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Targeting Nrf2/HO-1 Antioxidant Signaling Pathway in the Progression of Chronic Mild Stress and Influences on Testicular Dysfunctions

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Abstract: The nuclear factor erythroid 2-related factor (Nrf2)/hemoxygenase-1 (HO-1) pathway regulates cytoprotective genes coding for antioxidant, anti-inflammatory, and detoxifying proteins. In the current investigation, the significance of Nrf2/HO-1 pathway using dimethyl fumarate (DMF) was explored for its protective effect against testicular dysfunctions provoked by chronic unpredictable mild stress (CUMS) model for 8 weeks in rats. Group I (Control) rats received 0.5% CMC (2.5 ml/kg/day, orally). Group II (DMF) received DMF only (25 mg/kg/day, orally). Group III (Stress) rats were exposed to CUMS alongside a daily dosage of 0.5% CMC. Finally, group IV (DMF + stress) rats were exposed to CUMS alongside a daily dosage of DMF 1 h before the initial daily stress session. Rats were exposed daily to two of the following stressors: 45°cage tilting, food and water deprivation, cold water swimming, tail pinching, physical restraint, moist bedding, overnight lighting, level shaking, and a reversed light/dark cycle. DMF markedly lowered serum corticosterone levels. DMF significantly boosted testicular Nrf2 and HO-1 levels, concurrently with a marked decline in Keap-1 expression. DMF suppressed testicular NF-κB and IL-1β levels. DMF also restored a normal Bax/Bcl-2 ratio and significantly reduced testicular Beclin-1 and sequestosome 1/p62 levels. Furthermore, DMF substantially raised testicular levels of StAR and CYP11A1, leading to a raised serum testosterone level. In conclusion, histopathological alterations, oxidative stress, inflammation, and apoptosis of the testicles were all dramatically reduced when the Nrf2/HO-1 pathway was upregulated. Consequently, stimulators of the Nrf2/HO-1 pathway have the potential as a prophylactic approach for CUMS and associated testicular disorders.

Keywords: CUMS; Dimethyl fumarate; Nrf2, HO-1; Oxidative stress; Beclin 1.

1. INTRODUCTION

Stress is a substantial factor that negatively impacts people's quality of life. Stressful life situations exacerbate the psychological burden and hasten the progression of various diseases, including male infertility[1]. Male reproduction is significantly influenced by psychological stress in addition to genetic and environmental variables [2]. In animals' studies, the chronic stress model is highly prevalent. Clinical stress can be modeled using chronic unpredictable mild stress (CUMS), maternal deprivation, and immobilization stress [3-5].

Inflammation, disruptions of the blood-testis barrier (BTB), and malfunctions of the Sertoli and Leydig cells are among the numerous mechanisms that mediate infertility in males. Potential factors that can impair reproductive functions include oxidative stress and disruptions in the hypothalamic-pituitary-testicular and hypothalamic-pituitary-adrenal (HPA) axes [6]. The hypothalamic corticotropin-releasing hormone (CRH) stimulates adrenocorticotropic hormone (ACTH) release into the bloodstream in response to stress, which impedes gonadotropin-releasing hormone (GnRH) and hampers the

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pituitary's capability to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which impair testosterone secretion and sperm quality and quantity [7].

Oxidative stress substantially impacts chronic stress-related testicular membrane and steroidogenesis alterations [8]. Sperm cells are very vulnerable to damage caused by free radicals, which leads to lipid peroxidation. This is because their cell membranes contain a significant amount of polyunsaturated fatty acids [9]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential transcription factor that controls the production of anti-oxidant genes to regulate the cellular defense systems against reactive oxygen species (ROS) [10]. Detaching itself from Kelch-like ECH-associated protein 1 (Keap1), Nrf2 migrates from the cytoplasm to the nucleus when oxidative stress is induced [11]. When Nrf2 is activated, the antioxidant response element (ARE) is overexpressed. This element controls the synthesis of several antioxidant enzymes, including heme oxygenase-1 (HO-1), NADPH: quinone oxidoreductase-1 (NQO-1), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), and glutathione (GSH)-producing enzymes [12, 13].

Enhanced oxidative stress markers, which are associated with decreased Nrf2 activity, stimulate the nuclear factor kappa-B (NF-κB) pathway, which governs proinflammatory cytokines including interleukin-1beta (IL-1β) [14, 15]. Nrf2 suppresses the transcription of numerous proinflammatory cytokines, involving IL-Iβ, tumor necrosis factor-α (TNF-α), interleukin (IL-6), and interleukin (IL-8) [16, 17]. In addition, downregulation of Nrf2 has been demonstrated to stimulate the apoptotic signaling pathway [18].

Chronic stress can lead to a reduction in germ cells and trigger apoptosis in the testes, potentially due to a decline in testosterone levels, which can negatively impact fertility [19, 20]. In addition, autophagy plays a role in preserving cellular balance, marked by the formation of autophagosomes that ingest cellular debris and merge with lysosomes for breakdown [21]. Autophagy has been reported to regulate the survival of germ cells [22]. Oxidative stress has been shown to trigger abnormal autophagy signaling [23]. Aberrant autophagy has been implicated in impaired spermatid differentiation and acrosome formation during spermatogenesis [24], decreased serum testosterone levels [25], damage to the BTB, and apoptosis of Sertoli cells [26, 27].

The fumarate esters, of which dimethyl fumarate (DMF) is a member, have antioxidant and anti-inflammatory effects in different types of cells and tissues [28]. In vitro studies have demonstrated that DMF and its principal metabolite, monomethyl fumarate (MMF), enhance neuronal survival under oxidative stress [29]. Furthermore, DMF has already been approved for clinical application in the management of psoriasis [30] and multiple sclerosis [31]. DMF promotes Nrf2 activation 32 and suppresses the expression of NF-κB in neuronal cells 33, belaying its potential anti-inflammatory effect. Therefore, this study set out to assess the effect of the antioxidant Nrf2/HO-1 signalling pathway, using DMF, on testicular dysfunctions induced by CUMS in rats and the underlying inflammatory and apoptotic cascades.

2. METHODS

2.1. Drugs and chemicals

The St. Louis, MO, USA-based Sigma Chemical Company reached out to acquire DMF. We accomplished oral administration by mixing DMF with 0.5% carboxymethylcellulose (CMC). The chemicals used were all of the highest analytical quality.

2.2. Animals

The Egyptian Organization for Vaccines and Biological Products in Cairo, Egypt, supplied the male Wistar rats, which weighed 200-220 g and were 6-8 weeks old. The rats were kept in plastic cages that followed typical laboratory protocols, such as a 12-hour light-dark cycle, a relative humidity of 55%, and a temperature of 25°C ± 1. The rats were provided with pellet food and water *ad libitum*. A minimum of one week was allotted for the rats to acclimate to the laboratory environment before the commencement of the experiments. The tests followed the rules of the United Kingdom's Animals (Scientific Procedures) Act, 1986, and were approved by the Research Ethics Committee of the Faculty of Pharmacy (Girls) at Al-Azhar University in Cairo, Egypt (Permit number: 462).

2.3. CUMS procedures

The CUMS procedures were implemented as previously delineated [34-36] by subjecting rats to two of the subsequent stressors: 45°cage tilting for 23 hours, 24 hours of fasting without food or water, swimming for 5 minutes at 4°C, 1 minute of pinching the tail (one centimeter from the tip), 4 hours of physical restraint, 20 hours of wet bedding, 14 hours of overnight lighting, 10 minutes of level shaking, and 24 hours of inverting the light/dark cycle. For eight consecutive weeks, the stressors were applied daily in a randomized sequence, ensuring that no repeat of a particular stressor occurred twice in consecutive days. Throughout the investigation,

control rats were housed in groups and were not exposed to any of the aforementioned stressors.

2.4. Experimental design

Four groups of rats were allocated at random. Group I (Control) rats received oral administration of 0.5% CMC (2.5 ml/kg/day) for eight weeks, while the rats of group II (DMF) received DMF only (25 mg/kg/day, per oral) [37] for 8 weeks. Group III (Stress) rats were exposed to CUMS alongside a daily dosage of 0.5% CMC. Finally, group IV (DMF + stress) rats were exposed to CUMS as in group III, alongside a daily dosage of DMF 1 h prior to the initial stress session. The body weight of each rat was documented at the onset and termination of the experimental duration in order to ascertain the alteration in body weight by employing the subsequent formula: the result of subtracting the initial body weight (at week 0) from the ultimate body weight (at week 8).

2.5. Blood and tissue sampling

Blood samples were drawn from the retroorbital plexus of rats that had been anesthetized with sodium thiopental (50 mg/kg) at the end of the experiment [38]. After collecting blood samples in dry test tubes, serum was extracted by spinning the tubes at 3000 rpm for 15 minutes in order to undertake laboratory analysis. After serum separation, it was frozen at -80 °C. The next step was to sacrifice the rats by decapitation. The testes were swiftly removed from the animal after decapitation, washed in ice-cold isotonic saline, allowed to dry, and then weighed. A testicle was prepared for histological examination by preserving it in 10% neutral buffered formalin. Afterwards, the other testicle was quickly frozen at -80 °C until further examination.

