

M. AL-MAGHREBI¹, W.M. RENNO²

GENISTEIN ALLEVIATES TESTICULAR ISCHEMIA AND REPERFUSION INJURY-INDUCED SPERMATOGENIC DAMAGE AND OXIDATIVE STRESS BY SUPPRESSING ABNORMAL TESTICULAR MATRIX METALLOPROTEINASE SYSTEM VIA THE Notch 2/Jagged 1/Hes-1 AND CASPASE-8 PATHWAYS

¹Department of Biochemistry, Faculty of Medicine-Kuwait University, Jabriyah, Kuwait;

²Department of Anatomy, Faculty of Medicine-Kuwait University, Jabriyah, Kuwait

The aim of the study is to examine the role of matrix metalloproteinases (MMPs) and their inhibitors (TIMP) during testicular ischemia/reperfusion (t I/R). The involvement of the Notch pathway, and their modulation by the antioxidant genistein is also studied. Three groups of male Sprague-Dawley rats were used: sham rats, t I/R rats, and genistein-treated rats (10 mg/kg). The t I/R rat model underwent testicular artery occlusion of the left testis and was subjected to 60 min ischemia followed by 4 h reperfusion. Protein expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured in testicular tissue. Histological examination was performed to assess spermatogenesis. Protein levels of Notch 2, Jagged 1, and hairy/enhancer of split 1 (hes-1) was quantified. The degree of testicular oxidative stress, DNA damage and germ cell apoptosis were also evaluated. T I/R induced severe tubular damage, a significant increase in MMP-2 and MMP-9 expression and decreased expression TIMP-1 and TIMP-2. Genistein treatment normalized the MMP-TIMP imbalance. Rats subjected to t I/R had low total antioxidant capacity of the testis, decreased superoxide dismutase activity, and increased oxidative DNA damage. Enhanced activities of caspase 8, caspase 3 and PARP were also observed during t I/R. Genistein reversed the t I/R-induced suppression of the Notch 2/Jagged 1/hes-1 pathway. Genistein was also able to salvage the testicular structure and function through restoring the MMP-TIMP anti-proteolytic balance, suppressing spermatogenic damage, alleviating oxidative stress and apoptosis. The Notch pathway is partly involved in inhibiting the t I/R-induced testicular impairment.

Key words: *testicular ischemia/reperfusion, metalloproteinases, inhibitors of metalloproteinases, Notch pathway, genistein, spermatogenesis, oxidative stress, apoptosis*

INTRODUCTION

Testicular ischemia is a condition that can lead to permanent spermatogenic damage and critical complications. This condition is represented clinically as testicular torsion (TT), which creates a surgical emergency in order to salvage the testis from serious damage (1). Although the key aim of treatment after ischemia is to restore blood circulation and reperfusion of the testis, this action is also accompanied with negative consequences. Reperfusion may worsen the testicular ischemic injury due to its unfavorable effect on the delicate microenvironment of the testis (2, 3). Malfunction of the germ cells or Sertoli cells may lead to spermatogenic impairment that can be irreversible. Thus, such impairment resulting from testicular ischemia reperfusion (t I/R) injury can be corrected by either preconditioning, selective tissue reperfusion, or pharmacological intervention. The most plausible mechanism for t I/R and hence TT and detorsion (TT/D) is the generation of reactive oxygen species (ROS) that triggers oxidative stress associated with DNA damage followed by germ cell apoptosis (4, 5).

The microenvironment of the seminiferous tubules is protected by the testicular interstitium. An important interstitial component of the testis is the extracellular matrix (ECM), which provides structural integrity to the testis. (6). The ECM has been shown to play a vital role in controlling the morphology, differentiation, and migration of Sertoli cell and thus its overall function. Thus, playing an important role in male reproductive health. ECM remodeling is carried out by a family of calcium and zinc-dependent proteases known as metalloproteinases (MMPs), which are regulated by their specific tissue inhibitors of metalloproteinases (TIMPs) (7, 8). Since MMPs are involved in the degradation of the ECM, it has been suggested that it might have a crucial role in the pathophysiology of several diseases (9-11). More specifically, MMP-2, MMP-9, TIMP-1 and TIMP-2 were found to be highly expressed in the testis especially in the interstitium and in epididymis in a cell-specific expression (12). TIMP-1 associates with the latent form of MMP-9, while TIMP-2 complex with the latent form of MMP-2 (13, 7). This indicates their possible role in the testicular junction restructuring and spermatogenesis maintenance.

The differential expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 have been reported during I/R injury of the heart, liver, and kidney (14-16). However, to our knowledge, no investigations of the relationships between t I/R, MMP-2, MMP-9, TIMP-1 and TIMP-2 have been conducted. Therefore, the current study was designed to determine whether the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 is modulated during t I/R. We used the antioxidant, genistein (an isoflavone from soy extract) (17), to counteract the t I/R-induced oxidative stress, caspase-8 pathway, and to determine its effect on recovering damaged spermatogenesis. We also opted to investigate the role of the Notch 1/Jagged 2/hes-1 pathway for its reported involvement in the differentiation of male germ cells and their survival in the testis (18).

