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Research Article

Dimethyl Fumarate Protects Rats against Testicular Ischemia–Reperfusion Injury

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Ischemia–reperfusion injury (IRI) after testicular torsion is linked to significant damage in testicular tissue. Dimethyl fumarate (DMF) activates the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, thereby inducing antioxidant and anti-inflammatory effects. We analyzed the usefulness of DMF in preventing IRI following testicular torsion/detorsion in Sprague-Dawley rats ($n = 32$). The animals were classified into control (sham), DMF (200 mg/kg/day), IRI, and IRI+DMF (IRI with 200 mg/kg/day DMF) groups. Testicular IRI was induced by detorsion after 1.5 h of torsion. DMF was administered via oral gavage daily from 1 h before testicular detorsion until day 7, when orchiectomy was performed. The testis-to-body weight ratio was calculated. Histopathological evaluation was performed using the Johnsen and Cosentino scores for seminiferous tubules. Malondialdehyde, superoxide dismutase, and total glutathione levels were determined in testicular tissues. Moreover, Nrf2, heme oxygenase 1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), nuclear factor kappa B (NF- κ B), and inflammatory cytokine (interleukin 1b (IL1b), IL6, and tumor necrosis factor alpha (TNF- α)) levels were determined through quantitative polymerase chain reaction. Nuclear Nrf2 and cytoplasmic HO-1 and NQO1 protein levels were also evaluated. DMF significantly improved the testis-to-body weight ratio and reduced histopathological damage in the testes. Moreover, it significantly improved the concentration of malondialdehyde, superoxide dismutase, and total glutathione. Furthermore, it inhibited NF- κ B and inflammatory cytokine mRNA expression compared with the findings obtained in untreated rats with IRI (all $p < 0.05$). Nrf2, HO-1, and NQO1 expressions (mRNA and protein) were markedly elevated following DMF treatment in rats with IRI (all $p < 0.05$). DMF administration activated the Nrf2 signaling pathway and induced antioxidant and anti-inflammatory effects, thereby improving IRI-induced testicular damage. Thus, DMF may prevent IRI following testicular torsion.

1. Introduction

Testicular torsion is an acute condition resulting from spermatic cord twisting. In terms of incidence, it is more common in children and occurs at an annual rate of 3.8–4.5 per 100,000 individuals [1, 2]. Torsion release within 6–12 h preserves testicular function in 79–97% of patients [3]. However, rescue becomes difficult in approximately 80% of patients after 24 h [4], and testicular salvage should optimally be performed within 6 h of onset [5].

The pathogenesis of testicular torsion includes tissue damage attributable to hypoxia associated with ischemia

and ischemia–reperfusion injury (IRI), which occur after testicular detorsion. IRI is defined as the resumption of blood flow after ischemia, which results in elevated concentration of reactive oxygen species [6], induction of inflammatory responses by cytokines [7, 8], and increased interstitial pressure [9] caused by disruption of the vascular endothelium and increased permeability. This process leads to microcirculatory failure and tissue injury. In cardiac injury induced by ischemia (1 h) and reperfusion (3 h), the reported rate of cellular injury is 73%, versus 17% when ischemia persists for 4 h [10]. Tissue damage caused by IRI could be more severe than that caused by

ischemia. Thus, immediate testicular detorsion and prevention of IRI are crucial for the treatment of testicular torsion.

The relationship between testicular torsion and infertility remains controversial. Nevertheless, this risk continues to cause concern. Although there was no clear difference in semen quality between the orchiopexy and fertile sperm donor groups in prior research, semen quality was lower in patients who underwent orchiectomy than in fertile sperm donors [11]. In addition, only 17% of patients have normal semen despite testicular salvage by orchiopexy [12], suggesting that the procedure does not prevent infertility in all patients. Because IRI could be involved in the development of infertility after orchiopexy, a drug that alleviates testicular IRI could help prevent infertility.

Hence, it is extremely important to develop drugs that improve testicular IRI, and several such drugs have been reported [13–23]. However, currently, there are no established therapeutic options for clinical use.

The nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway induces antioxidant and anti-inflammatory effects. In the absence of stress, Nrf2 binds to cytoplasmic Kelch-like ECH-associated protein 1 (Keap1); this leads to rapid proteasomal degradation, preventing its activation [24]. Conversely, Keap1 activity is reduced under stress. This reduced activity allows the nuclear translocation of Nrf2 [25, 26], consequently forming a dimer with small Maf and binding to antioxidant response elements [27]. Subsequently, Nrf2 activates heme oxygenase 1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) [28, 29]. Rate-limiting enzyme HO-1 degrades heme into biliverdin/bilirubin, carbon monoxide, and iron ions [30]; notably, bilirubin inhibits free radicals [31]. NQO1 reduces endogenous quinone to stable hydroquinone, which has antioxidant properties [32]. Therefore, both HO-1 and NQO1 protect against cytotoxicity by exerting antioxidant effects when activated by the Nrf2 signaling pathway. Nrf2 exerts its inhibitory effect on inflammation via suppression of nuclear factor-kappa B (NF- κ B), which induces inflammation. This process prevents the induction of inflammatory cytokines, namely, interleukin 1b (IL1b), IL6, and tumor necrosis factor alpha (TNF- α) [33–40].