2.6. Determination of testicular reduced GSH, malondialdehyde (MDA) levels, serum catalase activity, and levels of total antioxidant capacity (TAC)

10% homogenate was prepared from a portion of the frozen testes samples by homogenizing them in cold phosphate buffered saline (PBS), pH 7.4, in preparation for the quantification of GSH and MDA. The quantification of GSH and MDA was performed colorimetrically utilizing commercial assay kits obtained from Bio-Diagnostic Co., Giza, Egypt (Cat. No. GR 25 11 and MD 25 29, respectively). Bio-Diagnostic Co., Giza, Egypt supplied the commercial assay kit (Cat. No. CA 25 17) used to measure serum catalase activity. The assessment of serum TAC levels was accomplished employing the Benzie and Strain methodology [39].

2.7. Enzyme-linked immunosorbent assays (ELISA)

The manufacturer-provided directions were followed to determine the serum corticosterone and testosterone concentrations using commercial ELISA kits (Cat. No: SL1204Ra and SL0668Ra, respectively) obtained from Sunlong Biotech Co., Ltd., China. To generate a 10% homogenate, another piece of frozen testes was mixed with protease inhibitors and cold PBS with a pH of 7.4. This homogenate was utilized for ELISA testing of oxidative stress, autophagy, and inflammatory biomarkers. In order to assess oxidative stress markers, a rat Nrf2 ELISA kit and a rat HO-1 ELISA kit were utilized (Sunlong Biotech Co., Ltd., China; Cat. No. SL0985Ra and SL0341Ra, respectively). The contents of NF-κB, IL-1β, B-cell lymphoma 2 (Bcl-2), and Bcl-2-associated X protein (Bax) were evaluated employing rat ELISA kits procured from Sunlong Biotech Co., Ltd., China (Cat. No. SL0537Ra, SL0402Ra, SL0108Ra, and SL0109Ra, respectively), in accordance with the guidelines provided by the kits' manufacturers. Furthermore, we used a rat Bcl-2-interacting protein 1 (Beclin 1) ELISA kit and a rat sequestosome 1/p62 ELISA kit (Sunlong Biotech Co., Ltd., China; Cat. No. SL1041Ra and SL1363Ra, respectively) for the estimation of autophagy.

2.8. Quantitative real-time polymerase chain reaction (qPCR)

Adhering to the instructions provided by the manufacturer, total RNA was extracted from testicular homogenates using the RNeasy Mini Kit (Cat. No. 74104, Qiagen, USA). Gene Quant 1300 from Uppsala, Sweden was used for spectrophotometry in order to measure and verify the amount of RNA that was extracted. Consistent with the guidelines provided by the manufacturer, cDNA for each sample was synthesized using the RevertAid Reverse Transcriptase Kit (Cat. No. EP0441, Thermo Fisher, USA). Quantitative PCR was used in conjunction with the SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Cat. No. 204141, Qiagen, USA) to relative quantify the mRNA level. The primer sequences that were designed according to the PubMed sequences of the following genes are displayed in Table 1: steroidogenic acute regulatory protein (StAR) [40], cytochrome P450 family 11 subfamily A member 1 (CYP11A1) [41], Nrf2 [42], HO-1 [43], Keap-1 [44], and β-actin [45] genes. As a constitutive control for the normalization process, the housekeeping gene β-actin was employed. In order to estimate the relative expression of each gene relative

to the β-actin gene, the $2^{-\Delta\Delta Ct}$ approach was employed [46].

Table 1. Oligonucleotide primer sequence of the genes used in real-time PCR.

Gene	Primer sequence (5'-3')
StAR	F-CGAAACCCTAGTCAGGCACA
	R-TGTCTCACTGTGGTCCAAGC
CYP11A1	F-AGGTGTAGCTCAGGACTT
	R-AGGAGGCTATAAAGGACACC
Nrf2	F-CACATCCAGACAGACACCAGT
	R-CTACAAATGGGAATGTCTCTGC
HO-1	F-GGCTTTAAGCTGGTGATGGC
	R-GGGTTCTGCTTGTTTCGCTC
Keap-1	F-CTGCATCCACCACAGCAGCGT
	R-GTGCAGCACACAGACCCCGGC
β-actin	F-TCCTCCTGAGCGCAAGTACTCT
	R-GCTCAGTAACAGTCCGCCTAGAA

2.9. Morphometry and spermatogenesis score

In each experimental group, the mean diameter of seminiferous tubules (µm) was calculated by using the most circular tubules in each region. The Johnsen score was used to evaluate the process of spermatogenesis [47].

2.10. Histopathological examination

Specimens from all experimental groups were obtained and preserved in 10% neutral buffered formalin. The specimens were then prepared for further analysis using the paraffin embedding procedure. To facilitate observations under a light microscope, transverse slices measuring 4-5 µm were prepared and stained with Haematoxylin and Eosin (H&E) [48].

2.11. Statistical analysis

Means plus or minus the SEM were used to present the data. Data analysis was carried out using GraphPad Prism (version 5.01, GraphPad Software Inc., CA, USA). For the purpose of comparing the means of the groups under study, a one-way ANOVA test and a multiple comparison were employed. To estimate post hoc differences between means, Tukey's test was employed. The difference was deemed statistically significant with a *p*-value below 0.05.

3. RESULTS

3.1. Corticosterone levels, body weight gain, and testes weight

Serum corticosterone levels in the stress model group were 280% higher than in the control group, indicating hyperactivity of the HPA axis. In contrast, DMF ameliorated the rise in corticosterone levels brought on by stress by 46.6%. Body weight gain and testicular weight were significantly decreased in the stress model rats by 158.4% and 30.1%, respectively, with respect to the rats in the control group. Compared to the stress model group, those treated with DMF had a 135% increase in testicular weight and a 195% rise in body weight gain Figure 1.

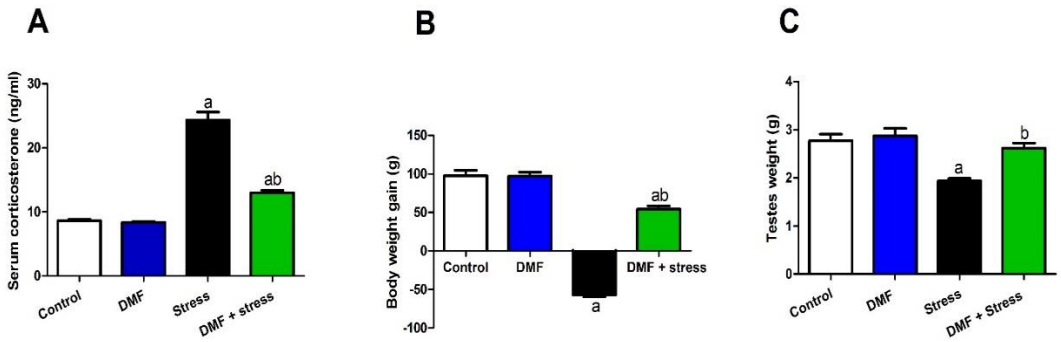

Figure 1. Effects of DMF on stress-induced changes in serum corticosterone (A), body weight gain (B), and testes weight (C) in the different studied groups. Values are expressed as the mean ± SEM. (a) Significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's test as a post-hoc test. SEM: standard error of the mean.

3.2. Involvement of the Nrf2/HO-1 antioxidant signaling pathway

In contrast to the control group, the stress model group exhibited a significant decrease in testicular gene expression and levels of Nrf2 by 78.8% and 55.3%, respectively, and of HO-1 by 92% and 44.2%. Conversely, treatment with DMF notably boosted testicular gene expression and levels of Nrf2 to about 374% and 185%, respectively, and HO-1 to about 1116% and 149%, respectively, relative to the stress model group. On the contrary, the stress model group had alarmingly high levels of testicular Keap-1 gene expression (1115%) relative to the control group. DMF treatment caused a momentous diminution in the Keap-1 testicular expression by 82% relative to the stress model group (Figure 2).

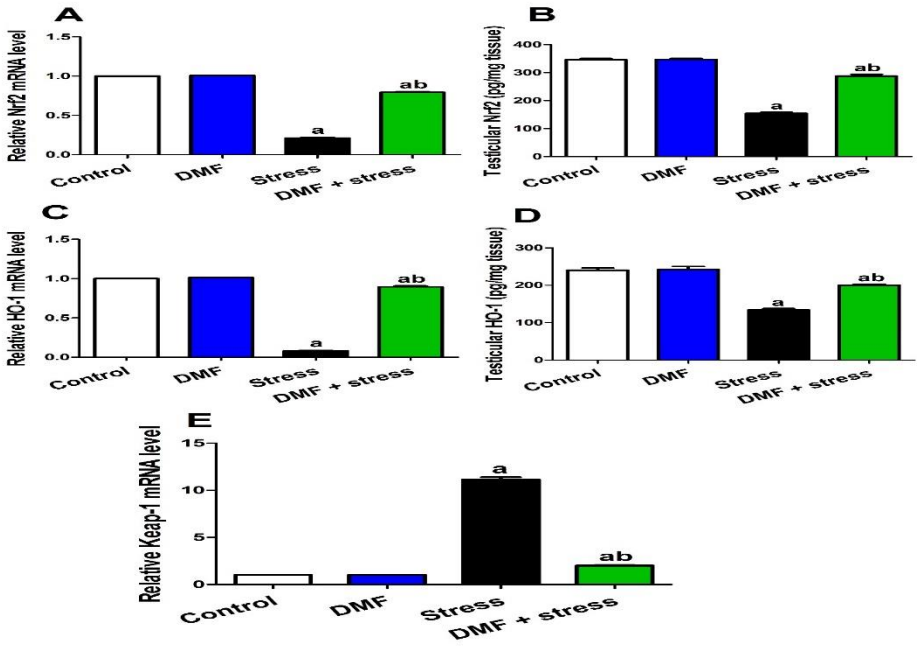

Figure 2. Effects of DMF on stress-induced changes in testicular gene expression and levels of Nrf2 (A&B), HO-1 (C&D), and gene expression of Keap-1 (E) in the different studied groups. Values are expressed as the mean ± SEM. (a) significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's test as a post-hoc test. Nrf2: nuclear factor erythroid 2-related factor 2; HO-1: heme oxygenase-1; Keap-1: Kelch-like ECH-associated protein 1; SEM: standard error of the mean.