MATERIALS AND METHODS

Animals

Eighteen adult male Sprague-Dawley rats (8 weeks old) were kept in a temperature controlled room with a 12 hour light/12 hour dark cycle in the Animal Facility of the Health Science Center, Kuwait University. The rats were provided with EURodent 14% diet (LabDiet, St. Louis, MO, USA) and water *ad libitum*. Animals were divided into three groups (6 rats/ groups): sham, t I/R + vehicle, and t I/R + genistein. Unilateral t I/R model was induced in the left testis with the contralateral testis as control.

All the experimental and animal handling procedures were approved by the ethics committee on animal research protocol at Kuwait University.

Testicular ischemia reperfusion model and drug treatment

The animal model of t I/R was induced using the testicular artery occlusion model that previously (19, 20). Briefly, animals were anesthetized (anesthetized with Tekam® 50 mg/kg and Rompun® 2 mg/kg administered intraperitoneally (i.p.)). The t I/R procedure consisted of 1 hour ischemia followed by 4 hours reperfusion. Except for sham rats, the testicular artery was occluded using a straight bulldog clamp (700 g pressure) without including the epididymis. Thirty minutes post ischemia, the t I/R group were injected i.p. with a 300 µl of saline, while the genistein-treated rats received an i.p. injection of genistein (10 mg/kg). The sham left testis were exposed through a standard ilioinguinal incision for 1 hour before returning it into the scrotal sac. Rats were sacrificed after the 4 hours of reperfusion by decapitation. Harvested testis were immediately cut into 2 halves. One half was fixed in 10% neutral-buffered formaldehyde solution for histological assessment and the other half was frozen at -80°C for further biochemical investigations.

Dosage

The genistein dosage and i.p. administration was determined as 10 mg/kg body weight based on earlier studies with various doses to reveal the biological effects of genistein (21-23).

Histological examination

The six ipsilateral testes from each rat group were processed for histological analysis. Deparaffinized and rehydrated testicular sections were stained with hematoxylin and eosin (H&E) to reveal the histology of the testis. Four H&E slides from each testis were scored blindly for morphological changes using the Johnsen scoring system (21). Testicular sections were

then decoded after blind examination and the scores were compared amongst the experimental groups.

Terminal 2-deoxyuridine 5-triphosphate nick end-labeling assay (TUNEL)

Paraffin testicular sections were dewaxed by heating and rehydrated with xylene and several grades of ethanol. Sections were then incubated with proteinase K followed by addition of the TUNEL reaction mixture at 37°C. Slides were then mounted with DAPI using the manufacturer's recommendations (Roche-Diagnostics, GmbH, Mannheim, Germany). Slides were analyzed with the LSM 700 confocal laser scanning microscope (Carl Zeiss Micro-Imaging GmbH, Jena, Germany). A total of one hundred seminiferous tubules from each group were assessed for DNA damage in circular cross sections using randomly selected microscopic fields from the six testes in each group. The number of TUNEL-positive nuclei per tubule were counted and expressed as the mean ± S.E.M. Images were taken at 400× magnification to allow for counting.

Biochemical analysis

Frozen testicular tissue was homogenized in a cold lysis buffer of 25 mM Tris-HCl, pH 7.5; 100 mM NaCl, and 1% Nonidet P-40 (NP-40). Just before use an EDTA free protease inhibitor cocktail (Roche-Diagnostics, GmbH, Mannheim, Germany) that inhibit serine, cysteine and acidic proteases but not MMPs was added. Homogenized tissues were incubated on ice for 10 minutes before centrifugation at 3,500 g for 5 minutes at 4°C to remove nuclei and large cell debris. The supernatants were collected in fresh tubes. Protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein lysate concentrations of 25 µg, 50 µg, and 100 µg were used to standardize the ELISA assays for optimum signal detection. A concentration of 50 µg from each protein lysate was used for all ELISA assays.

Evaluation of apoptosis markers

Activities of caspases-8 and -3 were evaluated using their specific colorimetric assays following their manufacturer's recommendations (Sigma Aldrich, St. Louis, MO, USA). Caspase-8 activity were expressed as nmol/min/ml of cell lysate, while caspase-3 activity is presented as µmol/min/ml of cell lysate. Poly (ADP-ribose) polymerase-1 (PARP-1) is involved in the cellular response mechanism to DNA damage. The CST's Pathscan® Cleaved PARP (Asp214) sandwich ELISA kit was used following the manufacturer's recommendations (Cell Signaling Inc., Danvers, MA, USA).

Assessment of testicular oxidative stress

Total antioxidant capacity (TAC) of testicular tissue was determined using a colorimetric assay (Sigma Aldrich, St. Louis, MO, USA) that measures the sample's antioxidative effect when incubated with potent free radical reactions initiated by hydroxyl free radicals. Antioxidant concentration (mM) is calculated relative to the concentration of the Trolox standard curve. Testicular tissue superoxide dismutase (SOD) activity was measured using the SOD determination kit (Sigma Aldrich, St. Louis, MO, USA). Data are expressed as percentage of SOD activity (inhibition rate %).

MMP-2, MMP-9, TIMP-1, and TIMP-2 assays

MMP-2, MMP-9, TIMP-1, and TIMP-2 were analyzed using an ELISA (enzyme-linked immunosorbent assay) commercial

kit (Cedarlane, Burlington, NC, USA). The assays allowed for quantitative measurements of MMP-2, MMP-9, TIMP-1, and TIMP-2 in rat tissue lysates. Data are expressed as ng/ml.