It was previously reported that Nrf2 signaling pathway reduced rat testicular damage by inducing antioxidant activity [41–43]. In mammals, the Nrf2 activator dimethyl fumarate (DMF) is hydrolyzed to monomethyl fumarate by esterase. In relapsing-remitting multiple sclerosis, DMF is clinically used to prevent disease relapse and progression of disability [44–46]. It is thought that DMF exerts its activity through multiple biological effects [47]. For example, DMF exerts neuroprotective effects by inducing an antioxidant response as an Nrf2 activator [48]. It also promotes T-helper cell type 1- (Th1-) to-Th2 transition as an immunomodulator [47, 49]. In addition, it has been reported that DMF is effective against renal and liver IRI [50, 51]. However, the ability of DMF to prevent testicular IRI has not been documented.

Therefore, we examined the effectiveness of DMF in preventing testicular IRI in rats by activating the Nrf2 signal-

ing pathway to induce antioxidant and anti-inflammatory effects.

2. Materials and Methods

2.1. Experimental Animals. The Institutional Animal Care and Use Committee of Kobe University approved this study (approval number: P200703-R1). The evaluations were conducted pursuant to the Kobe University Animal Experimentation Regulations and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (CLEA Japan Inc., Tokyo, Japan) (age: 6 weeks) were maintained under constant conditions for 1 week before experimentation and throughout the investigation.

2.2. Experimental Groups and Surgical Procedures. Figure 1 presents a summary of the experimental groups and schedules. Rats ($n = 32$; weight: 192–260 g) were randomized into four groups (8 animals/group): Ctrl (sham), DMF, IRI, and IRI+DMF. On day 1, all animals were anesthetized using isoflurane inhalation, and a midline vertical incision was performed on the scrotum, thereby opening the tunica vaginalis and exposing the right testis. In the IRI and IRI+DMF groups, the right testis was twisted clockwise 720° and sutured to the scrotum using a 7-0 nylon suture. Following 1.5 h of torsion, testicular detorsion was carried out, and the skin was sutured using 4-0 silk. The Ctrl and IRI groups were treated with methylcellulose, while the DMF and IRI+DMF groups were treated with DMF 200 mg/kg/day (mixed with methyl cellulose) via oral gavage for 7 days; the treatment was initiated 1 h before testicular detorsion. On day 7, orchiectomy was performed. Testicular tissues were collected after measuring the body and testicular weights.

2.3. Reagents. In this investigation, we used DMF (Sigma-Aldrich, St. Louis, MO, USA), as well as methylcellulose (0.5% weight/volume) and isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.4. Histopathological Analyses: Hematoxylin and Eosin (H&E) Staining. Testicular tissues were subjected to fixation using 10% formalin for 24 h, dehydration using ethanol, and paraffin embedding. Subsequently, the tissues were sectioned (thickness: 3 μ m) using a microtome, stained, and examined using a BZ-X710 microscope (KEYENCE Co., Osaka, Japan) at $\times 200$ magnification. The evaluation methods included scoring of the degree of spermatogenesis using the Johnsen score [52] and testicular tissue damage using the Cosentino score [53]. Histopathological analyses were performed by two investigators blinded to the group allocation.

2.5. Biochemical Parameters. Measurements were conducted to determine all biochemical parameters in testicular tissue. Malondialdehyde (MDA, oxidative stress marker) levels were calculated (nmol/g tissue) via an OxiSelect TBARS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA). The levels of superoxide dismutase (SOD, an antioxidant enzyme) were calculated (U/g tissue) via a SOD Assay Kit

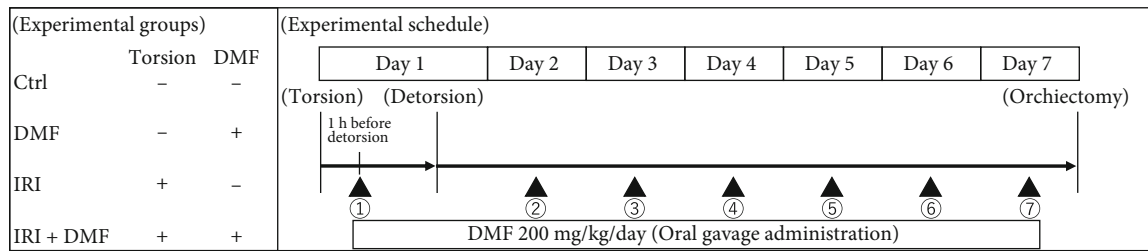


FIGURE 1: Experimental groups and schedule. Rats ($n = 32$) were classified into four groups. In the IRI and IRI+DMF groups, testicular IRI was created by 1.5 h of torsion. The DMF and IRI+DMF groups were treated with DMF via oral gavage. On day 7, orchiectomy was performed. Ctrl: sham; DMF: dimethyl fumarate; IRI: ischemia–reperfusion injury.