3.3. Oxidative and antioxidative parameters

Findings from the present study revealed increased oxidative stress as demonstrated by a substantial rise in testicular levels of MDA (157%) and a diminution of testicular GSH levels by 52.2%, along with serum TAC levels by 58.8% and catalase activity by 68.1% in the stress model group in contrast to the control group. DMF treatment has significantly decreased MDA levels by 25.5% and increased GSH (180%), TAC levels (172%), and catalase activity (277%) in contrast to the stress model group (Figure 3).

3.4. Expression levels of StAR, CYP11A1 genes, and serum testosterone levels

Rats exposed to stress had significantly lower levels of testicular StAR and CYP11A1 gene expression by 77.3% and 76.3%, respectively, than the control rats. Testicular StAR and CYP11A1 gene expression levels significantly increased after DMF treatment by about 339% and 318%, respectively, in comparison to the stress model group. According to our findings, the rats exposed to stress had significantly lower serum testosterone levels by 61.8% than the control rats. Relative to the stress model group, rats treated with DMF showed a substantial increase in serum testosterone levels (217%) (Figure 4).

3.5. Inflammatory cytokines and apoptotic cell death markers

In comparison to the control group, the stress model group showed substantially boosted contents of NF-κB and IL-1β expression in the testicles, with a surge of approximately 673% and 188%, respectively. However, DMF therapy nullified stress-induced alterations in these

inflammatory mediators by 57.5% and 33.6%, respectively.

Furthermore, stress caused a substantial 40.4% diminution of testicular Bcl-2 expression as well as a 315% increase in Bax levels and a 531% increase in the Bax/Bcl-2 ratio relative to the control rats. Treatment with DMF significantly increased Bcl-2 (145%) and markedly suppressed Bax levels and the Bax/Bcl-2 ratio by 56.8% and 70.2%, respectively, in contrast to the stress model rats (Figure 5).

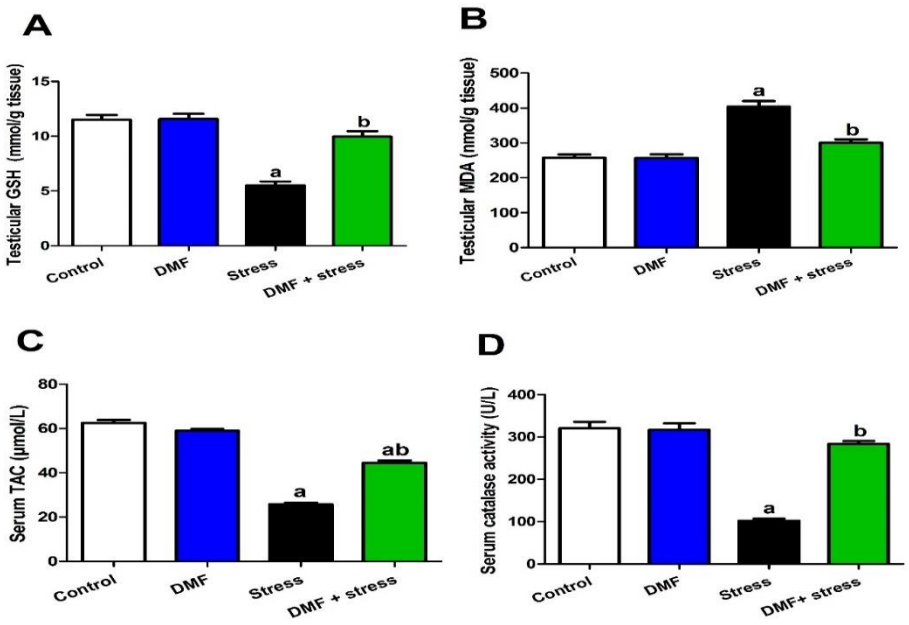

Figure 3. Effects of DMF on stress-induced changes in testicular GSH (A) and MDA (B) levels, serum TAC levels (C), and catalase activity (D) in the different studied groups. Values are expressed as the mean ± SEM. (a) significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's test as a post-hoc test. GSH: glutathione; MDA: malondialdehyde; SEM: standard error of the mean; TAC: total antioxidant capacity

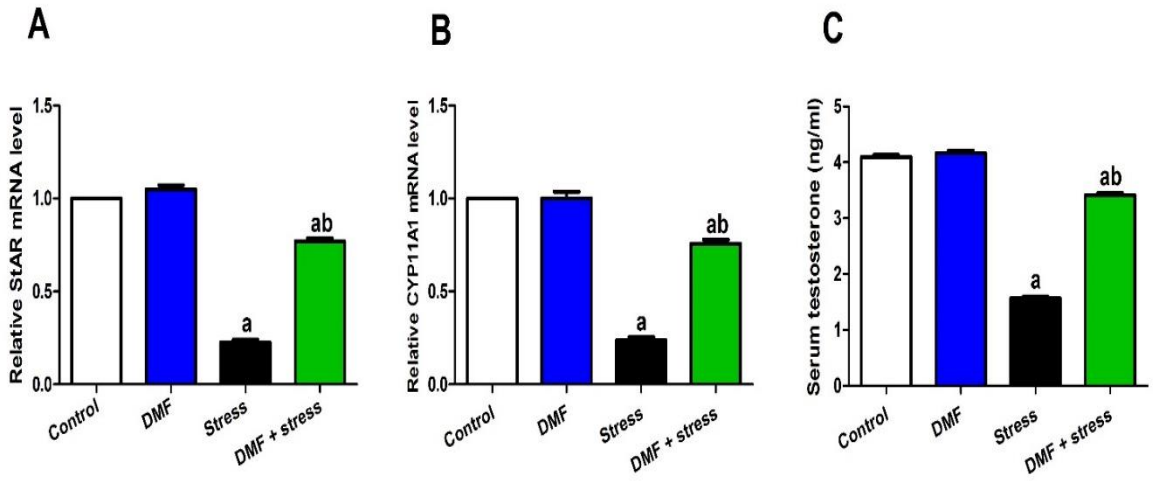

Figure 4. Effects of DMF on stress-induced changes in testicular gene expression levels of StAR (A) and CYP11A1 (B) and serum testosterone levels (C) in the different studied groups. Values are expressed as the mean ± SEM. (a) significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's test as a post-hoc test. CYP11A1: cytochrome P450 family 11 subfamily A member 1; StAR: steroidogenic acute regulatory protein; SEM: standard error of the mean.

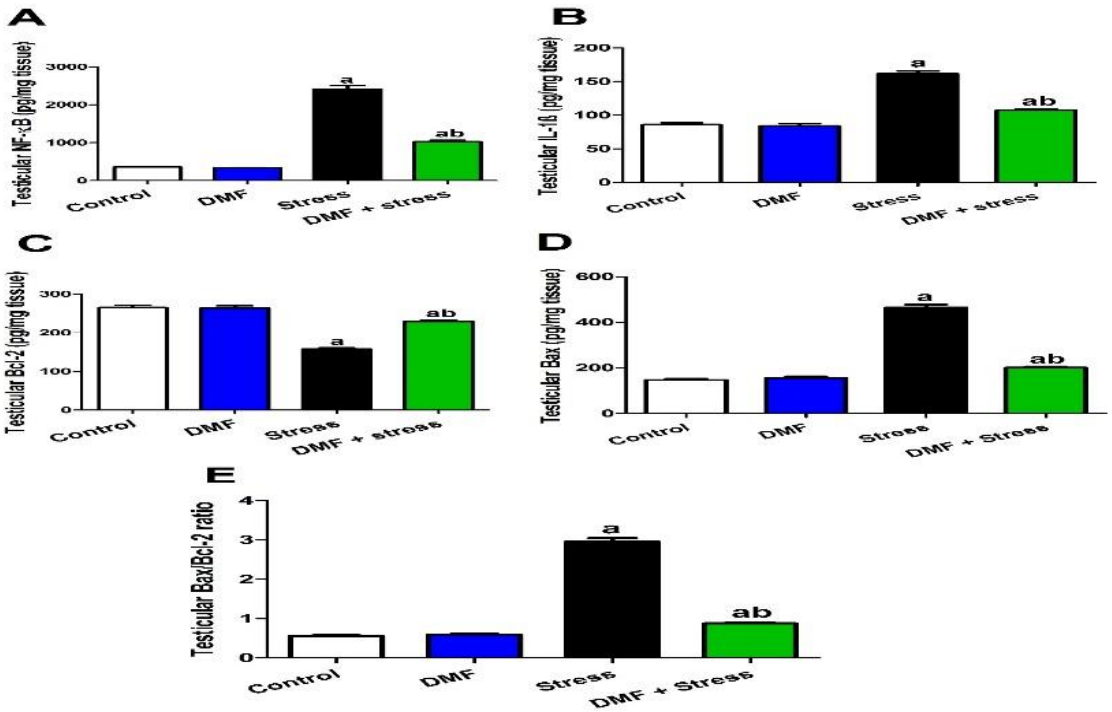

Figure 5. Effects of DMF on stress-induced changes in testicular levels of NF-κB (A), IL-1β (B), Bcl-2 (C), Bax (D), and Bax/Bcl-2 ratio (E) in the different studied groups. Values are expressed as the mean ± SEM. (a) significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's test as a post-hoc test. Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; IL-Iβ: interleukin-1beta; NF-κB: nuclear factor kappa-B; SEM: standard error of the mean.