Protein expression of Notch 2/Jagged 1/hes-1

Protein expression of members of the Notch signaling pathways were measured using a quantitative ELISA assay (Cedarlane, Burlington, NC, USA). Data are expressed as ng/ml.

Statistical analysis

Any subjective bias was avoided by following a blind approach during the experimental procedures and H&E scoring. Data are presented as mean \pm S.E.M. and analyzed using the GraphPad Prism v6.0 statistical program (GraphPad Software Inc., San Diego, CA). ANOVA followed by the Holm-Sidak's multiple comparisons test for comparison of means for all groups data. One-way ANOVA test was performed with Bonferroni *post hoc* for multiple comparisons (SPSS for Windows v. 12.0, Chicago, Ill., USA) was used to analyze the H&E scores. Statistically significant difference was set at $P < 0.05$.

RESULTS

Testicular histology and spermatogenesis

Light microscopy analysis of testicular sections were performed at lower (10 \times , Fig. 1A-1C) and at higher (40 \times , Fig. 1D-1F) magnifications. The sham testis revealed normal histology

with no disruption to the testicular interstitium nor to the structure of the seminiferous tubules (Fig. 1A and 1D). While, testicular tissues from the t I/R group displayed moderate to severe damage to the seminiferous epithelium with epithelial sloughing, luminal blockade by sloughed cells and debris, and lack of proper stages of spermatogenic organization (Fig. 1B + 1E). In the genistein-treated rats, the testis displayed an overall improved histological appearance with normal seminiferous tubule morphology, improved spermatogenic cellular organization, and recovery of interstitial space morphology (Fig. 1C + 1F).

The testis morphology from the three groups was parallel to the evaluation of spermatogenesis determined using the mean value of modified Johnsen scores. The t I/R testes had significant low scores compared to the sham group (5.75 ± 0.25 vs. 9.5 ± 0.34 ; $P < 0.0001$). In contrast, the genistein-treated testes exhibited significantly higher score compared to the t I/R testes (7.20 ± 0.37 vs. 5.75 ± 0.25 ; $P < 0.002$). This suggests that t I/R-induced disruption of the testicular structure and epithelium is accompanied with reduced spermatogenesis.

Expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in testicular tissue

The testicular tissue levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the left and right testes were determined by quantitative ELISA (Fig. 2). No statistically significant difference was found between proteins levels in the right contralateral testes from the 3 rat groups ($P > 0.05$). The tissue levels of MMP-2 and MMP-9 were significantly higher in the t I/R rat testis than in sham testis (18.23 ± 1.5 vs. 9.47 ± 0.63 ; $P < 0.0001$ and 113.5 ± 12.4 vs. 77.2 ± 3.74 ; $P = 0.0002$,

