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Protective role of Ferulic acid on acrylamide-induced testis toxicity in rats

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Abstract

It has become important over the last few years to reduce the detrimental effects of Acrylamide (ACR), which is a carcinogenic substance formed through the exposure of certain foods to high temperatures. The present investigation evaluated the defensive effect of ferulic acid (FA) against toxicity in testis tissue triggered by ACR in male rats. ACR (100 ppm) was administered orally in drinking water alone or with FA, for 30 days. This study revealed that treatment with FA could remarkably decrease MDA levels and significantly increase SOD levels in the testicular tissue of the ACR-treated rats. The intake of FA as a supplement is also supported by the increase in FSHR, LHR, and AR sex hormone receptor levels. FA also reduced the harmful effects leading to ACR by decreasing histopathological changes in the testicular tissue. This study demonstrates the probable protective mechanism of FA against testicular toxicity caused by ACR.

Keywords: acrylamide, ferulic acid, testis, rat

1. Introduction

Thermal processing, which humans have used for cooking for thousands of years, is of major significance in terms of preferenced sensory qualities such as texture, color, flavor, and desired nutritional quality. However, undesirable chemicals as a result of heat treatment negatively affect food processing operations (1, 2). One of the reasons for the emergence of chemical reactions that give rise to the creation of toxic materials in food is the heating process. Acrylamide (ACR), one of these compounds, is which is small and simple in structure but has the property of being a highly reactive substance (3). ACR is also found in non-dietary sources such as tobacco smoke and a wide range of other non-food industrial uses so humans can be tainted by ACR via oral, dermal, and inhalation routes (4, 5). Studies examining the effects of orally administered ACR concluded that it causes decreased sperm concentration in cauda epididymis, morphologically damaged sperm, testicular damage, raised lipid peroxidation, decreased superoxide dismutase (SOD) and glutathione (GSH), and infertility due to deoxyribonucleic acid (DNA) damage (6). These negative effects disrupt the oxidant/antioxidant balance, which results in oxidative stress (7).

Previous studies indicated that flavonoids are used to exert an efficient protective effect against oxidative damage and related toxic effects of ACR (8, 9). Ferulic acid (FA) is known that a water-soluble antioxidant. On the cell walls of various plants like grains, leaves, rice, seeds of coffee, tomato, wheat, oats, flowers, carrot, fruits, beans, peanuts, nuts, spinach, avocado, broccoli, and pineapple include FA in relatively high amounts (10). It can get rid of the excess ROS or straight scavenge free radicals and enzymes that produce free radicals to decrease the harm of inflammation caused by oxidative damage (11). FA shows several constructive effects, including anti-inflammatory (12), antiallergic (13), hepatoprotective (14), and antihyperlipidemic (15) activities. In addition to the benefits mentioned, FA is recommended as a potent antioxidant for the male genital system. It effectively improves sperm parameters such as motility, viability, and count in diabetic rats (16). Evidence indicates that FA is beneficial in preventing increased oxidative damage in the testis and repressed spermatogenesis in male rats (17).

Given these points, our study aims to investigate the protective role of FA on ACR toxicity in rat testis.

2. Materials and Methods

2.1. Drugs and Chemicals

Acrylamide (CAS no:79-06-1) and Ferulic acid (CAS no:

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1135-24-6) were purchased from Sigma (St. Louis, Missouri, USA). All the other reagents were of high analytical grade. The ACR dose (18) and FA dose (19) were determined by the previous investigation.

2.2. Animals, Experimental Design and Drug Administration

The study commenced following ethical committee approval (No. 115, dated 30.06.2022) in response to our application to the Atatürk University Animal Experiments Local Ethical Committee. The experiment was performed with 36 healthy, male Sprague Dawley rats weighing 225-250 g. Experimental applications and animal procurement were carried out at Atatürk University Medical Experiment Application and Research Center. The animals were kept in plastic transparent cages and 12 hours of a light-dark cycle. The room temperature and the relative humidity were set respectively at 22-24ºC and 55%.