3.6. Autophagy biomarkers

Testicular Beclin 1 and sequestosome 1/p62 levels were significantly elevated to about 440% and 369%, respectively, in the stress model rats in relation to the control rats. Relative to the stress model group, DMF treatment resulted in a 61.5% decline in testicular Beclin 1 levels and a 57.9% reduction in sequestosome 1/p62 levels Figure 6.

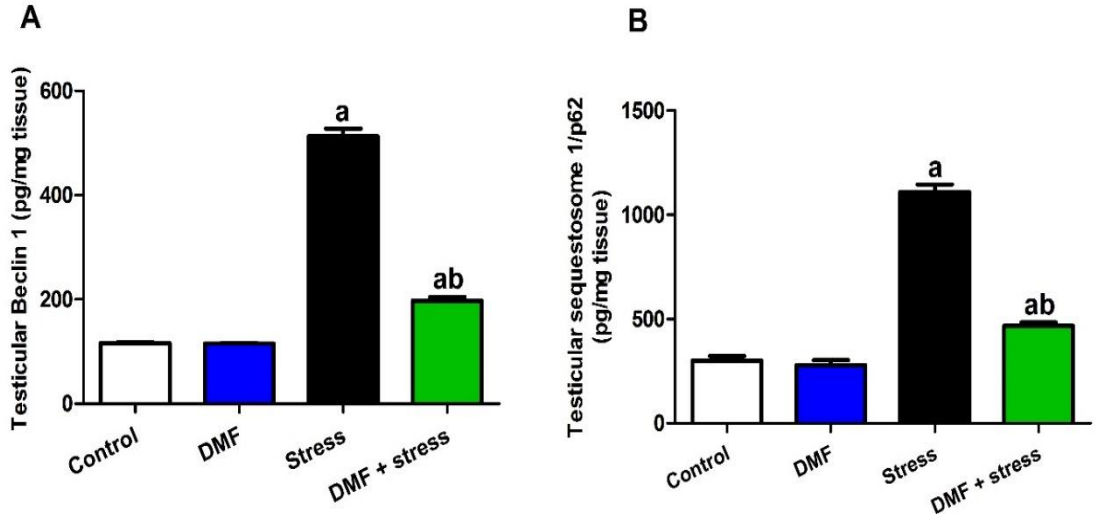

Figure 6. Effects of DMF on stress-induced changes in testicular Beclin 1 (A) and sequestosome 1/p62 (B) levels in the different studied groups. Values are expressed as the mean ± SEM. (a) significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's test as a post-hoc test. SEM: standard error of the mean.

3.7. Morphometry and spermatogenesis score

The diameter of seminiferous tubules was ominously diminished by 37.9% in the stress model group in contrast to the control group, as depicted in Figure 7. The DMF-treated group showed a momentous upsurge (157%) in the measured diameter relative to the stress model group. The Johnsen score for spermatogenesis revealed a notably lessened score in the stress model group by 67% relative to the control group. The DMF-treated group exhibited a higher score (280%) relative to the stress model group.

The present study's normal control and DMF control groups showed no statistically significant changes in any of the examined parameters, indicating that the dosage of DMF utilized was safe.

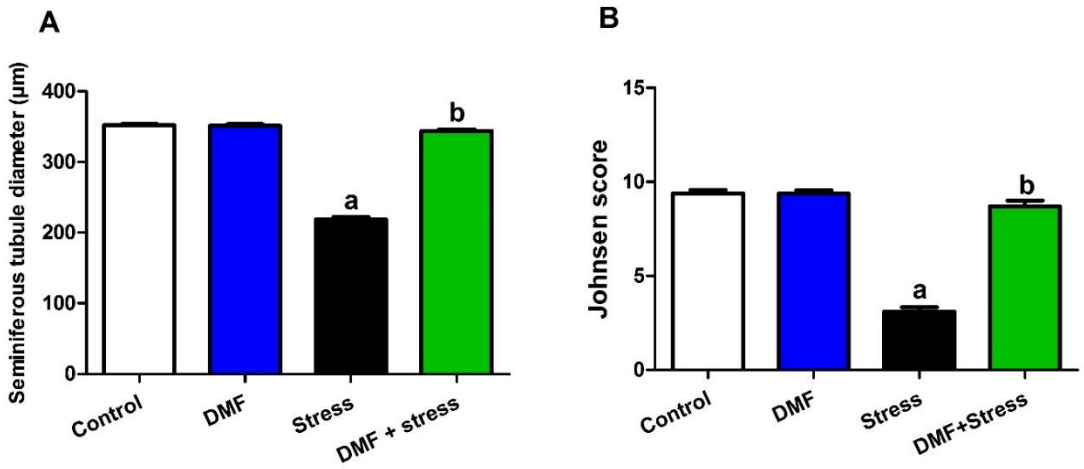

Figure 7. Effects of DMF on stress-induced changes in seminiferous tubule diameter (A) and Johnsen score (B) in the different studied groups. Values are expressed as the mean ± SEM. (a) significant difference from the control group at $p < 0.05$; (b) significant difference from the stress model group at $p < 0.05$. SEM: standard error of the mean.

3.8. Histopathological examination

The microscopic examination of tissue samples from the normal control group (A) revealed the normal histological structure of seminiferous tubules with complete spermatogonial cell series and numerous spermatids in their lumen. No histopathological findings were observed in the DMF-only group (B). On the contrary, the stress model group (C&D) showed marked histopathological changes; the seminiferous tubules were relatively smaller in diameter and showed a marked reduction in the spermatogonial cells population. The formed spermatids in the center of the tubules were fewer or completely absent. Some of the examined sections showed nearly empty tubules with a thickened basement membrane and mineralization in some instances. The DMF-treated group (E&F) showed marked improvement; almost all examined sections were apparently normal, while a few sections exhibited a mild reduction in the formed spermatids or a mild loss of spermatogonial cells (Figure 8).

4. DISCUSSION

Subfertility and infertility in males are just two of the many disorders that may have their roots in stress's ability to alter a wide range of physiological responses [49]. The CUMS is an animal stress model that replicates everyday life stress by subjecting animals to an array of mild stressors [50]. The actual chronic stress was evidenced in this study by a substantial upsurge in corticosterone levels in the stress model group, which coincided with others [51, 52]. This could be explained by stimulation of the HPA axis triggering increased releases of CRH, ACTH, and cortisol [53]. The hippocampus is a crucial component of the HPA axis negative feedback loop and has the highest concentration of glucocorticoid receptors in the brains of rats. Damage to the hippocampus from an overabundance of glucocorticoids throws off the inhibitory control of the HPA axis, which in turn triggers a cascade of events that ends with more glucocorticoids damaging the hippocampus [54].

Moreover, similar to the earlier CUMS rats, the stress model animals exhibited a substantial fall in both body and testicular weights [8]. Research by Jeong et al. showed that high corticosterone levels can inhibit the hypothalamic production of neuropeptide Y, a powerful neuropeptide that enhances eating behavior [55]. Also, elevated corticosterone stimulates the transcription of some proteins that accelerate muscle protein breakdown [56]. Furthermore, corticosterone has a direct impact on genes implicated in the lipolysis of adipocytes, including

hormone-sensitive and adipose triglyceride lipase [57]. An effect of stress on the gastrointestinal tract is a deceleration in the transit time of food through the intestines. Additionally, digestion and nutrient absorption in the intestines may be impacted [58]. Conversely, DMF substantially mitigated HPA axis stimulation, as shown by lower corticosterone levels and, as a result, enhanced body weight gain owing to slower protein catabolism and lipolysis.