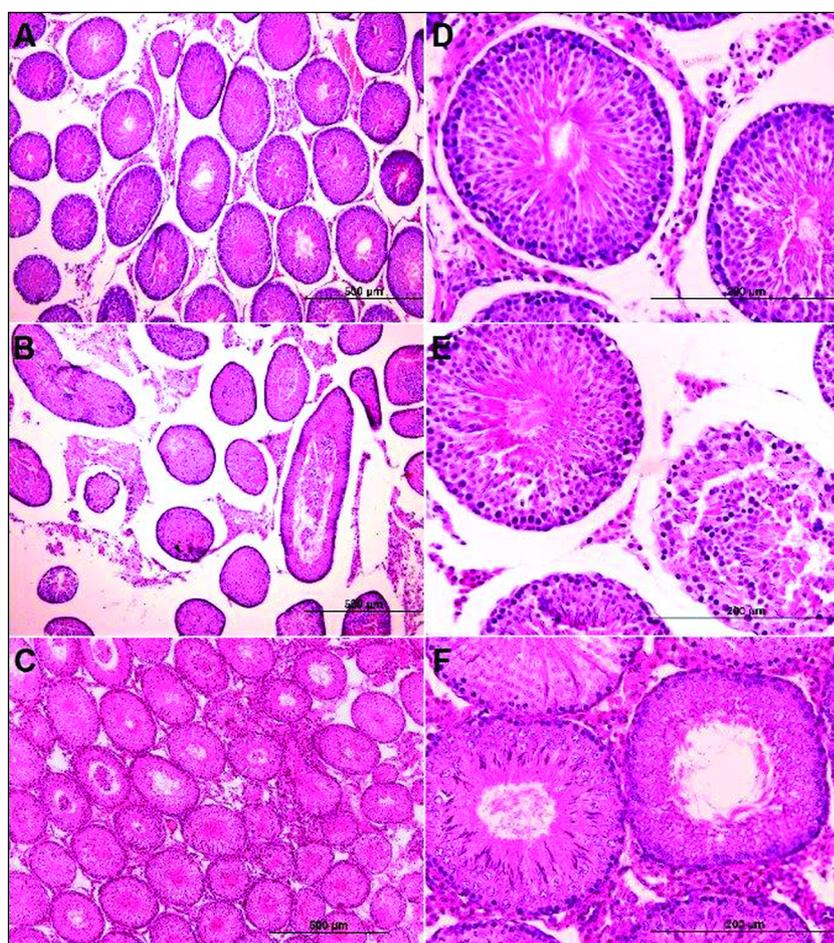


Fig. 1. Histopathological examination of the H & E sections of the ipsilateral testicular rat tissues. (A and D) Showing normal histological morphology of the testes from sham group. The images represent a Johnsen score 10 indicating normal spermatogenesis and numerous spermatozoa lining the central lumen. (B and E) The testes of the t I/R group showed histological changes in terms of disruptions in seminiferous epithelial cell associations, epithelial sloughing, and luminal blockade by sloughed cells and debris. The images represent a Johnsen score 5 indicating the presence of very few spermatocytes with no spermatids or spermatozoa present. (C and F) Showing the genistein-treated group (10 mg/kg, i.p.) with remarkable improvement in the structural and cellular morphology of the seminiferous tubules and interstitial space. The images represent a Johnsen score 7 indicating preliminary recovery of spermatogenesis with few spermatids present. The P value for the Johnsen score comparison between groups is: $P < 0.0001$ t I/R versus sham and genistein groups; $P < 0.002$ genistein-treated group versus t I/R group. A, B and C = 100 \times magnification; D, E and F = 400 \times magnification.

respectively) (Fig. 2A and 2B). Treatment with genistein significantly lowered the levels of MMP-2 and MMP-9 compared to the t I/R group (9.97 ± 0.42 vs. 18.23 ± 1.5 ; $P <$

0.0001 and 80.43 ± 5.7 vs. 113.5 ± 12.4 ; $P = 0.0003$, respectively). In contrast, the levels of both TIMP-1 and TIMP-2 were decreased during t I/R compared to sham (10.5 ± 0.35 vs.

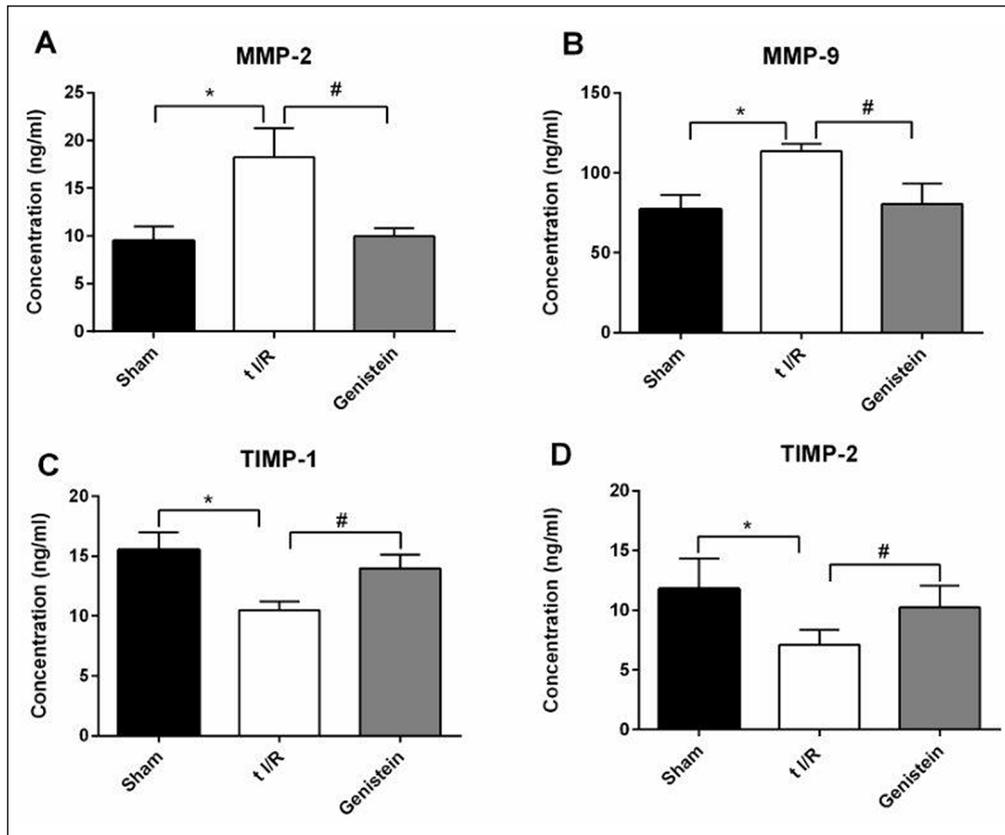


Fig. 2. Expression of MMP-2, MMP-9, TIMP-1, and TIMP2. The expression of MMP-2 (A) and MMP-9 (B) were significantly increased, while TIMP-1 (C) and TIMP-2 (D) were decreased during t I/R compared to sham (* $P < 0.0001$, $P = 0.0002$, $P < 0.0001$, and $P = 0.008$, respectively). Genistein treatment corrected the MMP/TIMP balance toward the anti-proteolytic direction with lowered MMP-2 (A) and MMP-9 (B) and increased TIMP-1 (C) and TIMP-2 (D) (# $P < 0.0001$, $P = 0.0003$, $P < 0.001$, and $P = 0.04$, respectively).

