Eurasian Journal of Molecular and Biochemical Sciences

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Protective Effects of Acacetin on Testicular Ischemia Reperfusion Injury

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Cite: *Kenger Y, Şimşek H. Protective Effects of Acacetin on Testicular Ischemia Reperfusion Injury. Eurasian Mol Biochem Sci 2024;3(2): 22-30*

Received: 12 July 2024, Accepted: 16 February 2025 *DOI: 10.5281/zenodo.14932929*

Abstract

Objective: Testicular torsion is a medical problem primarily seen in newborns and adolescents and requires urgent treatment. It is anticipated that the use of antioxidants may be an effective treatment option. Acacetin (ACA) is a natural flavonoid of plant origin and has attracted research attention in recent years with its powerful antioxidant properties. This study aims to investigate the therapeutic effects of Acacetin in testicular ischemia-reperfusion (IR) injury. Materials and Methods: Twenty-eight Wistar rats (male, 12 weeks, 250-300g) were used in the current study. After 1 hour of ischemia, 4 hours of reperfusion was performed. Acacetin was administered intraperitoneally at a single dose of 25mg/kg. Testicular tissues were analyzed using genetic, biochemical, and histological methods. Results: IR caused damage to testicular tissues by significantly increasing the levels of NF-κB ($p<0.05$), Caspase-3 ($p<0.01$), TOS ($p<0.001$), OSI ($p<0.001$) and decreasing the levels of Bcl-2 and increasing the levels of TAS, although not significantly. ACA exhibited protective properties against IR damage in testicular tissues by reversing the effects of IR. When ACA was applied together with IR, Caspase-3 ($p<0.01$), TOS ($p<0.001$), and OSI ($p<0.001$) levels decreased significantly compared to the IR group. While NF-κB levels decreased insignificantly, TAS and Bcl-2 levels increased insignificantly. Conclusions: ACA provides protective properties against testicular IR injury.

Keywords: Acacetin, apoptosis, ischemia reperfusion, oxidative stress, testis

Introduction

Testicular torsion (TT) is a medical emergency that primarily affects newborns and young adolescents. It causes testicular injury due to torsion of the spermatic cord and its components, initially in venous blood flow and finally in arterial blood flow. Prompt diagnosis and early surgical treatment are very important in managing this emergency. Despite successful surgical intervention, testicular torsion causes ischemic damage and detorsion causes reperfusion damage, causing some structural and biochemical changes in the tissue (1). The testicle is particularly prone to ischemic movements due to anatomical reasons (2). The annual incidence of TT is approximately 3.8 per 100,000 men under 18 years of age. Studies are showing that TT may be hereditary in cases of bilateral torsion. If not treated within 4 to 6 hours, spermatogenic cell loss occurs and undesirable

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consequences such as infertility, necrosis, and testicular loss may occur. The degree of bending of the spermatic cord is very effective in the occurrence of these conditions (1).

The term ischemia and reperfusion (IR) refers to the restoration of perfusion following a decrease in arterial blood flow to the tissues and, as a result, reoxygenation (3). Experimental studies examining the mechanisms and consequences of IR use surgical methods to block specific vessels in healthy animals, so these models are indicated to better understand the mechanisms involved in IR-induced damage (4). In IR injury, the overproduction of reactive oxygen species (ROS) is thought to play a critical role in the loss of ipsilateral testicular spermatogenesis. ROS can cause tissue damage through cell membrane lipid peroxidation, protein denaturation, and DNA disorder (5). Surgical repair of the testicle from IR damage, as well as the definition of antioxidants, free radical scavengers, and pharmacological agents that can be used in treatment, are significant clinical goals.