(DOJINDO Laboratories, Kumamoto, Japan). The levels of total glutathione (GSH, an antioxidant) were measured ($\mu\text{mol/g}$ tissue) using a GSH kit (DOJINDO Laboratories). An iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for all measurements.

2.6. Quantitative Polymerase Chain Reaction (qPCR). Testicular tissue was homogenized with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), followed by RNA extraction. SuperScript IV VILO Master Mix (Thermo Fisher Scientific) was used to generate cDNA from $1\ \mu\text{g}$ of RNA. qPCR was conducted using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays (Thermo Fisher Scientific) were employed for the preparation of cDNA. The primers and probes used for qPCR (TaqMan method) were Nrf2 (Rn00582415_m1), β -actin (Rn00667869_m1), HO-1 (Rn00561387_m1), NQO1 (Rn00566528_m1), NF- κ B (Rn01502266_m1), IL1b (Rn00580432_m1), IL6 (Rn01410330_m1), and TNF- α (Rn01525859_g1). The PCR conditions were 95°C for 20 s, 40 cycles of 95°C for 3 s, and 60°C for 30 s. The ΔCT method was used for evaluation, and the mRNA levels in all groups were quantitatively evaluated versus those detected in the Ctrl group.

2.7. Western Blotting Analyses. A Nuclear Extra Kit (Active Motif, Carlsbad, CA, USA) was utilized to extract and separate cytoplasmic and nuclear proteins. The nuclear fraction was used to evaluate Nrf2 expression, while the cytoplasmic fraction was used to investigate HO-1 and NQO1 expressions. Purified protein samples ($30\ \mu\text{g}$) in 2x Laemmli buffer (Bio-Rad Laboratories) were boiled at 95°C for 5 min. Electrophoresis was performed in Mini-PROTEAN TGX Gels (Bio-Rad Laboratories). Thereafter, the proteins were transferred onto polyvinylidene fluoride membranes, which were incubated with primary antibodies: anti-Nrf2 (ab92946, 1:1,000; Abcam, Cambridge, UK), anti-HO-1 (ab13243, 1:2,000; Abcam), anti-NQO1 (ab80588, 1:25,000; Abcam), anti-histone H3 (ab1791, 1:5,000; Abcam), and anti- β -actin (ab227387, 1:20,000; Abcam). Subsequently, they were incubated with the secondary antibody goat anti-rabbit IgG H&L (horseradish peroxidase) (ab7090, 1:10,000; Abcam). Finally, the membranes were exposed to ECL Prime chemiluminescent reagent (Cytiva, Tokyo, Japan) for 5 min, imaged using Imager 600 (Cytiva), and quantified using

ImageJ. For loading control, we used histone H3 (nuclear proteins) and β -actin (cytoplasmic proteins).

2.8. Statistical Analyses. Data are mean \pm standard deviation. EZR software was used for statistical analysis [54]. Normality was checked for each endpoint. For parametric statistics, one-way analysis of variance and Tukey's test were employed. For nonparametric statistics, the Kruskal–Wallis and Steel–Dwass tests were employed. A $p < 0.05$ denotes statistical significance.

3. Results

3.1. Testicular Weight and Testis-to-Body Weight Ratio. Excised testes, testicular weight, and the testis-to-body weight ratio are presented in Figures 2(a)–2(c). Testicular weight was statistically significantly lower in the IRI and IRI+DMF groups versus the Ctrl group (both $p < 0.05$). Testicular weight was similar in the IRI and IRI+DMF groups. The testis-to-body weight ratio was statistically significantly lower in the IRI group versus the Ctrl group. In contrast, this ratio was statistically significantly higher in the IRI+DMF group versus the IRI group (both $p < 0.05$).