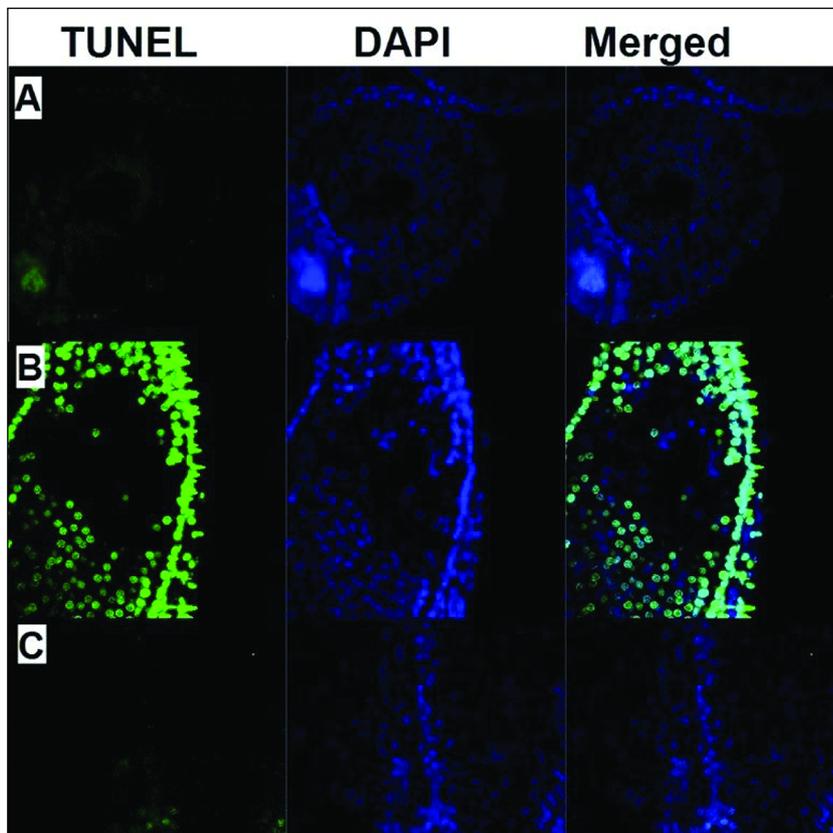


Fig. 3. Fluorescent images of TUNEL stained testicular nuclei. Higher number of TUNEL-positive nuclei were observed in the t I/R group (B) in comparison to the sham group (A) ($P < 0.0001$) and to the genistein-treated group (C) ($P < 0.007$). One hundred seminiferous tubules from each group were assessed for DNA damage using randomly selected microscopic fields from sections taken from the six testes in each group. Magnification at $\times 400$.

15.5 ± 0.6 ; $P < 0.0001$ and 7.09 ± 0.63 vs. 11.77 ± 1.04 ; $P = 0.0083$, respectively) (Fig. 2C and 2D). Their levels were reverted to almost sham levels upon genistein treatment (13.96 ± 0.52 vs. 10.5 ± 0.35 ; $P < 0.0011$ and 10.26 ± 0.80 vs. 7.09 ± 0.63 ; $P = 0.0403$, respectively).

Germ cell apoptosis

Induction of t I/R greatly increased the number of TUNEL-positive cells in comparison to sham (70.0 ± 5.0 vs. 3.0 ± 1.0 ; $P < 0.0001$) (Fig. 3). In the genistein-treated testis, there were significantly fewer apoptotic nuclei compared with those in the t I/R group (16.0 ± 2.0 vs. 70.0 ± 5.0 ; $P < 0.007$). The contralateral right testis of both t I/R and genistein-treated rats showed no TUNEL-positive cells (data not shown).

Mean testicular caspase-8 activity showed an 11-fold increase in activity upon t I/R injury when compared to sham (3.92 ± 0.27 vs. 0.35 ± 0.04 ; $P < 0.0001$) (Fig. 4A). This was significantly decreased after genistein treatment (3.92 ± 0.27 vs. 0.18 ± 0.03 ; $P < 0.0001$). Caspase-3 activity followed a similar pattern (Fig. 4B). In the t I/R group, a significant increase was observed in comparison to sham (17.75 ± 0.65 vs. 9.93 ± 0.47 ; $P < 0.0001$). After genistein treatment, caspase-3 activity was reduced to sham levels (12.35 ± 0.56 vs. 17.75 ± 0.65 ; $P < 0.0001$).

PARP-1 is an established substrate for caspase-3 (25). During apoptosis, PARP-1 is cleaved by caspase-3 into a 24 kDa

and 85 kDa fragments. Thus, measuring the levels of PARP-1 85 kDa fragment is indicative of apoptosis induction. Cleaved PARP-1 was detected in high levels upon t I/R injury compared to sham (5.99 ± 0.44 vs. 4.39 ± 0.32 ; $P < 0.0047$) (Fig. 4C), but was reverted to sham levels after genistein treatment (3.75 ± 0.19 vs. 5.99 ± 0.44 ; $P < 0.0011$).

Testicular oxidative status

The oxidative status of the ipsilateral left testis was severely hampered during t I/R as indicated by the significant decrease in the TAC levels and SOD activity (Fig. 5). The mean TAC concentrations (mM) were 0.343 ± 0.03 for sham, 0.047 ± 0.01 for t I/R, and 0.464 ± 0.03 for genistein treatment. The t I/R concentrations were significantly low compared to sham ($P < 0.0001$) and to genistein ($P < 0.0001$). The mean SOD values (% of inhibition rate) for the t I/R group were significantly lower than sham (81.4 ± 0.52 vs. 95.7 ± 1.46 ; $P = 0.0001$), and significantly lower than genistein treatment (81.4 ± 0.52 vs. 89.42 ± 2.15 ; $P = 0.0065$).