Experimental groups were formed by randomly assigned rats.

Group I (Control group; n=8): Saline was administered orally for 30 days.

Group II (FA group; n=8): 30 mg/kg FA was orally administered to rats for 30 days.

Group III (ACR group; n=8): 100 ppm ACR was received from drinking water by rats for 30 days.

Group IV (ACR+FA group; n=8): 30 mg/kg FA was orally administered to rats and 100 ppm ACR was received from drinking water by rats for 30 days.

The day after the experiment was completed (31st day), the animal's samples were taken by intracardiac route under 20mg/kg thiopental sodium (İ.E Ulagay İlaç Sanayi Türk A.Ş., İstanbul) and %5 sevoflurane anesthesia (Sevorane liquid 100%, Abbott Laboratory, İstanbul, Turkey). An overdose of general anesthetic (thiopental sodium, 50 mg/kg) was used in the sacrificial procedure of all rats. The left testicular tissues of the animals were stored at -80°C for biochemical analyses. The right testicular tissues were placed in 10% buffered neutral formaldehyde for immunohistochemical and histopathological analyses.

2.3. Histopathological Examination

The testes were taken out instantly and put in 10% neutral formalin for 24-48 hours. After the routine tissue process, paraffin blocks were prepared. Sections of 5 µm thickness were performed from paraffin blocks. The sections were stained with hematoxylin-eosin. Ten randomly selected tubules were inspected under the microscope (20x magnification). Histopathological evaluation of testicular tissues was performed using Johnsen's mean testicular biopsy score (MTBS) criteria (20). A score of 0-10 represented the level of epithelial maturation. According to this scoring system: 1 (no cells), 2 (Sertoli cells without germ cells),3 (only spermatogonia),4 (only a few spermatocytes), 5 (Many spermatocytes), 6 (Only a few early spermatids), 7 (Many early spermatids without differentiation), 8 (Few late spermatids), 9 (Many late spermatids), 10 (Full spermatogenesis).

2.4. Immunohistochemistry

Dehydration, clarification, and paraffinization processes were applied to the tissues first. Then, 5 µm thick sections were taken from the paraffin blocks. Deparaffinization and hydration procedures were applied to the prepared slides, respectively, and washed with PBS. Sections were then immersed in 3% H_2O_2 solution for 10 minutes followed by antigen retrieval solution (pH 6.0) and heated in the microwave for 10 minutes (2x5 minutes-500 watts), followed by washing with PBS. The primary antibodies Follicle-Stimulating Hormone Receptor (FSHR) (Cat No. bs-0895R, Bioss, USA), Luteinizing Hormone Receptor (LHR) (Cat No. bs-6431R, Bioss, USA), and Androgen Receptor (AR) (Cat No. bs-0118R, Bioss, USA) diluted 1/100 were added on each slide and were incubated in +4ºC overnight. The Seconder antibody (UltraVision Large Volume Detection System, horseradish peroxidase (ready-to-use), TP-125-HL, Thermo Scientific Inc., Waltham, MA, USA) was added and sections were incubated for 45 minutes at room temperature (20–25°C). Diaminobenzidine (DAB) was used as a chromogen. The preparations were covered, examined, and photographed under a light microscope (Eclipse E-600 Nikon, Japan). The results were divided into five categories as not determined (-), slight (+), moderate (++), intense (+++), and very intense (++++) classes according to immunopositive area percentile values.

2.5. Examination of SOD enzyme activities and lipid peroxidation

After the histopathological analysis, the testis tissues were stored at -80˚C until analysis day. The testis tissues were grinding with liquid nitrogen in a TissueLyser (Qiagen, Hilden, Germany). One ml of PBS buffer was included in 50 mg of dry tissues, and the homogenate was centrifugated.