Flavonoids, which are natural phenolic compounds found in many fruits and vegetables, have many beneficial effects, and antioxidant, anti-inflammatory, anti-mutagenic, anti-cancer, and anti-bacterial properties stand out among these effects (6). Flavonoids play a significant role in disease and satisfactory health management (7). Acacetin (5,7-dihydroxy-4'-methoxyflavone, ACA) is a plant-derived flavonoid that has recently attracted worldwide attention (8). This interest is because ACA is naturally abundant in acacia honey and citrus fruits and has a wide range of biological and pharmacological effects such as antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, neuroprotective, and cardioprotective (9,10). The most important distinguishing feature of ACA is that it has a good curative effect with almost no toxic reactions (11). The anti-inflammatory effect of ACA lies in its inhibition of NF-κB (12). Since ACA is a newly focused active ingredient, its testicular ischemia-reperfusion effect is not yet clearly understood.

The current study aims to investigate the therapeutic effect of ACA by modeling testicular torsion, which is very common in newborns and young adolescents, in rats.

Material and Method

Ethical Statement: To implement the study protocol, ethical permission was obtained from the Aksaray University Experimental Animals Local Ethics Committee (08.06.2022, 2022/5-19) and the Ethics Committee principles were meticulously followed in all experimental procedures.

Experimental Groups: In the current study, 28 Wistar rats (male, 12 weeks, 250–300g) obtained from Aksaray University Experimental Animal Application Research Center were used. During the study, rats were housed under standard conditions in ventilated rooms with constant temperature, 50±5% humidity, and laboratory conditions with a 12-hour light±dark cycle. The rats were randomly divided into four groups, 7 in each group:

1: Control

2: Acacetin (ACA)

3: Ischemia/Reperfusion (IR)

4: Ischemia/Reperfusion + Acacetin (IR+ACA)

All analyses were carried out in Aksaray University Faculty of Medicine laboratories and Aksaray University Scientific and Technological Application and Research Center (ASÜBTAM) laboratories.

Testicular Ischemia-Reperfusion Injury Model: Experimental IR models can be created in many organs (13). Rats were operated under Ketamine/Xylazine anesthesia and sterile conditions. Testicular torsion was created through a right scrotal incision, by finding the testicle and rotating it 720° clockwise (two full

turns), and it was fixed to the scrotum skin with 6.0 silk. At the end of the torsion period, the testicles were detorsioned and fixed to the scrotum skin with 6.0 silk. The scrotum skin was closed with 4.0 silk. The protocols lasted 5 hours, including 1 hour of ischemia and 4 hours of reperfusion (14).

Acacetin application: Acacetin (Cayman, CAS Number: 480-44-4, ≥98%, Michigan, U.S.A.) was dissolved in normal saline. A single dose of 25 mg/kg was administered intraperitoneally (15).

- In the ACA group, blood and testicular tissues were taken 2 hours after Acacetin application.

- In the IR+ACA group, Acacetin was administered at the 2nd hour of reperfusion, and blood and testicular tissues were taken 2 hours later. Thus, the reperfusion time was completed in 4 hours and the active ingredient was able to show its effect.

Collection of Samples: At the end of the whole experimental period, blood samples were taken from the abdominal aortas of rats anesthetized with Ketamine/Xylazine (75 mg/kg-10 mg/kg) and placed in anticoagulant-free biochemistry tubes. Serum samples obtained by centrifuging blood samples at 5,000 rpm for 15 minutes at +4°C were and stored at -80°C. Immediately after the rats were exsanguinated, the right and left testicular tissues of each rat were cleaned of the surrounding connective and fatty tissues and completely removed in a standard manner. The removed right testicular tissue was stored at –80°C for mRNA analysis. Testicular tissue was placed in 10% formaldehyde to be used in histopathological examinations.

Tissue Homogenization for Biochemical Analysis: To obtain 1:10 (w/v) homogenate from testicular tissues, they were homogenized in 1.15% potassium chloride (KCl) with a homogenizer (MiuLab, Zhejiang, China). Homogenates were centrifuged (+4°C, 1000 × g', 15 min), and the supernatant obtained was used for biochemical analysis. Inflammation and oxidative stress markers were analyzed from the obtained supernatants.