Expression of the Notch pathway

The expression level of Notch 2, jagged 1 and hes-1 (Fig. 6) were significantly down regulated during t I/R injury, compared with the sham but were normalized after genistein treatment. The

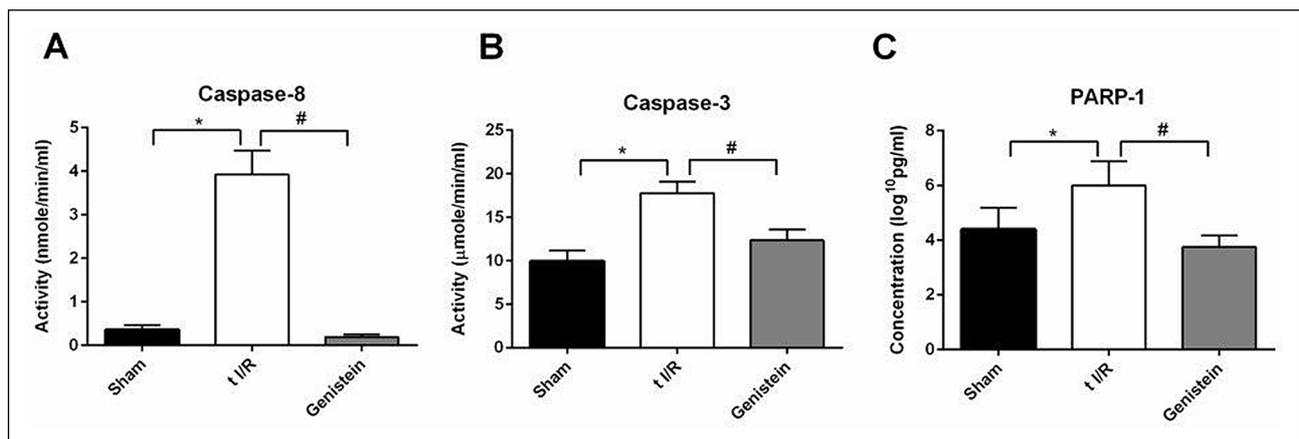


Fig. 4. Induction of the caspase-8 pathway during t I/R. Increased expression of the initiator caspase-8 (A), the executor caspase-3 (B), and cleaved PARP-1 (C) (caspase-3 substrate) were observed in the t I/R group (* $P < 0.0001$, $P < 0.0001$, and $P = 0.005$, respectively). Genistein treatment suppressed the activation of this apoptotic pathway (# $P < 0.0001$, $P < 0.0001$, and $P = 0.001$, respectively).

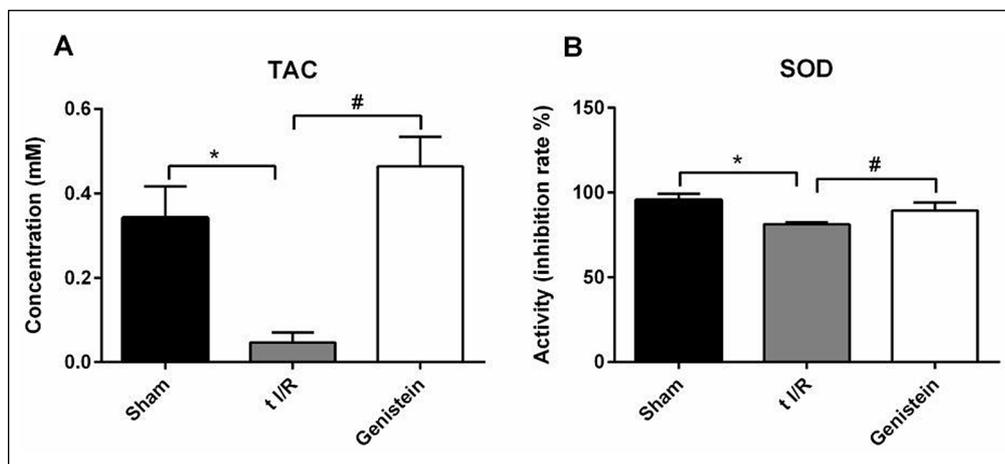


Fig. 5. Total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity in rat testicular tissue. The levels of TAC (A) and SOD (B) were lower in t I/R compared to sham (* $P < 0.0001$ and $P = 0.0001$, respectively), which were recovered by genistein treatment (# $P < 0.0001$, and $P = 0.006$, respectively).