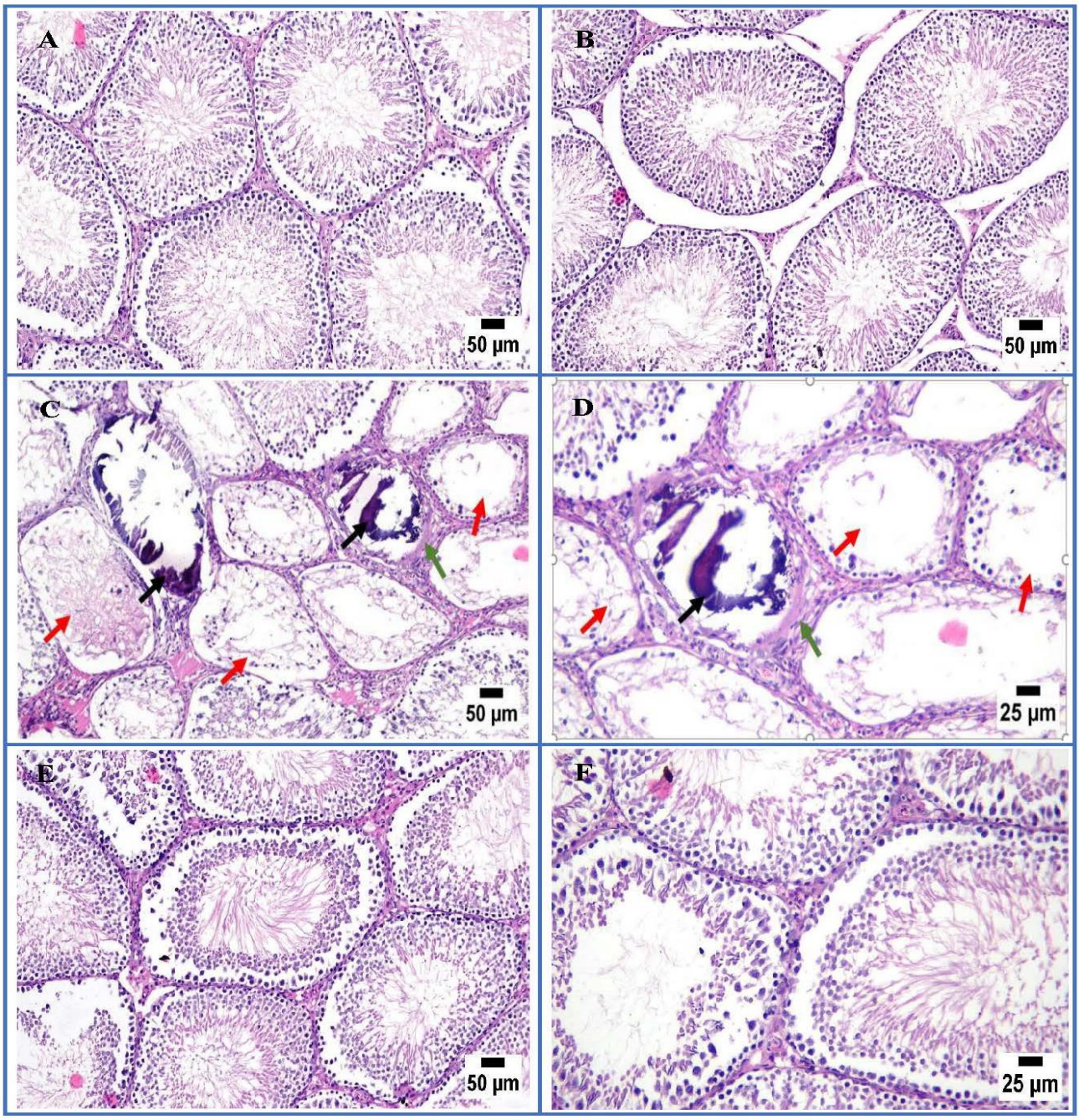

Figure 8. Photomicrographs of testicular tissue stained with H&E of the control group (A); the DMF group (B), showing normal seminiferous tubules; the stress group (C&D) showing testicular degeneration (red arrows), mineralization (black arrows), and thickened seminiferous tubule basement membrane (green arrow); and the DMF+ stress group (E&F) showing apparently normal seminiferous tubules.

In order to maintain redox equilibrium and cell viability, Nrf2 regulates the ARE-DNA sequences of phase II antioxidant genes such as HO-1, NQO1, and glutathione production enzymes [59]. Nrf2 is essential for limiting the emergence of oxidative stress during spermatogenesis, along with the involvement of HO-1 in the testicular reflex to stress [60]. In addition to being the most significant endogenous enzyme protecting against oxidative stress, HO-1 is the enzyme that governs the heme breakdown rate [61]. Nrf2 is normally bound and suppressed by the chaperone protein Keap1 in physiological circumstances. When Nrf2 is activated, it splits from Keap1 and floods to the nucleus to govern the targeted genes [62]. We found that chronic stress, which can cause oxidative stress, drastically decreased Nrf2 mRNA and protein expressions and its controlled HO-1, while dramatically increasing Keap-1 mRNA expression levels in testes relative to those in the control group. It implies that chronic stress may have impaired the Nrf2/HO-1 signaling pathway, leading to testicular oxidative stress. Our findings corroborate those of earlier studies [63].

The current study found that compared to the stress model rats, stressed rats treated with DMF had ominously elevated levels of Nrf2 and HO-1 mRNA and protein expression, and lower levels of Keap1 mRNA. These results show that DMF protects rats' testicles from oxidative stress caused by prolonged stress via regulating the Nrf2-Keap1 pathway. Results show that DMF and MMF do not increase Nrf2 production directly. They stop Keap1 from binding Nrf2, which frees Nrf2 to translocate to the nucleus and starts its signaling cascade [64]. DMF has been demonstrated in multiple investigations as an effective modulator of Nrf2 and HO-1. A recent study found that while DMF protected rats against testicular ischemia-reperfusion injury, it activated the Nrf2 signaling pathway [65]. DMF also activated the Nrf2/HO-1 signaling pathway, which alleviated complete Freund's adjuvant-provoked arthritis in rats [66]. Furthermore, DMF protected rats' kidneys from cisplatin-induced damage by regulating Nrf2 expression [67].

The rats in the stress model had lower levels of TAC and GSH, less catalase activity, and higher levels of MDA, which is a sign of lipid peroxidation. These findings clearly support the presence of oxidative stress in the testes. The preceding observations are in accordance with those of earlier reports [63, 68]. The proximity of Leydig cells to testicular macrophages renders them susceptible to extracellular sources of ROS [69]. DMF prevented lipid peroxidation in the testes by bringing back endogenous antioxidants (TAC, catalase, and GSH) to baseline levels and significantly lowered MDA levels. The beneficial impact of DMF is correlated with its antioxidant activity, which has previously been demonstrated [70, 71].

The most crucial organelle for cellular energy metabolism and a key player in testosterone production is the mitochondria. StAR and CYP11A1, two crucial mitochondrial proteins in Leydig cells, control the transport and metabolism of cholesterol to testosterone in mitochondria [1]. Our results revealed that the steroidogenic machinery proteins StAR and CYP11A1 are disrupted by chronic stress, which was in line with a previous report [68]. In the biosynthesis of steroid hormones, including sex steroids, the first reaction occurs when cholesterol is converted to pregnenolone by the mitochondrial enzyme CYP11A1 [72]. Also, the transport of cholesterol from the outer to the inner mitochondrial membranes limits the activity of CYP11A1. This phase is thought to be the real rate-limiting step in the synthesis of steroids, which is predominantly driven by the StAR protein [72].

Furthermore, when comparing the stress model rats to the control rats, we found that their serum testosterone levels were much lower. This may be explained by the marked decline in StAR and CYP11A1 expression levels. Our findings are correlated with previous studies [8, 68]. Inhibiting CYP450c17 activity via contact with the glucocorticoid receptor, glucocorticoids reduce testosterone production in Leydig cells [73]. Furthermore, β-endorphin plays a pivotal role in mediating male infertility linked to chronic stress. The pituitary gland is stimulated to produce and release ACTH and β-endorphin when the hypothalamus increases CRH production during times of stress. The molecular level production of FSH and LH by the pituitary is hindered by β-endorphin because it blocks the hypothalamus release of GnRH. This results in the inhibition of gonadal testosterone secretion and a subsequent reduction in sperm count and vitality [7].

It is possible that the reduced absolute weights of testes in stress model rats relative to control rats are due to the significant decrease in testosterone. Previous investigations have demonstrated that stress-related testicular weight loss in animals is caused by a lack of testosterone, which could hamper spermatogenesis [8, 63]. Conversely, administration of DMF prominently boosted testicular StAR and CYP11A1 expression levels and consequently increased serum testosterone levels, along with a substantial elevation in testes weights as compared to the stress model group.

Although pro-inflammatory cytokines are essential for maintaining the testicles' proper

homeostasis, testicular dysfunction could result from an increase in their expression levels [74]. Consistent with previous models of chronic stress, this study found that prolonged stress substantially boosted levels of NF-κB and IL-1β, indicating inflammation of the testicular tissues [63]. This might be caused by increased oxidative stress, which triggers NF-κB and then boosts the production of cytokines-mediated inflammation, like TNF-α and IL-Iβ, which could aggravate testicular damage [63]. An in vitro study revealed that key proteins involved in testosterone production, such as StAR, 3β-Hydroxysteroid dehydrogenase, and CYP11A1, were suppressed in Leydig cells by TNF-α or IL-1β treatment [75]. Also, steroidogenic factor 1, a key transcriptional regulator of several steroidogenic genes and controller of gonadal activities [59], is one of the orphan nuclear receptors that NF-κB is known to deactivate [76].