Oxidative Stress Index: Oxidant capacity (TOS) and antioxidant capacity (TAS) levels were analyzed from blood serum using commercial kits and according to the manufacturer's instructions (Rel Assay Diagnostic, Gaziantep, Turkey). The measurement method of the kits was developed by Erel. TAS was expressed as mmol Trolox Eq/mg protein and TOS as μmol H2O2 Eq/mg protein (16,17). Determination of oxidative stress index (OSI) was performed according to the previous study (18).

PCR: RNA was isolated from the testicular tissue samples taken with a commercial kit (Hydra Biotechnology, HY-GRNA-250, Van, Türkiye). A commercial kit (Atlas Biotechnology, Cat No: C03-01-05, Ankara, Türkiye) was used to obtain cDNA from RNAs. To demonstrate the inflammatory effect from cDNAs, the NF-κB gene expression level, as well as the expression levels of the apoptotic Caspase-3 and anti-apoptotic Bcl-2 genes in the cell death pathway, where NF-κB is effective, were determined by using specific primers with the quantitative PCR method. ß-actin was used as the reference gene. Expression levels of the studied genes (Table 1) were measured.

Table 1. Primer Sequence

Gene	Primer Sequence
NF-κB	F: 5'- CAC TGT CAA CAG ATG GCC C -3' R: 5'- GAT AAC CTT TGC AGG CCC CA -3'
Caspase-3	F: 5'- GAC TGC GGT ATT GAG ACA GA-3' R: 5'- CGA GTG AGG ATG TGC ATG AA-3'
Bcl-2	F: 5'- GTG GAT GAC TGA GTA CCT GAA C - 3' R: 5' - GCC AGG AGA AAT CAA ACA GAG G - 3'
β-Actin	F: 5'- CTA TCG GCA ATG AGC GGT TCC -3' R: 5'- TGT GTT GGC ATA GAG GTC TTT ACG -3'

Before the PCR stage, optimum PCR conditions specific to each gene region were determined, taking into account the %GC, Tm (Melting Temperature), and bp length values of each gene obtained as a result of the

design of the primers of the genes to be analyzed. The Gradient PCR step was carried out to determine the optimum conditions with the Applied Biosystems PCR device. Then, PCR was performed with the Applied Biosystems PCR device using the PCR conditions determined for each gene region. PCR mixtures (7 µl) were electrophoresed on a 2% agarose gel and then stained with SafeView Classic (ABM, G108, Richmond, Canada). Gels were scanned with a UV scanner and intensities were measured with the ImageJ program (19).

Light microscopy examination: Testicular tissues were kept in a 10% neutral formalin buffer for 24 hours. After tissue tracing, five µm thick sections were taken from the paraffin blocks with a microtome. The slices were placed on slides and stained with hematoxylin and eosin (H&E) stains. The stained samples were examined and photographed with an Olympus Cx 43 microscope (Japan).

Statistical analysis: Statistical analysis of the data obtained from testicular tissues was performed with SPSS 20.0 (IBM, NY) program. One-way ANOVA and Tukey's post hoc tests were used for group comparison. Data are presented as mean±SE. Statistical significance was accepted at three levels; $p<0.05$, $p<0.01$ and $p<0.001$.

Results

Inflammation Findings: NF-κB mRNA transcription level was determined from testicular tissues as an indicator of inflammation (Figure 1). NF-κB mRNA transcription level increased significantly in the IR group compared to the control group ($p<0.05$). Although there is a decrease in the NF-κB mRNA transcription level in the IR+ACA applied group, it is insignificant.