3.2. Histopathological Analyses. The results of H&E staining ($\times 200$) and the Johnsen and Cosentino scores are presented in Figures 3(a)–3(c). Normal seminiferous tubular structure and spermatogenesis were noted in the Ctrl and DMF groups. However, rats in the IRI group exhibited histopathological findings with testicular tissue damage (e.g., seminiferous tubule disruption and atrophy and loss of germ cells). The IRI+DMF group displayed improved histopathological findings versus the IRI group (Figure 3(a)). The Johnsen score was statistically significantly lower in the IRI group versus the Ctrl group ($p < 0.05$). Nevertheless, this score was statistically significantly higher in the IRI+DMF group versus the IRI group ($p < 0.05$, Figure 3(b)). Similarly, the Cosentino score was statistically significantly higher in the IRI group versus the Ctrl group ($p < 0.05$). The IRI+DMF group had a significantly lower Cosentino score versus the IRI group ($p < 0.05$) (Figure 3(c)). Therefore, histopathological parameters were improved by DMF treatment in rats with IRI, indicating that DMF administration might have alleviated tissue damage.

3.3. Biochemical Parameters. MDA, SOD, and total GSH levels are presented in Figures 4(a)–4(c). Statistically

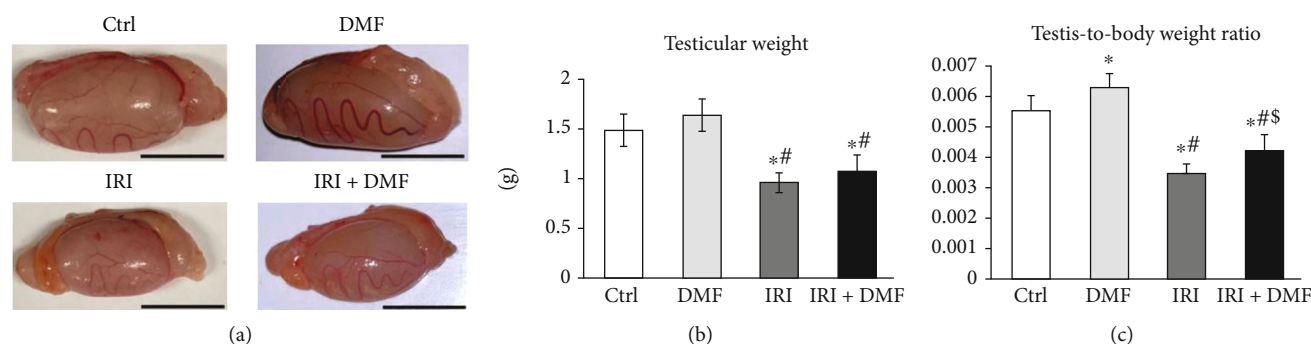


FIGURE 2: Testicular weight and testis-to-body weight ratio in all groups: (a) images of an excised testis from each group (scale bar = 1 cm); (b) testicular weight; (c) testis-to-body weight ratio. Data: mean \pm standard deviation. * $p < 0.05$, # $p < 0.05$, and \\$ $p < 0.05$ vs. Ctrl, DMF, and IRI groups, respectively. Ctrl: sham; DMF: dimethyl fumarate; IRI: ischemia-reperfusion injury.

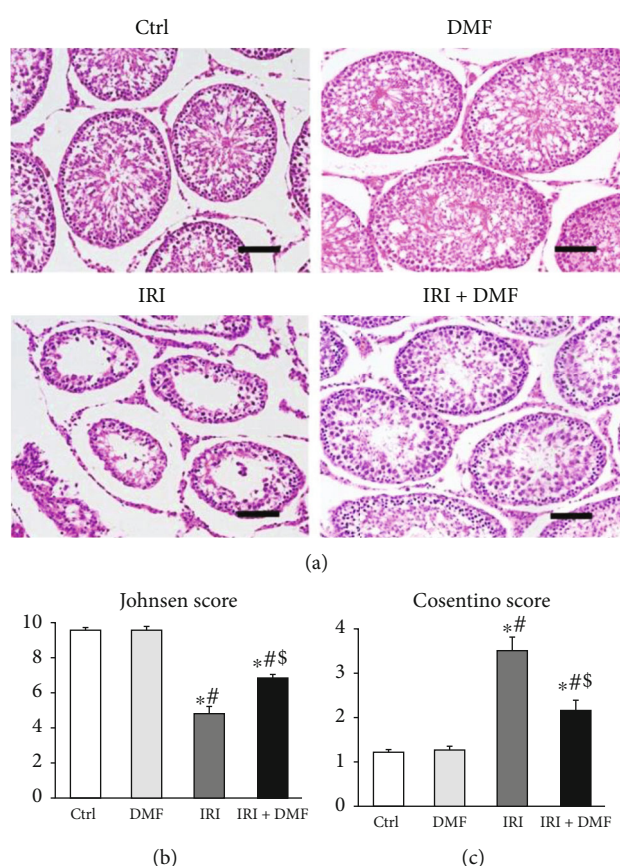


FIGURE 3: Histopathological analyses in the four groups: (a) H&E staining of tissue obtained from a single rat in each of the four groups ($\times 200$, scale bar = $100 \mu\text{m}$); (b) Johnsen score; (c) Cosentino score. Data: mean \pm standard deviation. * $p < 0.05$, # $p < 0.05$, and \\$ $p < 0.05$ vs. Ctrl, DMF, and IRI groups, respectively. Ctrl: sham; DMF: dimethyl fumarate; H&E: hematoxylin and eosin; IRI: ischemia-reperfusion injury.