As shown in this study, DMF prevented the stress-induced activation of NF-κB in the testes of rats. In addition, NF-κB activation led to elevated levels of IL-Iβ, a notable inflammatory cytokine; DMF later counteracted this impact. The activation of Nrf2 is responsible for DMF's anti-inflammatory effects, which are exerted by reducing NF-κB-mediated inflammation. This approach successfully blocks the generation of cytokines that promote inflammation, including IL-Iβ, IL-6, and TNF-α [65, 77]. Additionally, DMF can activate the histone deacetylase sirtuin 1 (SIRT1) [78], which is essential for controlling inflammatory signals through blocking NF-κB [79]. By deacetylating the RelA/p65 subunit, SIRT1 activation impedes NF-κB signaling [80].

The impact of apoptosis in the progress of stress-induced testicular injury has been confirmed in a large body of research, alongside oxidative stress and inflammation. In contrast to the control group, the stress model group exhibited an imbalance of apoptotic proteins, with the proapoptotic Bax being upregulated and the antiapoptotic Bcl-2 being downregulated. As a result, the Bax/Bcl-2 ratio increased. Comparable results have been documented in prior research [63, 81]. By triggering apoptotic signaling pathways, both elevated corticosterone levels in chronically stressed males and excessive ROS production are capable of eliciting apoptosis in testicular cells [63]. ROS may activate pro-apoptotic signaling proteins such as Bax, which initiate the mitochondrial apoptotic cascade. First, cytochrome c is released, and then caspases are activated in this process [63]. The downregulation of Nrf2, a protein participating in regulating the activity of an array of apoptotic proteins, may also contribute to increased apoptotic signaling [82]. Moreover, activation of NF-κB has been recognized as a factor that promotes apoptosis in testicular cells [83]. Furthermore, spermatocytes undergo apoptosis in response to testosterone deprivation [84]. The present results indicated that DMF rectified the disparity between pro- and antiapoptotic proteins provoked by chronic stress. The Bax/Bcl-2 ratio was notably lower in the DMF group than in the control group. The antiapoptotic potential of DMF can be elucidated by its capacity to suppress ROS and NF-κB, in addition to its inhibitory impact on serum corticosterone.

Our results showed that the testes of stressed rats had elevated levels of Beclin 1 and sequestosome 1/p62. These findings revealed stress-provoked testicular injury in conjunction with a surplus of autophagy that exhibited a correlation with oxidative stress [21]. A conserved cellular destruction mechanism, autophagy also goes by the name type II programmed cell death, involves the transport of organelles, lipids, and proteins to lysosomes for disintegration [85]. Gao et al. demonstrated that aberrant autophagy led to impairments in spermatogenesis and damage to the testes [86]. Autophagy is an intricate metabolic process that is governed by genes that are specific to autophagy. An indication that autophagy has begun is the separation of light chain 3 (LC3)-I into LC3-II. The autophagic breakdown of ubiquitinated protein aggregates is facilitated by LC3-II after it binds to the adaptor protein p62 [85]. LC3-II, Beclin l, and p62 are biomarkers that are frequently employed in the assessment of autophagy levels [87, 88]. Yi et al. reported that p62 aggregation impedes the decomposition of autophagosomes [27]. We found that stressed rats' testes had significantly elevated p62 levels, which may indicate that autophagosome degradation was impaired. Consistent with our findings, Di-(2-ethylhexyl) phthalate caused impairment of the BTB and hampered the growth of spermatogenic cells via exaggerated ROS-provoked accumulation of autophagosomes, along with elevated p62 expression, which hindered autophagy breakdown [27]. Our results are consistent with those of another study that established that aflatoxin B1 accelerates autophagy while blocking autophagy flux; this, in turn, increases the toxic effects of aflatoxin B1 on Leydig cells in rats, leading to a significant decrease in Leydig cell number and testosterone biosynthesis [89]. In this work, rats treated with DMF had lower testicular levels of Beclin 1 and p62 because DMF suppressed ROS formation. Duan et al. demonstrated that autophagy can be suppressed by halting the formation of ROS [26].

In addition, the stress model group exhibited degeneration, loss of spermatogenesis, and a thicker

basement membrane in addition to dispersed, deformed, empty seminiferous tubules in histological sections of testicular tissue. Chronic stress causes oxidative injury and profound apoptosis, which mainly lead to this impairment of testicular architecture. The assessed tubule diameter and the Johnsen score for spermatogenesis were both substantially boosted after DMF treatment, and all histopathological abnormalities were significantly reduced.

5. CONCLUSIONS

In the present study, treatment with DMF significantly improved the HPA axis hyperactivity in CUMS rats. Moreover, DMF could mitigate CUMS-induced testicular dysfunction by reducing oxidative stress, inflammation, apoptosis, and autophagy in testicular tissues. Based on our results, we demonstrated that DMF had therapeutic effects on the CUMS-induced testicular dysfunction in rats, and these effects might be related, in large part, to the activation of the Nrf2/HO-1 signaling pathway. Hence, candidates for treatment of CUMS and related testicular issues include boosters of the Nrf2/HO-1 pathway.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Ethical Statement: The experiments of the present study were conducted in accordance with the approval of ethics committee of Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt (Permit number: 462) and conform to the rules of the United Kingdom's Animals (Scientific Procedures) Act, 1986.

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List of Abbreviations: ACTH: adrenocorticotropic hormone, ARE: antioxidant response element, Bax: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2, Beclin 1: Bcl-2-interacting protein 1, BTB: blood-testis barrier, CRH: corticotropin-releasing hormone, CMC: carboxymethylcellulose, CUMS: chronic unpredictable mild stress, CYP11A1: cytochrome P450 family 11 subfamily A member 1, DMF: dimethyl fumarate, ELISA: Enzyme-linked immunosorbent assay, FSH: follicle-stimulating hormone, GnRH: gonadotropin-releasing hormone, GPx: glutathione peroxidase, GSH: glutathione, GST: glutathione-S-transferase, HO-1: heme oxygenase-1, HPA: hypothalamic-pituitary-adrenal, IL-1β: interleukin-1beta, Keap1: Kelch-like ECH-associated protein 1, LH: luteinizing hormone, MDA: malondialdehyde, MMF: monomethyl fumarate, NQO-1: NADPH: quinone oxidoreductase-1, NF-κB: nuclear factor kappa-B, Nrf2: Nuclear factor erythroid 2-related factor 2, ROS: reactive oxygen species, SEM: standard error of the mean, SIRT1: sirtuin 1, SOD: superoxide dismutase, StAR: steroidogenic acute regulatory protein, TAC: total antioxidant capacity, TNF-α: tumor necrosis factor-α.

REFERENCES

1. Xiong X, Wu Q, Zhang L, Gao S, Li R, Han L, et al. Chronic stress inhibits testosterone synthesis in Leydig cells through mitochondrial damage via Atp5a1. Journal of Cellular and Molecular Medicine. 2022;26(2):354-63.

2. Nordkap L, Jensen TK, Hansen ÅM, Lassen TH, Bang AK, Joensen UN, et al. Psychological stress and testicular function: a cross-sectional study of 1,215 Danish men. Fertility and sterility. 2016;105(1):174-87. e2.

3. Wang X, Xie Y, Zhang T, Bo S, Bai X, Liu H, et al. Resveratrol reverses chronic restraint stress-induced depression-like behaviour: Involvement of BDNF level, ERK phosphorylation and expression of Bcl-2 and Bax in rats. Brain Res Bull. 2016 Jul;125:134-43.

4. Geng C, Guo Y, Wang C, Liao D, Han W, Zhang J, et al. Systematic impacts of chronic unpredictable mild stress on metabolomics in rats. Sci Rep. 2020 Jan 20;10(1):700.

5. Gall Z, Farkas S, Albert A, Ferencz E, Vancea S, Urkon M, et al. Effects of Chronic Cannabidiol Treatment in the Rat Chronic Unpredictable Mild Stress Model of Depression. Biomolecules. 2020 May 22;10(5).

6. Nargund VH. Effects of psychological stress on male fertility. Nature Reviews Urology. 2015;12(7):373-82.

7. Xiong X, Zhang L, Fan M, Han L, Wu Q, Liu S, et al. β-Endorphin induction by psychological stress promotes Leydig cell apoptosis through p38 MAPK pathway in male rats. Cells. 2019;8(10):1265.

8. Salami SA, Salahdeen HM, Moronkola OT, Murtala BA, Raji Y. Vitamin C supplementation during chronic variable stress exposure modulates contractile functions of testicular artery and sperm parameters in male Wistar rats. Middle East Fertility Society Journal. 2020;25(1):1-10.

9. Agarwal A, Virk G, Ong C, Du Plessis SS. Effect of oxidative stress on male reproduction. The world journal of men's health. 2014;32(1):1-17.

10. Upadhayay S, Mehan S. Targeting Nrf2/HO-1 anti-oxidant signaling pathway in the progression of multiple sclerosis and influences on neurological dysfunctions. Brain Disorders. 2021;3:100019.

11. Dong W, Yang B, Wang L, Li B, Guo X, Zhang M, et al. Curcumin plays neuroprotective roles against traumatic brain injury partly via Nrf2 signaling. Toxicology and Applied Pharmacology. 2018;346:28-36.