The MDA determination method is based on measuring the reaction of lipid peroxides in animal tissues with thiobarbituric acid (21). The SOD determination method is based on the reduction of NBT by superoxide produced by the xanthine/xanthine oxidase system (22). The MDA levels were expressed as nmol/mg protein and SOD activities were expressed as U/mg protein.

2.6. Statistical Analysis

The data were analyzed using the SPSS (version 25.0; IBM SPSS Inc, Chicago, IL, USA) package program. Descriptive statistical analyses (mean ± standard deviation) were used. One-way ANOVA test and post hoc Tukey test were performed to compare groups. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Histopathology

There was a statistically significant difference between the

groups in histopathological inspections (Table 1).

Table 1. Testicular injury scores (Johnsen's mean testicular score) for all groups

Groups	Johnsen's mean testicular score (MTBS)
Control (C)	8.44±0.40[a]
Ferulic Acid (FA)	8.12±0.60[a]
Acrylamide (ACR)	6.33±0.51[b]
Ferulic Acid+Acrylamide(FA+ACR)	7.42±0.50[c]

[a,b,c] The letters indicate the statistical differences between the groups ($p < 0.05$)

Testicular tissues of Control and FA group rats showed normal histological appearance. The degenerative changes observed in the seminiferous tubules in the ACR group were severe. In addition, advanced degenerative and necrotic changes and vacuolizations were observed in the spermatogenic cells of the seminiferous tubules in this group. In the FA + ACR group, degenerative changes in the seminiferous tubules were observed to be lighter compared to the ACR group. Furthermore, degenerative and necrotic changes and vacuolizations were seen in spermatogenic cells in the tubules. These degenerative and necrotic changes were observed milder than in the ACR group (Fig. 1-2).

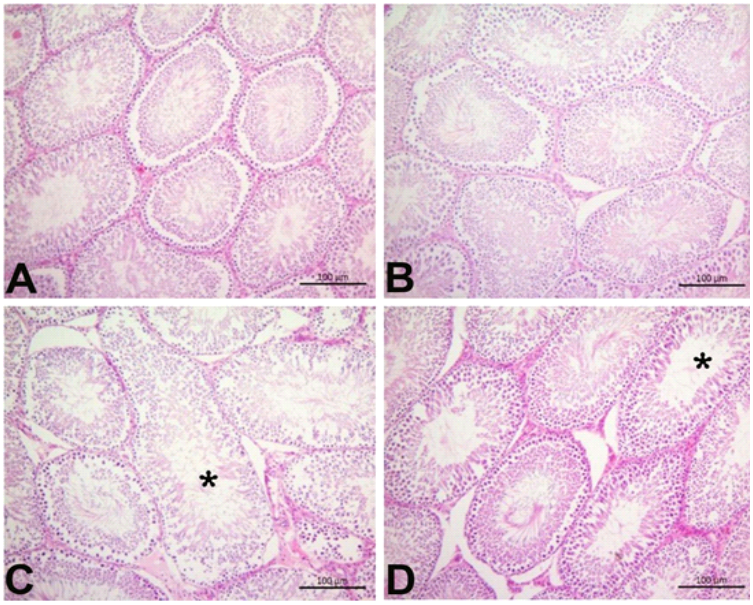

Fig 1. A- Control group, B- FA group: Normal histological structure of testis tissues. **C- ACR group:** Severe degeneration in the seminiferous tubules (*), **D- FA+ACR group:** Moderate degeneration in the seminiferous tubules (*). H-E

