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

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