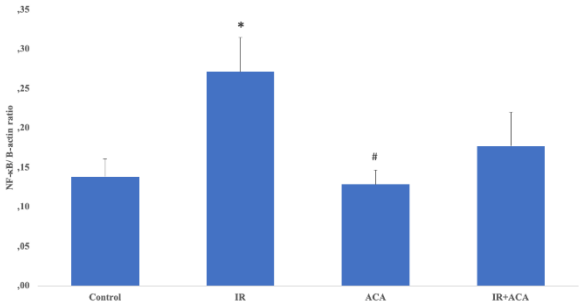

Figure 1. Effects of ischemia-reperfusion (IR) and Acacetin (ACA) applications on NF-κB mRNA transcription levels in rat testicular tissues. Values are given as mean ± SE. Control vs. others: *p < 0.05, Others with IR: #p < 0.05

Apoptotic Findings: To determine the level of apoptosis in testicular tissues, apoptotic Caspase-3 and anti-apoptotic Bcl-2 mRNA transcription levels were determined (Figure 2). Caspase-3 mRNA transcription level increased significantly in the IR group compared to the control group ($p<0.01$). Compared with the IR group, there was a significant decrease in Caspase-3 mRNA transcription level in the ACA and IR+ACA group ($p<0.01$). There was no significant change between groups in Bcl-2 mRNA transcription level.

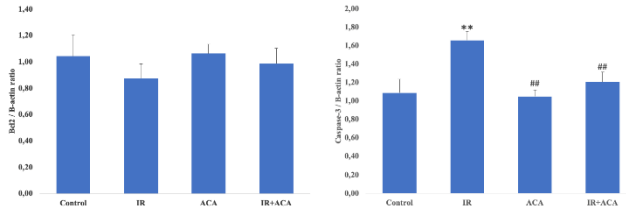

Figure 2. Effects of ischemia-reperfusion (IR) and Acacetin (ACA) applications on Caspase-3 and Bcl-2 mRNA transcription levels in rat testicular tissues. Values are given as mean ± SE. Control vs. others: **p < 0.01; Others with IR: ##p < 0.01

Oxidative Stress Findings: TAS, TOS, and OSI were determined to determine the oxidative stress level from testicular tissues (Figure 3). It was observed that there was no significant difference between the TAS values of the Control, IR, and IR+CA groups, but the TAS values of the ACA group increased significantly compared to

control groups. TOS values increased in the IR and IR+ACA groups compared to the control group ($p<0.001$). TOS values decreased in the ACA and IR+ACA groups compared to the IR group ($p<0.001$). There is also a difference between ACA and IR+ACA groups ($p<0.05$). OSI values increased in the IR and IR+ACA groups compared to the control group ($p<0.001$). OSI values decreased in the ACA and IR+ACA groups compared to the IR group ($p<0.001$). There is also a difference between ACA and IR+ACA groups ($p<0.01$).

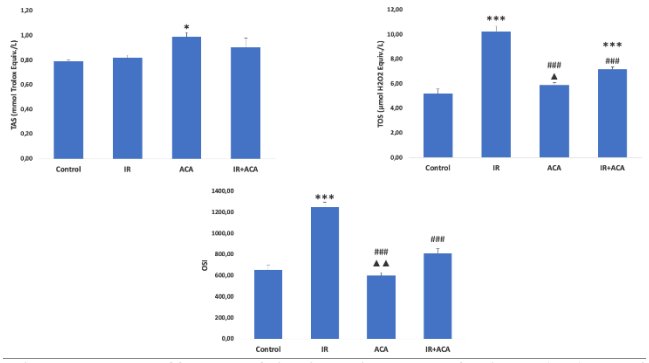

Figure 3. Effects of ischemia-reperfusion (IR) and Acacetin (ACA) applications on oxidative stress levels in rat testicular tissues. Values are given as mean ± SE. Control vs. others: *p < 0.05, ***p < 0.001; Others with IR: ###p < 0.001, ACA vs. IR+ACA: ▲p < 0.05, ▲▲ p < 0.01

Light Microscope Findings: Seminiferous tubules, germinal epithelial cells, and interstitial cells were evaluated by H&E staining in rat testicular sections taken from control, IR, ACA, and IR+ACA groups. When the control group's testicular sections were examined, they showed healthy tissue architecture. Sertoli cells, spermatogonia, primary spermatocytes, spermatids, and spermatozoa within the seminiferous tubule appeared normal (Figure 4A). Atrophy in the seminiferous tubules and deterioration of the epithelium were observed in the testicular tissues of the IR group. Loss and vacuolization in the germ cells of some tubules were noted. Specifically, edematous areas with increased congestion and bleeding were detected (Figure 4B). No pathological changes were observed in the group treated with ACA alone. Sperm cells with normal morphology were seen in the lumen of the seminiferous tubules (Figure 4C). When the IR+ACA group was evaluated, the majority of tubules had a normal histological appearance and there was an increase in germ cell density. Further, congestion, bleeding, and edema were significantly reduced (Figure 4D).