significant elevations in MDA were recorded in the IRI group versus the Ctrl group ($p < 0.05$); these increases were reversed by DMF ($p < 0.05$) (Figure 4(a)). Conversely, statistically significant reductions in SOD and GSH were detected in the IRI group versus the Ctrl group ($p < 0.05$). Statistically

significant increases in SOD and GSH were recorded in the IRI+DMF group versus the IRI group ($p < 0.05$) (Figures 4(b) and 4(c)). The observed improvements in oxidative stress markers in the IRI+DMF group suggested that DMF exerted antioxidant effects in rats with testicular IRI.

3.4. qPCR. As highlighted in Figures 5(a)–5(c), the mRNA expression of Nrf2, HO-1, and NQO1 levels did not vary significantly between the Ctrl and IRI groups. Conversely, Nrf2, HO-1, and NQO1 mRNA expressions exhibited statistically significant elevations in the IRI+DMF group versus the IRI group (all $p < 0.05$) (Figures 5(a)–5(c)). NF- κ B mRNA expression showed statistically significant elevations in the IRI group versus the Ctrl group ($p < 0.05$). Of note, NF- κ B mRNA expression demonstrated statistically significant reductions in the IRI+DMF group versus the IRI group ($p < 0.05$) (Figure 5(d)). The mRNA concentration of IL1b, IL6, and TNF- α showed statistically significant elevations in the IRI group versus the Ctrl group (all $p < 0.05$); these increases were reversed by DMF (all $p < 0.05$) (Figures 5(e)–5(g)). These findings revealed that the administration of DMF activated the Nrf2 signaling pathway and exerted anti-inflammatory effects in rats with testicular IRI.

3.5. Western Blotting Analyses. Results are shown in Figures 6(a)–6(d). As presented in Figure 6(b), the expression of Nuclear Nrf2 protein exhibited statistically significant decreases in the IRI group versus the Ctrl group ($p < 0.05$). The IRI+DMF group displayed statistically significant elevations in the levels of Nuclear Nrf2 protein versus the IRI group ($p < 0.05$) (Figure 6(b)). Cytoplasmic HO-1 expression was similar between the IRI and Ctrl groups. Cytoplasmic HO-1 protein demonstrated a statistically significant increase in the IRI+DMF group versus the IRI group ($p < 0.05$) (Figure 6(c)). The expression of cytoplasmic NQO1 protein was similar between the IRI and Ctrl groups. However, a statistically significant increase was detected in the IRI+DMF group versus the IRI group ($p < 0.05$) (Figure 6(d)). Collectively, the observed changes in nuclear and cytoplasmic protein expressions following DMF treatment further supported that this agent activated the Nrf2 signaling pathway in rats with testicular IRI.

