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REPRODUCTIVE BIOLOGY: ORIGINAL ARTICLE

Activation of Nrf-2 Transcription Factor and Caspase Pathway with Royal Jelly Reduces Fluoride Induced Testicular Damage and Infertility in Rats

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Abstract

This study was carried out to investigate the protective properties of royal jelly on the testicular tissue of rats with testicular damage by giving fluoride. Sperm motility, epididymal sperm density and abnormal sperm ratios were examined and visualized with a light microscope. Expression levels of Caspase-3, Bcl-2, Nrf-2, NF-κB, COX-2, TNF-α and IL1-α proteins in testis tissue were determined by western blot technique. As a result of the study, MDA level, expression level of Bcl-2, NFκB, COX-2, TNF-α and IL1-α proteins, abnormal sperm rates were found higher in Fluoride-50 and Fluoride100 groups compared to other groups. In addition GSH, Catalase enzyme levels, expression levels of Caspase-3 and Nrf-2 proteins were found to be higher in Fluoride + Royal Jelly groups compared to Fluoride-50 and Fluoride-100 groups. In addition, lower degeneration of testicular tissue was found in the histological evaluation in the Fluoride + Royal Jelly groups compared to the other groups. When the data are evaluated royal jelly provides effective protection against testicular damage. From this point of view, we hope that similar results will be obtained when royal jelly is tested on humans.

Keywords Fluoride · Proteins · Royal jelly · Sperm parameters · Testicular damage

Introduction

People can be exposed to many different types of chemicals in their environment that can endanger their health. A wide variety of industrial and environmental toxicants are known to affect male reproductive functions negatively [1]. Today, there is increasing concern about the effects of environmental changes on male reproduction. And the importance of studies on this subject is increasing [2]. Fluorine is an element with high reactivity and electronegativity. For this reason, it is generally found in molecular (fluoride) structure in nature by forming compounds with many other elements other than noble gases (inert gases) and oxygen. Fluoride has been reported to be a cumulative toxicant in the literature. Cumulative toxicity can be defined as the toxic effects appearing after a certain period of time, with increased dose due to accumulation [3, 4]. In literature, it has been reported that low-concentration fluoride contributes to the development of teeth and bones, while excessive fluoride intake has a negative effect on various tissues and organs such as the thyroid, bones, teeth, liver, kidneys, brain, and intestines [5–12]. It has also been reported that prolonged exposure to excess

Highlights
- In recent years, royal jelly has been widely used in traditional and modern medicine.
- Royal jelly application by gavage for eight weeks reduced testicular tissue damage.
- Fluoride exposure has caused pathological changes in reproductive tissues, especially in the male reproductive system.

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fluoride can lead to hematological, hepatic, renal and neurological diseases in various tissues [13–15]. Because of these effects, fluoride can be classified as an environmental toxic substance that has negative effects on human health [10].

However, despite many studies, the toxicity mechanism of fluoride has not been clearly explained. In general, oxidative stress occurs when the body's own natural antioxidant defenses are overrun by the production of potentially destructive reactive oxygen species. Free radicals constitute the lipid peroxidation process in an organism. Increased levels of free radicals cause an overproduction of MDA. Malondialdehyde level is known as an indicator of oxidative stress and antioxidant status in cellular damage [16]. The GSH form of glutathione, which is in the reduced structure, plays a role in eliminating the effects of free oxygen species or free radicals that damage the body. Reduced Glutathione level can also be considered as an indicator of oxidative stress level. It is used as an important indicator to identify toxicity in cells or tissues [17, 18]. Catalase (CAT) enzyme is a type of antioxidant enzyme that protects cellular tissue from free radicals. It plays an important role in reducing the harmful effects of free radicals that cause diseases. As a result, it protects tissues and organs from free radicals [19]. In our study, the activity of catalase (CAT), one of the antioxidant enzymes, is analyzed while identifying the levels of GSH and MDA, which are also shown as oxidative stress bioindicators in testicular tissue. In addition, some reactive oxygen species can target cellular proteins. During this event, reactive oxygen species can react with the amino acid chains of protein molecules and cause oxidative damage to the protein. This situation may also lead to changes on the release levels of proteins [20]. Expression levels of Caspase-3, Bcl-2, Nrf-2, NF-κB, COX-2, TNF-α and IL1-α proteins, which play a role in the body's response to toxic effects, are also very useful clinically. It is very important to reveal these levels in experimental research. Therefore, in our study, we investigated the changes in the expression of some proteins involved in the regulation of apoptosis.