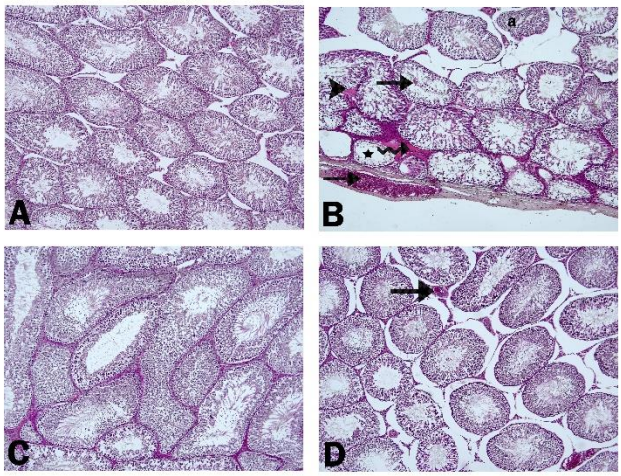

Figure 4. (A) Control group with normal histological appearance, (B) Atrophy in the seminiferous tubules (a), loss of germ cells (asterisk), congestion and bleeding areas in the vessels (arrow), edema (curved arrow) and vacuolization (arrowhead). (C) ACA group with a normal histological appearance, (D) IR+ACA group with reduced congestion (arrow), and normal tubules in the majority.

Discussion

Testicular torsion is a urologic emergency. The major pathophysiology of testicular torsion appears to be IR injury of the testis caused by twisting and releasing the spermatic cord (20). Mechanisms such as oxidative stress, inflammation, and apoptosis as a result of ROS production are effective in IR damage (21). IR injury is responsible for a pathophysiologic cascade involving the activation of neutrophils, inflammatory cytokines, adhesion molecules, increased thrombogenicity, massive intracellular Ca2+ release, and formation of oxygen-derived free radicals (20). Bioflavonoids have many pharmacological and biological effects, including antioxidant effects (22). ACA is a naturally occurring

flavonoid known to possess numerous pharmacological properties, including neuroprotective, cardioprotective, anticancer, anti-inflammatory, antidiabetic, and antimicrobial activities (8). In the present study, the therapeutic effects of ACA in testicular reperfusion injury were detected.

Oxidative stress resulting from an imbalance between ROS and antioxidants may be primarily responsible for tissue damage (23). Lipids are an important component of the cellular membrane and are directly negatively affected by increased ROS (24). Lipid peroxidation, which occurs as a result of increased free oxygen radicals, causes damage to the cell membrane, leading to disruption of membrane integrity and ultimately cell lysis (25). Testicular tissue is highly sensitive to ROS-induced oxidative stress and spermatogenesis stages are directly affected by oxidative stress (26). Akaras et al. (27) reported that testicular IR damage caused a significant increase in the OSI index, primarily by increasing the TOS level. Simsek and Akaras (28) reported that TOS and OSI levels, which increased in different tissues, decreased significantly with ACA application. In the current study, it was determined that IR injury caused oxidative stress damage by increasing TOS and OSI levels in testicular tissues. It is estimated that especially the increase in oxidants is effective here. Because TAS levels did not change, whereas TOS levels increased significantly. On the other hand, ACA treatment contributed significantly to the antioxidant capacity, mainly by increasing TAS.