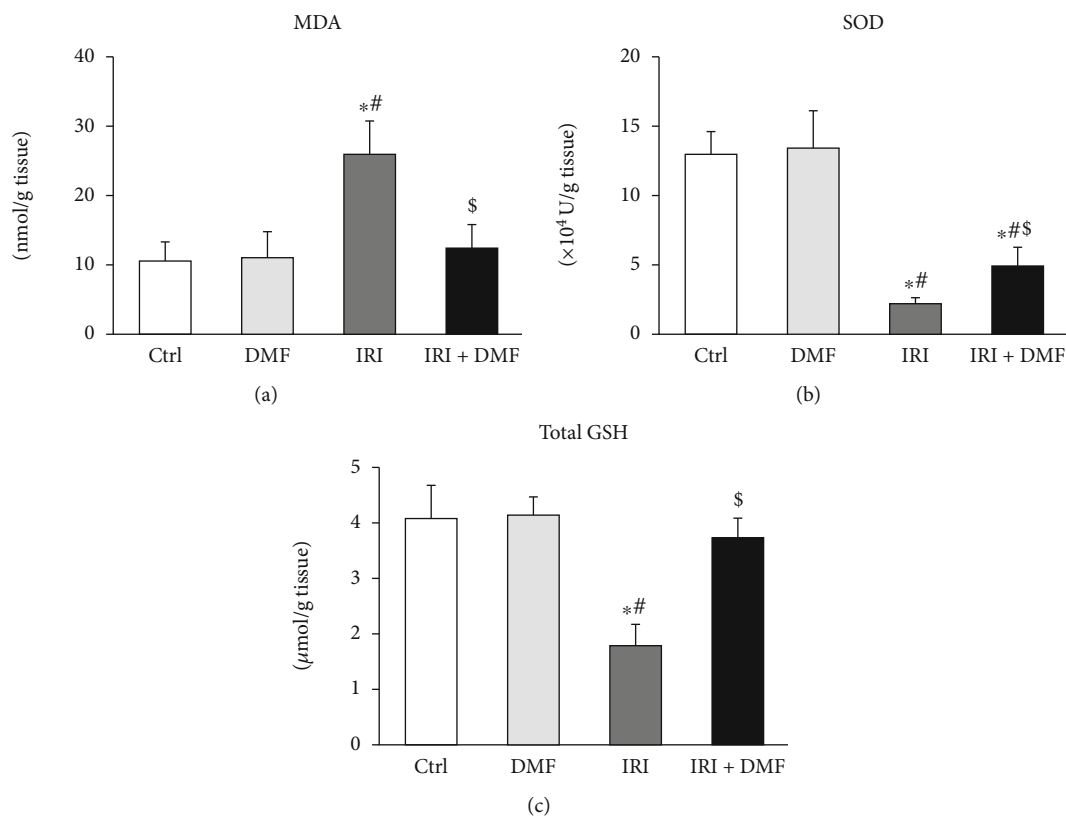


FIGURE 4: Biochemical parameters in rat testicular tissues: (a) MDA levels; (b) SOD levels; (c) total GSH levels. Data: mean \pm standard deviation. * $p < 0.05$, # $p < 0.05$, and \$ $p < 0.05$ vs. Ctrl, DMF, and IRI groups, respectively. Ctrl: sham; DMF: dimethyl fumarate; GSH: total glutathione; IRI: ischemia-reperfusion injury; MDA: malondialdehyde; SOD: superoxide dismutase.

4. Discussion

Oxidative stress reportedly contributes to DNA damage in sperm [55] and is associated with infertility in males [56]. Although cytokines are required for spermatogenesis, the presence of excessive levels of inflammatory cytokines may impair spermatogenesis [57, 58]. Notably, testicular IRI is characterized by oxidative stress and increased concentration of inflammatory cytokines [59, 60]. Therefore, the current study examined whether DMF alleviates testicular IRI via Nrf2 signaling pathway activation.

In prior research studies, treatment with DMF led to Nrf2 signaling pathway activation in multiple organs [50, 51]. Moreover, Nrf2 signaling pathway activation reportedly alleviates testicular damage [41, 61, 62]. The administration of DMF in rats with testicular IRI increased Nrf2, HO-1, and NQO1 expressions (protein and mRNA), confirming Nrf2 signaling pathway activation. Therefore, DMF could be a candidate for the activation of Nrf2 in the testes.

It has been reported that DMF and monomethyl fumarate do not directly enhance Nrf2 production. It is thought that they prevent Keap1 from binding Nrf2, thereby enabling the nuclear translocation of Nrf2 and activating its signaling pathway [63]. The administration of DMF in rats with testicular IRI increased the Nrf2 mRNA levels. It is hypothesized that the nuclear translocation of Nrf2 leads

to its consumption in the cytoplasm, which may indirectly result in increased Nrf2 mRNA levels. Similarly, Yao et al. also recorded increases in Nrf2 expression (mRNA and protein) following the administration of DMF in mice with cerebral ischemia [64].

The administration of DMF in rats with testicular IRI resulted in increased HO-1 and NQO1 expression (protein and mRNA) and affected the levels of MDA, SOD, and GSH. According to these findings, the Nrf2 signaling pathway induced antioxidant activity and alleviated testicular damage. These findings were in line with previous evidence regarding the association of Nrf2, HO-1, and NQO1 with testicular damage in rats [41, 61, 62, 65]. For example, Qin et al. demonstrated that low-dose zinc in rats with testicular IRI elevated the protein expression of Nrf2, HO-1, and NQO1, thereby reducing oxidative stress and preventing tissue damage [62]. Therefore, Nrf2 signaling pathway activation by DMF appears to promote antioxidant activity in rats with testicular IRI.