In addition to study about the effect of fluoride toxicity on testicular tissue, we also examined its effects on some spermatogenic parameters. In addition, changes in body and reproductive organ weights were analysed. Adequate amounts of antioxidants or products with antioxidant properties should be consumed in order to minimize the effect of toxicity on metabolism, which may occur due to different reasons. Royal jelly is a valuable food secreted from the hypo-pharyngeal glands of worker bees. Royal jelly has been reported to strengthen memory, increase physical performance, relieve fatigue, and help tissue and skin regeneration. In addition, royal jelly has been reported to have anti-tumor, anti-oxidative, anti-diabetic, immune-stimulating, anti-allergic, antibacterial, antiulcer, estrogenic, anti-inflammatory, antihypertensive, reproductive and enhancing effects among males and females [21]. There is an increasing interest in studies on the use of some food supplements and nutrients to increase the fertilization potential of sperm. Worker bees are sterile and live seven to eight weeks. In contrast, the queen bee fed with royal jelly produces reproductively fertile offspring and can live for five to seven years.

The genotype of queen bees fed only with royal jelly differs from the same worker bees in terms of structural and physical characteristics, their long-life span, superior reproductive ability, and the thought that the same effects may occur in the human population has greatly increased the interest in the product [21–23]. These properties that royal jelly brings to the queen bee have made royal jelly (RJ) one of the most valuable therapeutic products listed by natural medicine scientists for its promising medical and nutritional purposes. On the other hand, the physiological effects of royal jelly have not yet been fully explained. Since royal jelly is generally effective on cell renewal, production and metabolism in the body, it can create vitality in all tissues of the organism and, thus, important changes in health, energy and immune system [24]. Royal jelly has received great attention because of increasing data on its beneficial effects on animal and human reproduction.

This study was planned to find out the damage caused by fluoride, which we can be exposed to from various sources throughout our lives, and to determine the protective effect of royal jelly against this damage and When we look at the existing literature, we think that there are limited studies on the effect of fluoride on the expression of some apoptotic proteins. therefore, we especially preferred the apoptotic protein pathway.

Material-Method

Chemicals

Royal jelly was obtained from Apilife company and Fluoride was obtained from Chemsolute brand. All other chemicals used for the experiments were of analytical purity.

Animals

All the animal experiments were carried out at the Fırat University Experimental Animal Research Center with the approval of the Firat University Animal Experiments Ethics Committee, with the protocol number 2018/57 dated 30.05.2018 and the decision number 101. In our study, a total of 42 Wistar albino male rats, 8 weeks old, were used 7 in each group. A daily lighting period was applied to the rats with 12 h of light and darkness. Feed and water were provided ad libitum throughout the study. Rats were divided into 6 groups with their live weights close to each other.

Groups: (1) Control; fed with a standard diet and not given fluoride and royal jelly, (2) Royal jelly 100 mg/kg group; fed with a standard diet, not given fluoride, given 100 mg/kg royal jelly, (3) Fluoride 50 mg/kg group; fed with a standard diet and given 50 mg/kg fluoride, (4) Fluoride 50 mg/kg + Royal jelly 100 mg/kg group; fluoride fed on a standard diet (50 mg/kg) and royal jelly (100 mg/kg), (5) Fluoride 100 mg/kg group; fed a standard diet and given 100 mg/kg fluoride, (6) Fluoride 100 mg/kg + Royal jelly 100 mg/kg group; fluoride fed on a standard diet (100 mg/kg) and royal jelly (100 mg/kg).