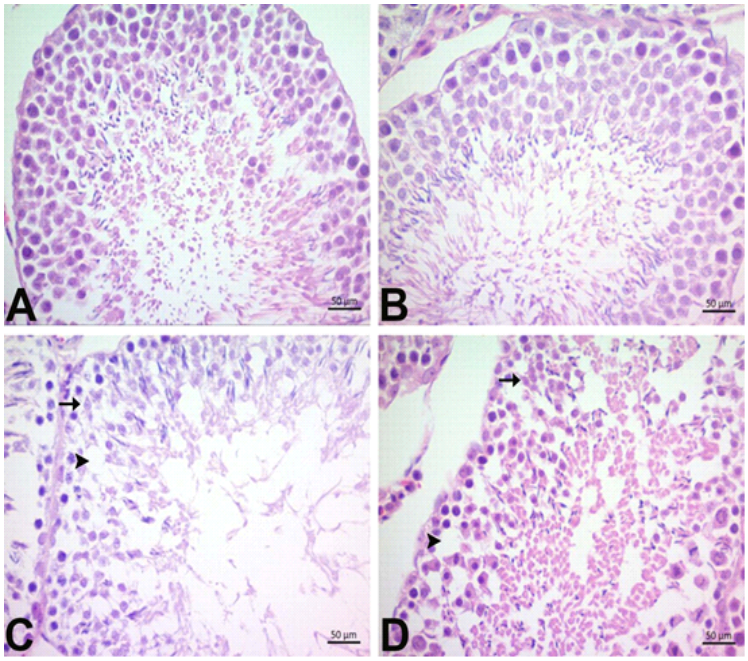

Fig 2. A- Control group, B- FA group: Normal histological structure of testis tissues. **C- ACR group:** Severe vacuolization (arrowhead) in seminiferous tubules and severe degenerative/necrotic changes in spermatogenic cells (arrow), **D- FA+ACR group.** Moderate vacuolization (arrowhead) in seminiferous tubules and moderate degenerative/necrotic changes in spermatogenic cells (arrow), H-E.

3.2. Immunohistochemistry

There was a statistically significant difference between the groups regarding FSHR-, LHR-, and AR- immunopositivity (-ip) (Table 2).

Table 2. FSHR, LHR, and AR immunoreactivity scores

Groups	FSHR	LHR	AR
Control (C)	2.83±0.40[a]	3.66±0.51[a]	2.16±0.40[a]
Ferulic Acid (FA)	2.66±0.51[a]	3.83±0.40[a]	1.83±0.40[a]
Acrylamide (ACR)	1.16±0.40[b]	2.16±0.40[b]	1.00±0.00[b]
Ferulic Acid + Acrylamide (FA+ACR)	1.83±0.40[c]	2.83±0.40[c]	1.83±0.40[a]

[a,b,c] The letters indicate the statistical differences between the groups ($p < 0.05$)

FSHR-, LHR-, and AR-ip were found similar in the control and FA group rats. FSHR-, LHR- and AR-ip were observed intense, very intense, and moderate in these two groups respectively. In the ACR group, FSHR- and AR-ip were seen as slight, while LHR-ip occurred moderately. In the FA+ACR group, the IP was increased compared to the ACR group. In the ACR+FA group, FSHR- and AR-ip were seen as moderate, and LHR-ip was found very intense. FSHR- and LHR- ip were detected in interstitial areas, while AR-ip was detected in spermatogenic cells in both interstitial areas and seminiferous tubules (Fig. 3-5).