Inflammation is one of the reactions to damage occurring in the body (29). Increased ROS also increases inflammation by triggering the expression of NF-κB, which is involved in the regulation of pro-inflammatory cytokines (30). NF-κB is an inducible transcription factor involved in inflammation, immune response, and malignant transformation. NF-κB is retained in the cytoplasm through interactions with an NF-κB inhibitor (IκB), but after dissociation, it moves to the nucleus and triggers inflammatory processes (31). Inhibiting NF-κB is an important step in reducing inflammation (32). Prasad et al. (33) reported that ACA exerts its anti-inflammatory effects through regulation of the NF-κB pathway. In the present study, IR injury significantly increased NF-κB mRNA transcription levels in rat testicular tissue. When ACA was applied together with IR, the increased NF-κB transcription level tended to decrease.

Mitochondria-induced ROS production also causes apoptotic effects in the cell (28,34). Apoptosis is a programmed cell death pathway and is effective in removing damaged or dangerous cells from the body. On the other hand, apoptosis is undesirable in normal healthy cells but is risky in case of cellular stress or damage (35,36). Apoptosis is among the most important causes of damage in ischemia-reperfusion in different tissues (37). In the apoptotic process, cell death is induced when the caspase family is active (38,39). Caspase-3, the most important member of the caspase family, also known as executioner caspase, is apoptotic (40,41). Once activated, caspase-3 causes proteolytic degradation of most cellular targets and eventually cell death. Bcl-2 shows an anti-apoptotic effect by preventing the opening of mitochondrial membrane pores (42). Liu et al. (43) reported that the Caspase-3 protein level was significantly increased in IR injury in different tissues and this situation was reversed with ACA application and the Caspase-3 protein level decreased. In the present study, IR caused apoptotic damage in testicular tissues by increasing Caspase-3 mRNA transcription levels and partially decreasing antiapoptotic Bcl-2 mRNA transcription levels. When ACA was administered together with IR, ACA reversed these changes and protected against apoptotic damage by decreasing apoptotic factors and partially increasing anti-apoptotic factors.

ROS destroys macromolecules in the cell membrane and negatively affects the selective permeability of the membrane (44). ROS, including superoxide anions,

hydrogen peroxide or hydroxyl radical, and nitric oxide or peroxynitrite, lead to both DNA and endothelial damage and germinal cell necrosis (20). Apoptosis is effective in the loss of germ cells (45). This damage may occur at the cellular level disrupting tissue integrity and loss of function. Many studies have reported that IR damages the structural integrity of the tissue by increasing the level of NF-κB, Caspase-3, and oxidative stress in both testicular tissues and different tissues and causes tissue damage (21, 27, 36, 38). The other side, studies have reported that ACA exhibits tissue protective properties by reducing structural damage in different tissues (28). Indeed, Tanrıverdi et al. reported that using antioxidants may exhibit protective properties against structural and functional disorders that occur in testicular tissue due to IR damage (46). In the present study, disruptions in seminiferous tubule structures were observed in IR-treated testicular tissues. Severe hemorrhage foci and edema were observed. The reason for tissue damage is that increased oxidative stress and oxidative stress-induced inflammation, and apoptosis cause cellular membrane damage and structural disorders in the tissue. When ACA was administered together with IR, these damage levels decreased. This protective effect of ACA may be due to its strong antioxidant properties.

Conclusions

In conclusion, the current study findings suggest that ACA administration may have a protective effect on experimental testicular IR damage in rats. Further studies are needed to elucidate the protection mechanisms.

Acknowledgments: The authors thank Dr. Nurhan Akaras for her support in the histological examination. This study was supported by Tubitak 2209 project (2209/A, Project: 1919B012105612).

Authorship Contributions: Concept – H.Ş.; Design – Y.K., H.Ş.; Supervision - H.Ş.; Materials – Y.K., H.Ş.; Data Collection and/or Processing - Y.K., H.Ş.; Analysis and/or Interpretation - Y.K., H.Ş.; Literature Search Y.K., H.Ş.; Writing Manuscript Y.K., H.Ş.; Critical Review - Y.K., H.Ş.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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