DMF reduced the NF- κ B, IL1b, IL6, and TNF- α mRNA levels, suggesting that Nrf2 induced anti-inflammatory effects by inhibiting NF- κ B. Similarly, Qi et al. reported that the consumption of omega-3 polyunsaturated fatty acid improved testicular IRI via Nrf2 activation and inhibition of NF- κ B, resulting in antioxidant and anti-inflammatory effects [41]. Fahim et al. also demonstrated that DMF

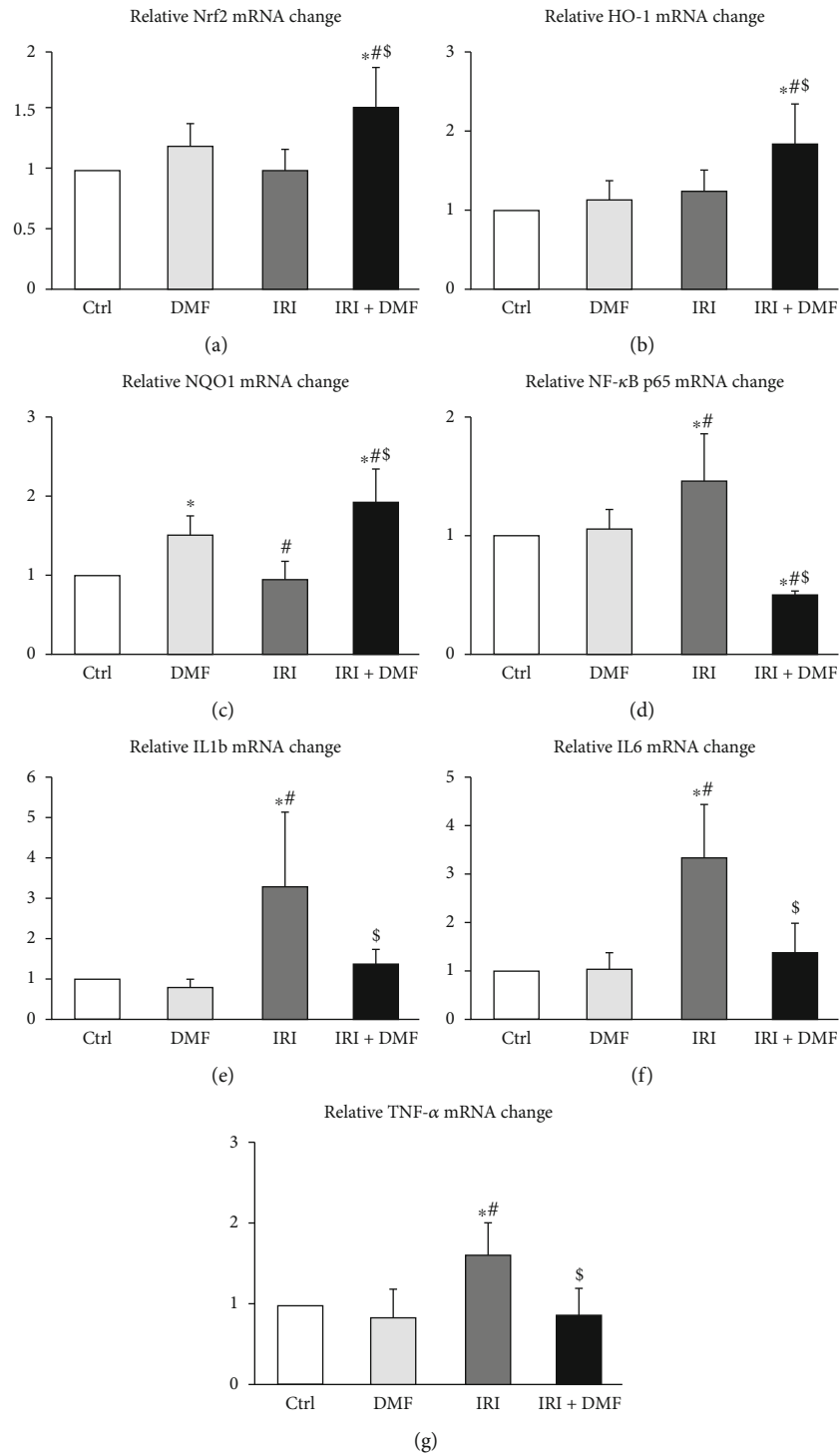


FIGURE 5: mRNA expression in rat testicular tissues: (a) Nrf2; (b) HO-1; (c) NQO1; (d) NF-κB p65; (e) IL1b; (f) IL6; (g) TNF-α. Data: mean ± standard deviation. * $p < 0.05$, # $p < 0.05$, and \$ $p < 0.05$ vs. Ctrl, DMF, and IRI groups, respectively. Ctrl: sham; DMF: dimethyl fumarate; HO-1: heme oxygenase 1; IL: interleukin; IRI: ischemia-reperfusion injury; NF-κB: nuclear factor kappa B; NQO1: NAD(P)H quinone dehydrogenase 1; Nrf2: nuclear factor erythroid 2-related factor 2; TNF-α: tumor necrosis factor alpha.

improved testicular damage in rats with chronic stress-induced depression [66]. Moreover, the NF-κB, IL1b, and TNF-α mRNA levels were reduced, confirming the anti-inflammatory property of DMF. These findings accorded with the data of the present study. Activation of Nrf2 by

DMF might have suppressed inflammation in rats with testicular IRI by suppressing NF-κB.