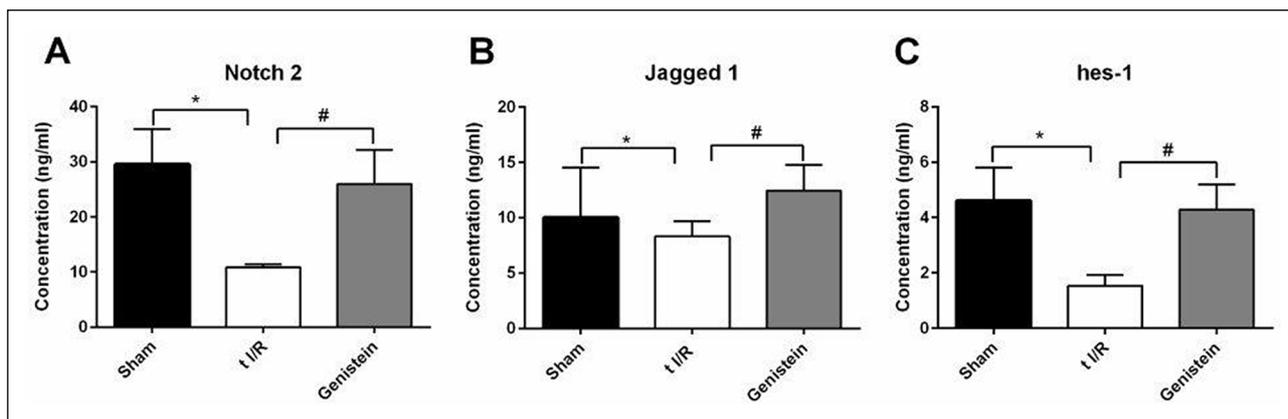


Fig. 6. Testicular tissue levels of Notch 2/Jagged 1/hes-1. The expression level of Notch 2/Jagged 1/hes-1 decreased significantly in the t I/R group, compared with the sham group (* $P = 0.0004$, $P = 0.0158$, and $P = 0.0017$, respectively), but were normalized in the genistein-treated group (# $P = 0.0014$, $P = 0.0158$, and $P = 0.0007$, respectively).

level of Notch 2 in the t I/R group were lower than in the sham (10.86 ± 0.28 vs. 29.54 ± 2.62 ; $P = 0.0004$) and genistein treated rats (10.86 ± 0.28 vs. 25.99 ± 2.678 ; $P = 0.0014$). The protein expression of the Notch 2 ligand, Jagged 1, was also reduced in rats subjected to t I/R when compared to sham (8.31 ± 0.67 vs. 12.11 ± 0.77 ; $P = 0.0158$), which was also elevated to sham level after genistein treatment (12.11 ± 0.77 vs. 12.44 ± 1.03 ; $P = 0.0158$). The hes-1 expression decreased significantly in the t I/R group when compared to sham (1.53 ± 0.2 vs. 4.60 ± 0.49 ; $P = 0.0007$), but then peaked in the genistein-treated group (4.28 ± 0.41 vs. 4.6 ± 0.49 ; $P = 0.0012$).

DISCUSSION

The damage to the germinal epithelium caused by t I/R injury include tubular atrophy, atrophy of Leydig cells, malformation at the spermatid level, and decreased spermatogenesis (26). The ECM is vital to the structure and function of the testis. It regulates the expression of the tight junction proteins between Sertoli cells (27). These tight junctions are responsible for creating the testis-blood barrier, which is in turn responsible for the translocation of the elongated mature spermatids. In the testis, MMP-2 and MMP-9 are secreted by inflammatory and Sertoli cells and are capable of destroying all of the ECM components of the basement membrane (12, 28). Thus, remodeling of the testicular ECM is a delicate process that requires fine tuning of the levels of both MMP-2 and MMP-9 and their specific TIMPs (TIMP-2 and TIMP-1, respectively).

Over expression of these MMPs and their TIMPs has been associated with several ischemia reperfusion models. MMP2 overexpression has been detected in I/R of the heart (16). Renal I/R injury is also reported to increase MMP-2 and MMP-9 protein activities (14). MMP-2 and MMP-9 were also significantly up-regulated in skeletal muscle I/R associated with degradation of muscle basement membranes (29). Inhibition of MMPs by a non-selective MMP inhibitor diminished I/R after experimental lung transplantation (30). Similarly, the novel MMP inhibitor ONO-4817 was able to suppress hepatic I/R injury-induced expression of MMP-2 and MMP-9 but not TIMP-2 mRNA expression (31). Results from our study are in agreement with other I/R models in terms of MMP-2 and MMP-9 up-regulation during t I/R. In addition, the expression of TIMP-1 and TIMP-2 were also significantly reduced during our t I/R model.

Another hallmark of t I/R is the structural damage to the seminiferous tubules seen with histological analysis. Usually, prominent ECM degradation is observed in tissues when an imbalance is created between MMPs and TIMPs, where MMP activity becomes more noticeable. In the present study, irregular structure of seminiferous tubules, absence of mature spermatids, and interstitial compartment filled with ECM were observed with H&E stained testicular tissue sections. This suggests that t I/R-induced MMP expression could have shifted the protease-protease inhibitor balance in the direction of proteolytic activity and ECM degradation. A knockout mice of the extracellular matrix metalloproteinase inducer showed altered gene expression of MMPs and their inhibitors leading to an aberrant tissue ECM remodeling (32). Besides degrading the ECM components, MMPs can also regulate the secretion of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) (33) and interleukin 1 β (IL-1 β) (34), adhesion molecules (35), and E-cadherin (36). An earlier study by our group have demonstrated the release of TNF- α , IL- β and IL-6 during t I/R injury (20). This further consolidates the fuller picture of the underlying mechanism of t I/R. Furthermore, a recent work by our group had also demonstrated an association between increased levels of the adhesion molecules, ICAM and VCAM, with increased inflammation and spermatogenic damage during t I/R (unpublished data).