12. He F, Ru X, Wen T. NRF2, a Transcription Factor for Stress Response and Beyond. Int J Mol Sci. 2020 Jul 6;21(13).

13. Pérez S, Taléns-Visconti R, Rius-Pérez S, Finamor I, Sastre J. Redox signaling in the gastrointestinal tract. Free Radical Biology and Medicine. 2017;104:75-103.

14. Ekici S, Ekici AID, Öztürk G, Aksungar FB, Sinanoğlu O, Turan G, et al. Comparison of melatonin and ozone in the prevention of reperfusion injury following unilateral testicular torsion in rats. Urology. 2012;80(4):899-906.

15. Bhowmick S, D'Mello V, Caruso D, Abdul-Muneer P. Traumatic brain injury-induced downregulation of Nrf2 activates inflammatory response and apoptotic cell death. Journal of molecular medicine. 2019;97:1627-41.

16. Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, et al. Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. Nature communications. 2016;7(1):1-14.

17. Quinti L, Dayalan Naidu S, Träger U, Chen X, Kegel-Gleason K, Llères D, et al. KEAP1-modifying small molecule reveals muted NRF2 signaling responses in neural stem cells from Huntington's disease patients. Proceedings of the National Academy of Sciences. 2017;114(23):E4676-E85.

18. Zheng K-m, Zhang J, Zhang C-l, Zhang Y-w, Chen X-c. Curcumin inhibits appoptosin-induced apoptosis via upregulating heme oxygenase-1 expression in SH-SY5Y cells. Acta Pharmacologica Sinica. 2015;36(5):544-52.

19. Kanter M, Aktas C, Erboga M. Heat stress decreases testicular germ cell proliferation and increases apoptosis in short term: an immunohistochemical and ultrastructural study. Toxicol Ind Health. 2013 Mar;29(2):99-113.

20. Retana-Marquez S, Vigueras-Villasenor RM, Juarez-Rojas L, Aragon-Martinez A, Torres GR. Sexual behavior attenuates the effects of chronic stress in body weight, testes, sexual accessory glands, and plasma testosterone in male rats. Horm Behav. 2014 Nov;66(5):766-78.

21. Huang W, Cao Z, Zhang J, Ji Q, Li Y. Aflatoxin B1 promotes autophagy associated with oxidative stress-related PI3K/AKT/mTOR signaling pathway in mice testis. Environmental Pollution. 2019;255:113317.

22. Shen Y, He D, He L, Bai Y, Wang B, Xue Y, et al. Chronic Psychological Stress, but Not Chronic Pain Stress, Influences Sexual Motivation and Induces Testicular Autophagy in Male Rats. Front Psychol. 2020;11:826.

23. Li L, Tan J, Miao Y, Lei P, Zhang Q. ROS and autophagy: interactions and molecular regulatory mechanisms. Cellular and molecular neurobiology. 2015;35:615-21.

24. Shang Y, Wang H, Jia P, Zhao H, Liu C, Liu W, et al. Autophagy regulates spermatid

differentiation via degradation of PDLIM1. Autophagy. 2016;12(9):1575-92.

25. Zhao X, Xu W, Wu J, Zhang D, Abou-Shakra A, Di L, et al. Nicotine induced autophagy of Leydig cells rather than apoptosis is the major reason of the decrease of serum testosterone. The International Journal of Biochemistry & Cell Biology. 2018;100:30-41.

26. Duan P, Hu C, Quan C, Yu T, Zhou W, Yuan M, et al. 4-Nonylphenol induces apoptosis, autophagy and necrosis in Sertoli cells: involvement of ROS-mediated AMPK/AKT-mTOR and JNK pathways. Toxicology. 2016;341:28-40.

27. Yi W, Xiang-Liang T, Yu Z, Bin L, Lian-Ju S, Chun-Lan L, et al. DEHP exposure destroys blood-testis barrier (BTB) integrity of immature testes through excessive ROS-mediated autophagy. Genes & diseases. 2018;5(3):263-74.

28. Linker RA, Haghikia A. Dimethyl fumarate in multiple sclerosis: latest developments, evidence and place in therapy. Ther Adv Chronic Dis. 2016 Jul;7(4):198-207.

29. Albrecht P, Bouchachia I, Goebels N, Henke N, Hofstetter HH, Issberner A, et al. Effects of dimethyl fumarate on neuroprotection and immunomodulation. J Neuroinflammation. 2012 Jul 07;9:163.

30. Kunze R, Urrutia A, Hoffmann A, Liu H, Helluy X, Pham M, et al. Dimethyl fumarate attenuates cerebral edema formation by protecting the blood-brain barrier integrity. Exp Neurol. 2015 Apr;266:99-111.

31. Fox RJ, Miller DH, Phillips JT, Hutchinson M, Havrdova E, Kita M, et al. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. N Engl J Med. 2012 Sep 20;367(12):1087-97.

32. Hammer A, Waschbisch A, Kuhbandner K, Bayas A, Lee DH, Duscha A, et al. The NRF2 pathway as potential biomarker for dimethyl fumarate treatment in multiple sclerosis. Ann Clin Transl Neurol. 2018 Jun;5(6):668-76.

33. Peng H, Guerau-de-Arellano M, Mehta VB, Yang Y, Huss DJ, Papenfuss TL, et al. Dimethyl fumarate inhibits dendritic cell maturation via nuclear factor kappaB (NF-kappaB) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mitogen stress-activated kinase 1 (MSK1) signaling. J Biol Chem. 2012 Aug 10;287(33):28017-26.

34. Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology. 2005;52(2):90-110.

35. Bhatt S, Shukla P, Raval J, Goswami S. Role of Aspirin and Dexamethasone against Experimentally Induced Depression in Rats. Basic Clin Pharmacol Toxicol. 2016 Jul;119(1):10-8.

36. Karagiannides I, Golovatscka V, Bakirtzi K, Sideri A, Salas M, Stavrakis D, et al. Chronic unpredictable stress regulates visceral adipocyte-mediated glucose metabolism and inflammatory circuits in male rats. Physiol Rep. 2014;2(5):e00284.

37. Robles L, Vaziri ND, Li S, Masuda Y, Takasu C, Takasu M, et al. Dimethyl fumarate protects pancreatic islet cells and non-endocrine tissue in L-arginine-induced chronic pancreatitis. PLoS One. 2014;9(9):e107111.

38. Abdel-Sattar E, Mehanna ET, El-Ghaiesh SH, Mohammad HM, Elgendy HA, Zaitone SA. Pharmacological action of a pregnane glycoside, russelioside B, in dietary obese rats: impact on weight gain and energy expenditure. Frontiers in pharmacology. 2018;9:990.

39. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem. 1996 Jul 15;239(1):70-6.

40. Naciff JM, Hess KA, Overmann GJ, Torontali SM, Carr GJ, Tiesman JP, et al. Gene expression changes induced in the testis by transplacental exposure to high and low doses of 17{alpha}-ethynyl estradiol, genistein, or bisphenol A. Toxicol Sci. 2005 Aug;86(2):396-416.

41. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology. 2004 Feb;145(2):592-603.

42. Yamashita Y, Ueyama T, Nishi T, Yamamoto Y, Kawakoshi A, Sunami S, et al. Nrf2-inducing anti-oxidation stress response in the rat liver-new beneficial effect of lansoprazole. PLoS One. 2014;9(5):e97419.

43. Chu X, Zhang X, Gong X, Zhou H, Cai C. Effects of hyperoxia exposure on the expression of Nrf2 and heme oxygenase-1 in lung tissues of premature rats. Molecular and cellular probes. 2020;51:101529.

44. Nakai K, Fujii H, Kono K, Goto S, Kitazawa R, Kitazawa S, et al. Vitamin D activates the Nrf2-Keap1 antioxidant pathway and ameliorates nephropathy in diabetic rats. American journal of hypertension. 2014;27(4):586-95.

45. Banni M, Messaoudi I, Said L, El Heni J, Kerkeni A, Said K. Metallothionein gene expression in liver of rats exposed to cadmium and supplemented with zinc and selenium. Archives of environmental contamination and toxicology. 2010;59(3):513-9.

46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

47. Hozyen HF, Khalil HMA, Ghandour RA, Al-Mokaddem AK, Amer MS, Azouz RA. Nano selenium protects against deltamethrin-induced reproductive toxicity in male rats. Toxicol Appl Pharmacol. 2020 Dec 1;408:115274.

48. Bancroft JD, Gamble M. Theory and practice of histological techniques: Elsevier health sciences; 2008.

49. Meng X, Peng L, Xu J, Guo D, Cao W, Xu Y, et al. Betaine attenuate chronic restraint stress-induced changes in testicular damage and oxidative stress in male mice. Reproductive Biology and Endocrinology. 2022;20(1):80.

50. Antoniuk S, Bijata M, Ponimaskin E, Wlodarczyk J. Chronic unpredictable mild stress for modeling depression in rodents: Meta-analysis of model reliability. Neuroscience & Biobehavioral Reviews. 2019;99:101-16.

51. Andric SA, Kostic TS. Regulation of Leydig cell steroidogenesis: intriguing network of signaling pathways and mitochondrial signalosome. Current Opinion in Endocrine and Metabolic Research. 2019;6:7-20.

52. Zou P, Wang X, Yang W, Liu C, Chen Q, Yang H, et al. Mechanisms of stress-induced spermatogenesis impairment in male rats following unpredictable chronic mild stress (uCMS). International journal of molecular sciences. 2019;20(18):4470.