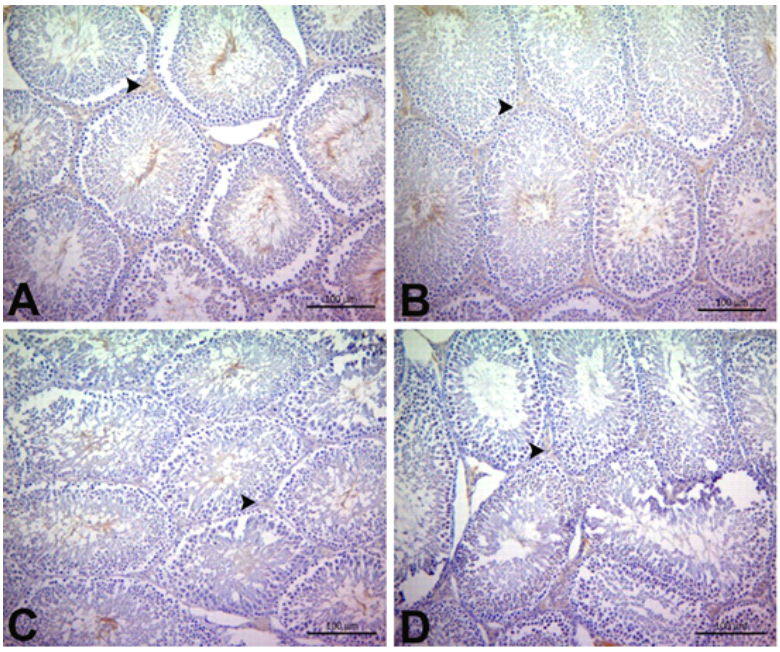

Fig 3. FSHR immunopositivity in interstitial areas (arrowhead). **A- Control group, B- FA group:** intense immunopositivity, **C- ACR group:** slight immunopositivity, **D- FA+ACR group:** moderate immunopositivity, IHC

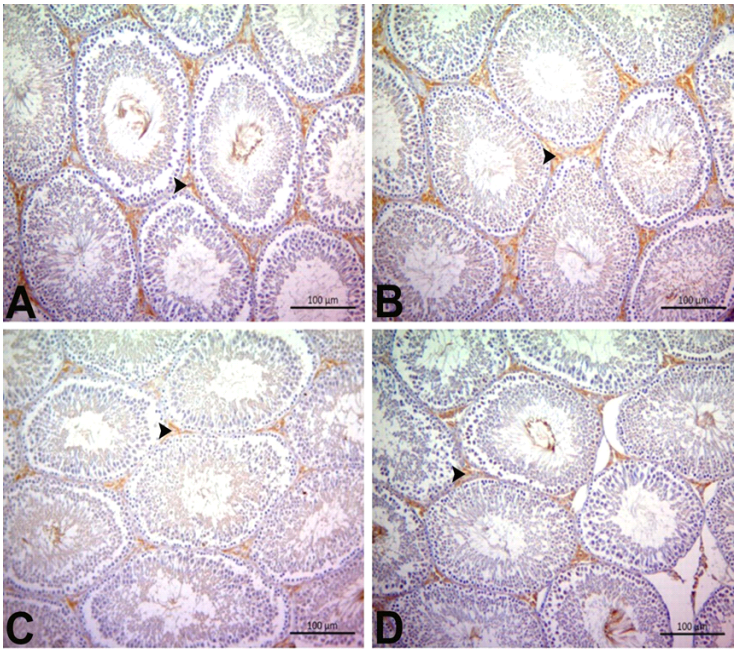

Fig 4. LHR immunopositivity in interstitial areas (arrowhead). **A- Control group, B- FA group:** very intense immunopositivity, **C- ACR group:** moderate immunopositivity, **D- FA+ACR group:** very intense immunopositivity, IHC