Application of Fluoride and Royal Jelly

Royal jelly was made by gavage at 100 mg/kg five times a week for eight weeks [25]. Fluoride was made by adding 50 mg/kg A and 100 mg/kg doses to drinking water [26].

Measuring Body and Reproductive Organ Weights

Before decapitation, the final body weights of the rats in the experimental groups, whose initial weights were not statistically significant, were measured. After the experimental applications were completed, the rats were decapitated, their testicles with epididymis were cleared of adipose tissue and weighed. The prostate was removed from the appendage glands with v. seminalis and weighed. Absolute and relative weights of all reproductive organs were determined and recorded. Relative organ weights calculated [27] with the formula:

Relative organ weight = Absolute reproductive organ weight (g) / Body weight (g) × 100.

Tissue Homogenization

After 8 weeks of fluoride and royal jelly applications, testicular tissues of the rats were taken and necessary analyzes were made. Testicular tissues were dissected and digested in buffer solution and homogenates were prepared. Centrifugation was done at 20,379 g for 45 min. Supernatant was kept at -80 °C for later use [28].

SDS-PAGE and Western Blot Technique for Protein Analysis

Primary antibodies were diluted 1/500, secondary antibodies were diluted 1/1000. Protein densities were measured using the Lowry kit and 25 µg of protein was loaded into each well. Tissue protein samples were run on 12% gel by SDS-PAGE method. Then, the protein synthesis rates of the proteins named Caspase-3 (sc-70497, Santa cruz biotechnology), Bcl-2 (sc-509, Santa cruz biotechnology), Nrf-2 (ab137550, Abcam, UK), NF-κB (sc-8008, Santa cruz biotechnology), Beta-actin (sc-47778, Santa cruz biotechnology), secondary antibodies (sc-516102; Santa cruz biotechnology, ab97023; Abcam, UK) were determined using Western blot technique and protein levels were evaluated according to density analysis [28].

MDA Analysis in Testicular Tissue Samples

4.5 ml of 1.15% KCl was added to the testicular tissue pieces, which were cut into small 0.5-g pieces. MDA was determined from this prepared homogenization. During the measurement process, 0.1 ml of tissue homogenate was taken and added to 0.1 ml of 8.1 ml of 8.1% sodium dodecyl sulfate (SDS) + acetic acid solution (750 µl). Then, thiobarbuturic acid solution (750 µl) was added with distilled water and the final volume of the mixture was adjusted to be 4 ml. After this mixture was kept in a boiling water bath (45 min at 95 °C), 1 ml of distilled water and 5 ml of the mixture were added at a rate of 15:1 (v/v), cooled and vortexed. After n-butanol-pyridine was centrifuged (10 min at2599 g), the supernatant was collected and measured spectrophotometrically (at 532 nm wavelength). Results recorded in nmol/g [28, 29].

MDA Analysis in Testicular Plasma Samples

200 µl of sample was taken from the groups and 200 µl of 8.1% SDS was added. 1.5 ml of 20% acetic acid (pH: 3.5), and 1.5 ml of 0.8% (pH: 3.5) TBA was added, and the final volume was made up to 4 ml with distilled water. It was then heated in a water bath (1 h at 95 °C) and then cooled. It was vortexed with 1 ml of distilled water and 5 ml of n-butanol pyridine at 15:1 (v/v). After centrifugation (15 min at 4000 rpm), the upper organic layer was removed and measured spectrophotometrically (at 532 nm). Results recorded in nmol/ml [28, 29].

GSH (Glutathione) Analysis in Testicular Tissue Samples

GSH obtained from testicular homogenate was measured spectrometrically. Briefly, 0.1 ml of cell homogenate was added to 0.4 ml of TCA (10% trichloroacetic acid) solution. After centrifugation (3000 rpm, 5 min) the supernatant (0.1 ml) was taken and mixed with a solution of distilled water (0.9 ml), Tris buffer (2 ml, 0.4 M pH 8.9) and DTNB (0.1 ml, also known as Ellman's reagent). The results were measured spectrophotometrically at a wavelength of 412 nm [30, 31].