Testicular oxidative stress is considered as a central cause leading to infertility. This association has been attributed to the increased levels of ROS in infertile men (37, 38). Testicular dysfunction and impaired spermatogenesis has also been associated with increased oxidative stress during stress-induced depression, which is considered a major factor contributing to male infertility (39). High levels of TAC and advanced oxidation protein products (AOPP) associated with high levels of seminal MMP-9 but not seminal MMP-2 were detected in infertile than in fertile men (40). Thus, utilization of exogenous, food-extracted compounds with antioxidant properties could confer testicular protection. Genistein is an isoflavone that is abundant in soy extracts (41). It is known for its antioxidant and antiinflammatory properties, it acts as a protein kinase inhibitor (42), and it exhibits a neuroprotective effect in subjects feeding on soya-rich diet (43). It has been shown that it has an estrogen-like activity and is an estrogen receptor agonist (44). It was also reported that genistein plays an important role in steroidogenesis by inhibition of progesterone synthesis and reducing secretion of cortisol and corticosterone (45). Genistein has attracted scientific interest due to its potential benefits in protecting against several types of cancer

like cervix (46), prostate (47), and breast (48). Genistein was also shown to have a protective role against gamma irradiation-induced testicular dysfunction through a mechanism that involves its strong antioxidant property (49). As a soy isoflavone, genistein is suggested to partition the lipid bilayer of the plasma membrane where it would dramatically decrease lipid fluidity, delay the diffusion of ROS, and thus result in reducing of the kinetics of free radical reactions. Interestingly, low doses of genistein was suggested to augment fertility by stimulating acrosome reaction without affecting sperm morphology (50). It has also been suggested that genistein is capable of shifting the MMP/TIMP proteolytic balance toward the antiproteolysis in the media of actively proliferating Ehrlich tumor cells (51). Furthermore, genistein treatment down-regulated the mRNA expression of all MMP in the metastatic breast cancer cell line MDA-MB-231 and most of MMPs in MCF-7 breast cancer cell line (52). In this study, genistein treatment reversed the testicular damage induced by the t I/R injury through suppressing the overexpression of MMP-2 and MMP-9 and enhancing the levels of TIMP-1 and TIMP-2. This effect has tipped off the MMP/TIMP balance in favor of the anti-proteolytic activity and improved spermatogenesis.

As a consequence of t I/R-induced ROS generation, the caspase-dependent apoptosis pathways are activated leading to germ cell death. Here we have shown that caspase-8 pathway was activated by the t I/R injury. High levels of the initiator caspase-8 and the executioner caspase-3 were measured during t I/R, which were reduced by genistein treatment. Caspase-8 deficient mice were found to be resistant to death receptor-induced apoptosis (53). Cerebral I/R is thought to be partly mediated by caspase-3 activation resulting in DNA fragmentation (54).

The Notch pathway is thought to regulate the rate of spermatogenesis and coordinate the intricate cell proliferation and differentiation during the spermatogenic cycle (55). The localization of several Notch signaling proteins in the adult mouse testis is indicative of the crucial role for Notch in the regulation of spermatogenesis (56). The three Notch receptors (Notch 1, 2, and 3) were localized to the cell surface of Spermatogonia, whereas Jagged 1, a Notch ligand, is only expressed in Sertoli cells. Thus, Jagged 1 can activate Notch by mediating the interaction between Sertoli cells and Spermatogonia through any of its three Notch receptors (57). Upon binding of Jagged 1 to the Notch receptor, the latter translocates to the nucleus resulting in up-regulation of the hairy/enhancer of split 1 (*hes-1*) gene expression, which in turn activates NF- κ B gene transcription (58). Cultured rat testis treated with either anti-Notch 1 or anti-Jagged 2 antibody disrupted spermatogenesis, which supports their important role in the process of spermatogenesis (18). The same study also demonstrated that patients diagnosed with spermatogenic arrest did not express any Notch 1 in their testicular tissues, whereas Jagged 2 was detected. This shows that lack of Notch 1 but not its ligand, Jagged 1, is a must for germ cell survival. In the current study, the decline in protein expression of Notch 2/Jagged 1/*hes-1* in the t I/R group could have partly been responsible for the decrease in the number of spermatocytes/ spermatids, the low Johnsen score, and the induced germ cell apoptosis. Genistein treatment restored proper spermatogenesis by up-regulating the expression of the Notch 2/Jagged 1/*hes-1* proteins.

In conclusion, the results of this study suggest that t I/R-induced spermatogenic damage could be mediated by the proteolytic activity of the MMP/TIMP system resulting in the degradation of the testicular ECM and down regulation of the Notch 1/Jagged 2/*hes-1* pathway. The testicular protection demonstrated by genistein offered testicular protection via attenuation of oxidative stress and inhibition of the caspase-8 apoptosis pathway. Thus, it can be suggested that genistein could

have clinical applicability in testicular torsion patients, however, further clinical studies will be needed.

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Author's address: Dr. May Al-Maghrebi, Department of Biochemistry, Faculty of Medicine, Kuwait University, P.O. Box: 24923, Safat 13110, Kuwait
E-mail address: malmaghrebi@hsc.edu.kw