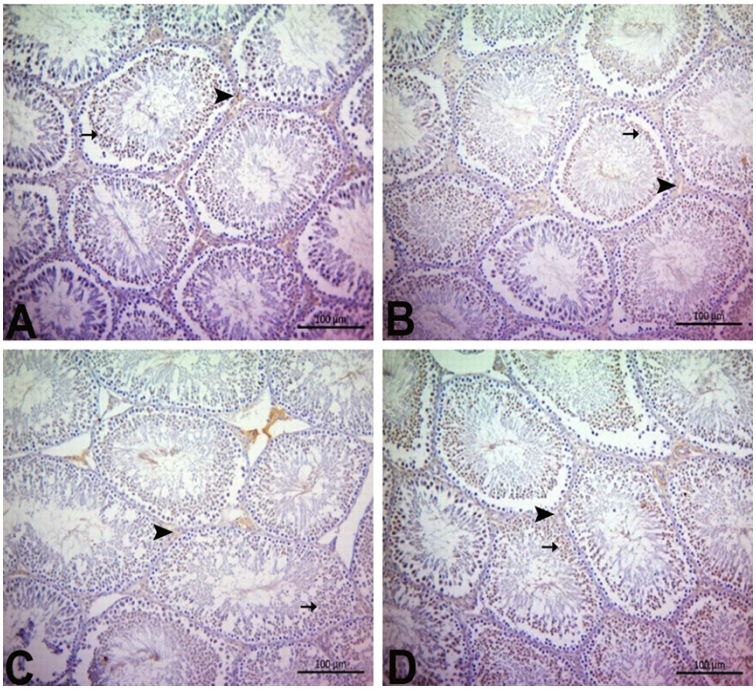

Fig 5. AR immunopositivity in spermatogenic cells in the interstitial tissue (arrow) and seminiferous tubules) (arrowhead). **A- Control group, B- FA group:** moderate immunopositivity, **C- ACR group:** slight immunopositivity, D**- FA+ACR group.** Moderate immunopositivity, IHC

3.3. Results of Biochemical Analysis

The SOD activities and MDA levels of animals are presented in Table 3. One-way ANOVA tests showed significant differences between groups in MDA levels and SOD activities (p<0,001 for both MDA and SOD). The post hoc test revealed significant differences between all pairwise comparisons in MDA levels, except control and FA groups (p<0.05 for all comparisons). A significant difference between all pairwise comparisons in SOD levels, except control and FA groups and control and FA+AD groups, was determined by post hoc test. (p<0.05 for all comparisons).

Table 3. Oxidative stress markers of groups in over-tissues

	Control	FA	ACR	FA+ACR	*p*
MDA (mmole/gram protein)	1719.02±267.86^{a,b}	1530.65±262.32^{c,d}	17820.74±2444.26^{e}	4476.45±1003.18	<0.001
SOD (U/mg protein)	1.51±0.33^{a}	1.73±0.42^{c,d}	0.75±0.29^{e}	1.24±0.40	<0.001

MDA: malondialdehyde, SOD: superoxide dismutase, FA: Ferulic Acid (1ml/kg) group, AD: Acrylamide (100 ppm) group.
Results are expressed as standard deviation. P: One-way ANOVA test *P* value. $^{a, b, c, d, e}$ shows the post hoc Tukey test results of the one-way ANOVA test. ^{a}Significant difference (p<0.05) between control and FA, ^{b}Significant difference (p<0.05) between control and FA+ACR, ^{c}Significant difference (p<0.05) between FA and ACR, ^{d}Significant difference (p<0.05) between FA and FA+ACR, ^{e}Significant difference (p<0.05) between ACR and FA+ACR

4. Discussion

ACR is a colorless and soluble in-water composite with very high chemical activity and is widely used (23). In addition to its widespread use in the industrial field, it is also formed during the cooking of foods (24). ACR formation in foods is associated with high-temperature cooking processes for some carbohydrate-rich foods. When some carbohydrate-rich foods are cooked at high temperatures, asparagine reacts with sugars, which is a recipe for the formation of ACR in foods (25, 26).

Male infertility has become a health problem that has concerned the whole world in recent years. Testicular damage and functional disorders of the testis induced by ACR are problems that should be taken seriously for male reproductive health (27, 28). Recently, it has been widely used compounds that include antioxidant and anti-inflammatory effects have been widely used to prevent or reduce the testicular damage caused by ACR (29).

Reactive oxygen species (ROS) can affect various cellular reactions by inhibiting many cytosolic enzymes (30). When ACR exposure exists, overproduction of ROS occurs, resulting in oxidative stress and DNA damage in cells (31). Antioxidant agents reinforce the body's endogenous antioxidant defense mechanism. It plays a critical role in reducing tissue damage by eliminating the level of ROS that causes cellular degeneration and ultimately death. Supplementary antioxidant agents, like endogenous antioxidants, provide an important contribution to reducing damage to tissues by eliminating the level of ROS (32). The ability of ROS to oxidize structural proteins in tissues causes protein oxidation and amino acid degradation (33). The FA has been demonstrated to improve

the cell stress response by regulating several key enzymes such as catalase (CAT) and superoxide dismutase (SOD) (34).