53. Choowong-in P, Sattayasai J, Poodendaen C, Iamsaard S. Decreased expression of AKAP4 and TyrPho proteins in testis, epididymis, and spermatozoa with low sexual performance of mice induced by modified CUMS. Andrologia. 2021;53(3):e13977.

54. Dronse J, Ohndorf A, Richter N, Bischof GN, Fassbender R, Behfar Q, et al. Serum cortisol is negatively related to hippocampal volume, brain structure, and memory performance in healthy aging and Alzheimer's disease. Frontiers in aging neuroscience. 2023;15:1154112.

55. Jeong JY, Lee DH, Kang SS. Effects of chronic restraint stress on body weight, food intake, and hypothalamic gene expressions in mice. Endocrinology and metabolism. 2013;28(4):288-96.

56. Schakman O, Gilson H, Thissen J-P. Mechanisms of glucocorticoid-induced myopathy. The Journal of endocrinology. 2008;197(1):1-10.

57. Xu C, He J, Jiang H, Zu L, Zhai W, Pu S, et al. Direct effect of glucocorticoids on lipolysis in adipocytes. Molecular Endocrinology. 2009;23(8):1161-70.

58. Mireille KP. Protective effects of Nymphaea lotus Linn.(Nymphaeaceae) aqueous extract against chronic unpredictable mild stress induced testicular lipid peroxidation. Asian Journal of Biomedical and Pharmaceutical Sciences. 2016;6(54):01.

59. Shi X, Fu L. Piceatannol inhibits oxidative stress through modification of Nrf2-signaling pathway in testes and attenuates spermatogenesis and steroidogenesis in rats exposed to cadmium during adulthood. Drug

Design, Development and Therapy. 2019:2811-24.

60. Bae WJ, Ha U, Choi JB, Kim KS, Kim SJ, Cho HJ, et al. Protective effect of decursin extracted from Angelica gigas in male infertility via Nrf2/HO-1 signaling pathway. Oxidative medicine and cellular longevity. 2016;2016.

61. Luo W, Li J, Li Z, Lin T, Zhang L, Yang W, et al. HO-1 nuclear accumulation and interaction with NPM1 protect against stress-induced endothelial senescence independent of its enzymatic activity. Cell death & disease. 2021;12(8):738.

62. Kansanen E, Kuosmanen SM, Leinonen H, Levonen A-L. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Redox biology. 2013;1(1):45-9.

63. Guo Y, Sun J, Li T, Zhang Q, Bu S, Wang Q, et al. Melatonin ameliorates restraint stress-induced oxidative stress and apoptosis in testicular cells via NF-κB/iNOS and Nrf2/HO-1 signaling pathway. Scientific reports. 2017;7(1):9599.

64. Cores Á, Piquero M, Villacampa M, León R, Menéndez JC. NRF2 regulation processes as a source of potential drug targets against neurodegenerative diseases. Biomolecules. 2020;10(6):904.

65. Onishi A, Chiba K, Kawamura S, Sato K, Kaku Y, Okada K, et al. Dimethyl Fumarate Protects Rats against Testicular Ischemia–Reperfusion Injury. Andrologia. 2023;2023.

66. Lal R, Dhaliwal J, Dhaliwal N, Dharavath RN, Chopra K. Activation of the Nrf2/HO-1 signaling pathway by dimethyl fumarate ameliorates complete Freund's adjuvant-induced arthritis in rats. Eur J Pharmacol. 2021 May 15;899:174044.

67. Zhao K, Wen L-B. DMF attenuates cisplatin-induced kidney injury via activating Nrf2 signaling pathway and inhibiting NF-kB signaling pathway. European Review for Medical & Pharmacological Sciences. 2018;22(24).

68. Sakr HF, Abbas A, Elsamanoudy A, Ghoneim F. Effect of fluoxetine and resveratrol on testicular functions and oxidative stress in a rat model of chronic mild stress-induced depression. J Physiol Pharmacol. 2015;66(4):515-27.

69. Riris AAIDC, I'tishom R, Khaerunnisa S. Role of antioxidant to protect Leydig cells induced by reactive oxygen species: a literature review. Qanun Medika-Medical Journal Faculty of Medicine Muhammadiyah Surabaya. 2021;5(1):49-60.

70. Manai F, Amadio M. Dimethyl Fumarate Triggers the Antioxidant Defense System in Human Retinal Endothelial Cells through Nrf2 Activation. Antioxidants (Basel). 2022 Sep 28;11(10).

71. Li J, Ma J, Lacagnina MJ, Lorca S, Odem MA, Walters ET, et al. Oral Dimethyl Fumarate Reduces Peripheral Neuropathic Pain in Rodents via NFE2L2 Antioxidant Signaling. Anesthesiology. 2020 Feb;132(2):343-56.

72. Lavoie HA, King SR. Transcriptional regulation of steroidogenic genes: STARD1, CYP11A1 and HSD3B. Exp Biol Med (Maywood). 2009 Aug;234(8):880-907.

73. Czuchlej SC, Volonteri MC, Regueira E, Ceballos NR. Effect of glucocorticoids on androgen biosynthesis in the testes of the toad Rhinella arenarum (Amphibia, Anura). Journal of Experimental Zoology Part A: Ecological and Integrative Physiology. 2019;331(1):17-26.

74. Rizk SM, Zaki HF, Mina MA. Propolis attenuates doxorubicin-induced testicular toxicity in rats. Food and chemical toxicology. 2014;67:176-86.

75. Hong CY, Park JH, Ahn RS, Im SY, Choi H-S, Soh J, et al. Molecular mechanism of suppression of testicular steroidogenesis by proinflammatory cytokine tumor necrosis factor alpha. Molecular and cellular biology. 2004;24(7):2593-604.

76. Lysiak JJ. The role of tumor necrosis factor-alpha and interleukin-1 in the mammalian testis and their involvement in testicular torsion and autoimmune orchitis. Reproductive Biology and Endocrinology. 2004;2(1):1-10.

77. Chen L-G, Zhang Y-Q, Wu Z-Z, Hsieh C-W, Chu C-S, Wung B-S. Peanut arachidin-1 enhances Nrf2-mediated protective mechanisms against TNF-α-induced ICAM-1

expression and NF-κB activation in endothelial cells. International Journal of Molecular Medicine. 2018;41(1):541-7.

78. Parodi B, Rossi S, Morando S, Cordano C, Bragoni A, Motta C, et al. Fumarates modulate microglia activation through a novel HCAR2 signaling pathway and rescue synaptic dysregulation in inflamed CNS. Acta neuropathologica. 2015;130:279-95.

79. Lv H, Wang L, Shen J, Hao S, Ming A, Wang X, et al. Salvianolic acid B attenuates apoptosis and inflammation via SIRT1 activation in experimental stroke rats. Brain Research Bulletin. 2015;115:30-6.

80. Schug TT, Xu Q, Gao H, Peres-da-Silva A, Draper DW, Fessler MB, et al. Myeloid deletion of SIRT1 induces inflammatory signaling in response to environmental stress. Molecular and cellular biology. 2010;30(19):4712-21.

81. Juárez-Rojas AL, Garcia-Lorenzana M, Aragón-Martínez A, Gómez-Quiroz LE, del Socorro Retana-Márquez M. Intrinsic and extrinsic apoptotic pathways are involved in rat testis by cold water immersion-induced acute and chronic stress. Systems biology in reproductive medicine. 2015;61(4):211-21.

82. Xiong W, Garfinkel AEM, Li Y, Benowitz LI, Cepko CL. NRF2 promotes neuronal survival in neurodegeneration and acute nerve damage. The Journal of clinical investigation. 2015;125(4):1433-45.

83. Rasoulpour RJ, Boekelheide K. NF-kappaB activation elicited by ionizing radiation is proapoptotic in testis. Biology of reproduction. 2007;76(2):279-85.

84. Kim JM, Ghosh SR, Weil AC, Zirkin BR. Caspase-3 and caspase-activated deoxyribonuclease are associated with testicular germ cell apoptosis resulting from reduced intratesticular testosterone. Endocrinology. 2001 Sep;142(9):3809-16.

85. Shen N, Wang L, Wu J, Chen X, Hu F, Su Y. Meta-analysis of the autophagy-associated protein LC3 as a prognostic marker in colorectal cancer. Experimental and Therapeutic Medicine. 2023;26(4):1-9.

86. Gao F, Li G, Liu C, Gao H, Wang H, Liu W, et al. Autophagy regulates testosterone synthesis by facilitating cholesterol uptake in Leydig cells. Journal of Cell Biology. 2018;217(6):2103-19.

87. Zhang J, Zhu Y, Shi Y, Han Y, Liang C, Feng Z, et al. Fluoride-induced autophagy via the regulation of phosphorylation of mammalian targets of rapamycin in mice leydig cells. Journal of agricultural and food chemistry. 2017;65(40):8966-76.

88. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, Abdellatif M, Abdoli A, Abel S, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2021;17(1):1-382.

89. Chen X, Li C, Chen Y, Ni C, Chen X, Zhang L, et al. Aflatoxin B1 impairs leydig cells through inhibiting AMPK/mTOR-mediated autophagy flux pathway. Chemosphere. 2019;233:261-72.