DMF induced antioxidant and anti-inflammatory effects, which improved testicular histopathological findings and the Johnsen and Cosentino scores. Although testicular weight

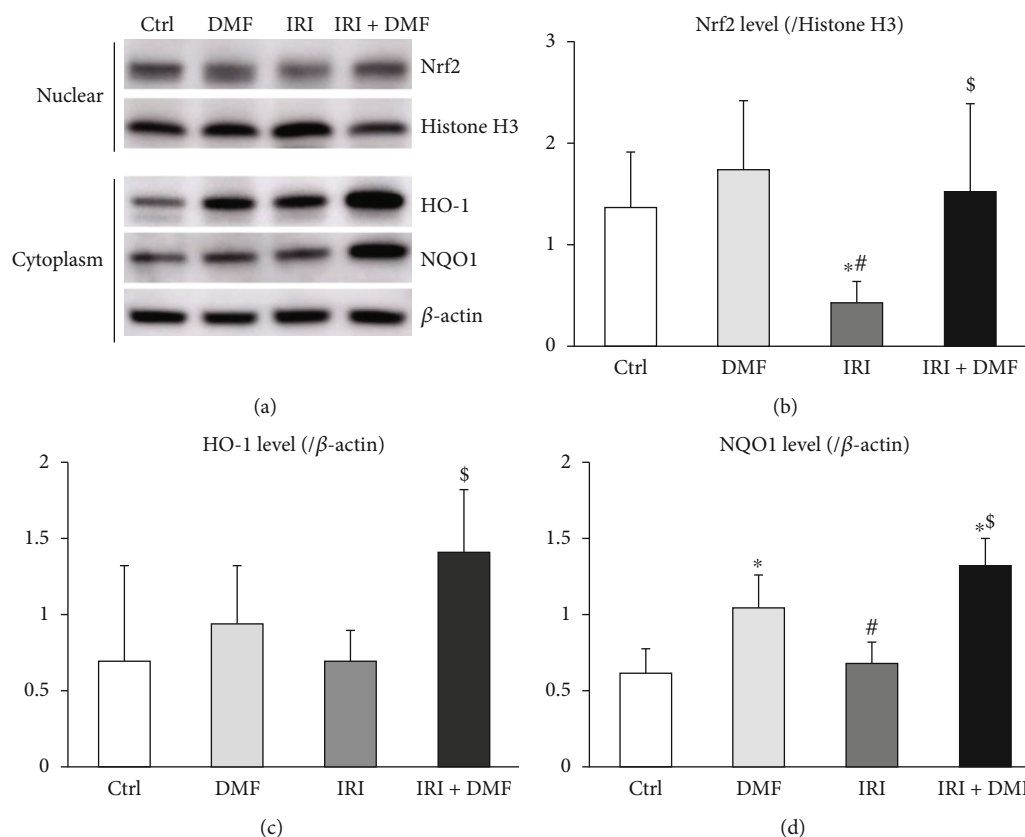


FIGURE 6: Protein expression in rats: (a) representative western blotting for Nrf2, histone H3, HO-1, NQO1, and β -actin; (b) Nuclear Nrf2 levels; (c) cytoplasm HO-1 levels; (d) cytoplasm NQO1 levels. Data: mean \pm standard deviation. * $p < 0.05$, # $p < 0.05$, and \$ $p < 0.05$ vs. Ctrl, DMF, and IRI groups, respectively. Ctrl: sham; DMF: dimethyl fumarate; HO-1: heme oxygenase 1; IRI: ischemia–reperfusion injury; Nrf2: nuclear factor erythroid 2-related factor 2; NQO1: NAD(P)H quinone dehydrogenase 1.

was not improved, the testis-to-body weight ratio was increased by DMF treatment in rats with testicular IRI, suggesting that DMF protects spermatogenesis.

The present study had a few limitations. Firstly, owing to the study duration, it was not possible to conduct a long-term evaluation of spermatogenesis. Secondly, although DMF is clinically used in patients with multiple sclerosis, its optimal concentration and duration of treatment for testicular torsion have not been studied. Thirdly, DMF was administered at a relatively high dosage. Therefore, additional investigation is necessary to determine the most effective dosage and duration of DMF use in the treatment of testicular IRI.

5. Conclusions

DMF activated the Nrf2 signaling pathway. The treatment led to antioxidant and anti-inflammatory effects, which improved testicular damage induced by IRI. DMF may exert protective effects in the testes, emphasizing its potential efficacy against testicular torsion.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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