In a study showing an increase in LPO and ROS content in the testis and kidneys in the lead acetate-treated group, a decrease in LPO and ROS content was found as a result of FA application (35). The 3-methoxy and 4-hydroxyl groups on the benzene ring and the carboxylic acid group with the adjacent unsaturated carbon-carbon double bond in FA probably have a great effect on the scavenging of free radicals. (35).

In our study, we investigated the protective effects of FA application on testis toxicity induced by ACR in rats.

Many studies have examined the effects of orally administered ACR on the testis and have shown that testicular damage occurs with increased lipid peroxidation, resulting in infertility (7, 36, 37). In the present study, ACR exposure caused testicular injury determined by seminiferous tubule degeneration, necrotic changes, and vacuolizations. In the FA + ACR group, the degenerative changes in the seminiferous tubules were less than in the ACR group. In keeping with the given results, previous studies revealed that FA could reduce tissue damage in testis tissue such as atrophy, exfoliating, degeneration, and apoptosis in germ cells (38-40).

Hypothalamic-pituitary-gonadal axis (HPG) axis is directly affected by genotoxic substances that cause male infertility (41). In the HPG axis, the binding of gonadotropin-releasing hormone (GnRH) to anterior pituitary gonadotrophs triggers LH and FSH secretion. FSH binds to FSHR in Sertoli cells to support spermatogenesis. Leydig cells excrete testosterone in pulses in answer to LH-LHR binding (42). Mutational defects of AR, the primary receptor for androgens such as testosterone and 5a dihydrotestosterone, cause androgen insensitivity, resulting in a deficiency in spermatogenesis (43). In this study, the -ip levels of AR, LHR, and FSHR were significantly reduced in the testicular tissue of rats that underwent ACR genotoxicities. In keeping with the given results, previous investigations revealed that environmental toxicants could induce AR (44), FSHR, LHR (45, 46) expression, and -ip compared to the Control group. Nevertheless, treatment with FA noticeably prohibited the low -IP of these receptors. These results propose that FA plays a critical role in preserving critical levels of sex hormone receptors such as AR, LHR, and FSHR.In addition, FA provides the proper functioning of androgens and spermatogenesis. Various natural compounds were also reported to inhibit ACR-induced reproductive hormone disorders in previous studies (47). We determined that our findings are consistent with this literature and that FA reduces ACR-induced reproductive hormone disorders.

In our study, SOD levels decreased and MDA levels were increased via ACR exposure. These parameters in the FA-administered group reached levels similar to the control group. The present study revealed that FA remarkably reduces the MDA level and induces SOD activity during ACR exposure in the testis. In the study of Saçık et al. (38) increased SOD levels and decreased MDA levels in FA groups compared to torsion groups were significant, which is in agreement with this study.

The results of the present study are coherent with the previous investigations regarding oxidant/antioxidant indicators (MDA, SOD), sex hormone receptors (AR, LHR, and FSHR), and histopathological parameters. As a final point, ACR increased oxidative stress and accordingly induced testicular damage. FA revealed strong antioxidant properties that alleviate the destructive effects of oxidative stress and support the antioxidant effect.

Ethical Statement

The study commenced following ethical committee approval (No. 115, dated 30.06.2022) in response to our application to the Atatürk University Animal Experiments Local Ethical Committee.

Conflict of interest

The authors declare no conflict of interest.

Funding

None to declare.

Acknowledgments

None to declare.

Authors' contributions

Concept: A.G., S.Y., N.K.B., B.T.G., Design: A.G., S.Y., N.K.B., B.T.G., Data Collection or Processing: A.G., S.Y., N.K.B., B.T.G., Analysis or Interpretation: A.G., S.Y., N.K.B., B.T.G., Literature Search: S.Y., B.T.G., Writing: S.Y., B.T.G.

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