Determination of Catalase Activity in Testicular Tissue Samples

To use in the measurement of catalase, a mixture was prepared by adding concentrated hydrogen peroxide to a solution

of 1/15 M Na–K-phosphate buffer (Na_2HP0_4-KH_2PO_4, pH 7) at a wavelength of 240 nm by spectrophotometric method until the absorbance was 0.7–0.9. 1.000 µl of this prepared mixture were taken and placed in the incubator to measure the catalase activity of the samples. After adding increasing concentrations of supernatant starting from 30 µl, the difference in absorbance in H_2O_2 was recorded in the spectrophotometer (during 30 s) [32, 33].

Histopathological Evaluation

After staining with Hematoxylin – Eosin (H&E), the sections were examined under the research microscope (Olympus BH-2), and histopathological evaluations were made and photographed [34, 35].

Sections of 5–6 µm thick from paraffin blocks were transferred to a polylysine microscope slide. Testicular tissues taken from all groups were fixed in the appropriate solution, passed through routine histological follow-up series, and then embedded in paraffin blocks. 5–6 mm thick sections were taken from these paraffin blocks. The ratio of apoptotic cells to total (normal + apoptotic) cells and Apoptotic index (AI) were calculated and statistical analyzes were performed. Apoptotic cells were photographed using a Leica DM500 microscope after identifying Apoptotic cells using the Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA). As a result of TUNEL [Tunnel] staining, cell nuclei stained with Harris hematoxylin were considered normal-looking, and those stained brown were considered apoptotic cells [36].

Spermatological Analysis

Spermconcentration, motility, and abnormal sperm rates have been determined [27].

Epididymal Spermatozoa Density

The right epididymis was divided in 1 ml of physiological saline (0.9% NaCl) in the petri dish with the help of scalpel and scissors, and the particles were crushed with a forceps for 2 min. It was then incubated for 4 h at room temperature to allow all spermatozoa in the epididymal tissue to pass into the liquid. After the waiting period, the supernatant containing spermatozoa was drawn up to the 0.5 line of the red blood cell pipette and the eosin solution up to the 101 lines (5 g sodium bicarbonate, 1 ml formalin, 25 mg eosin and 100 ml distilled water). Therefore, the supernatant is diluted 1:200. Approximately 10 µl of diluted supernatant was placed on both counting areas of a pre-covered Neubauer slide (0.1 mm depth, 0.0025 mm^2 area, Labart, Munich, Germany) by touching the edge of the coverslip with the tip of the pipette. The Neubauer slide was placed under the light microscope and waited for 5 min to ensure that the spermatozoa in the solution were homogeneously distributed over the entire area. The spermatozoa falling into all squares in both counting areas were counted and calculated at 200 magnifications of the light microscope [27].

Spermatozoa Motility

For analysis, a slide was placed on the heating plate of the microscope and its temperature was ensured to reach 37 ^{0}C. 200 µl of Tris buffer solution [Tris (hydroxymethyl) aminomethane 3.63 g, glucose 0.50 g, citric acid 1.99 g and distilled water 100 ml] was dropped onto the slide on the heating plate. Then, 5–10 µl of suspension containing spermatozoa, taken by sectioning from the left cauda epididymis, was dropped onto Tris buffer solution and mixed with the help of a coverslip to ensure a homogeneous state. Progressive and non-progressive motile spermatozoa were visually determined by examining at least three different fields for each sperm suspension by an expert at 400 magnification of the light microscope. Values were given as 10% and multiples of 10% for visual motility estimates. The average of the motile spermatozoa examined in at least three microscope fields was given as the final motility score and the results were presented as the percentage of total motility (progressive + non-progressive) [27].

Abnormal Rate of Spermatozoa

To determine the abnormal sperm ratio, 20 µl of Tris buffer-spermatozoan mixture used in motility determination was taken and dropped onto a dry, clean and pre-warmed (37 ^{0}C) slide. 2 drops of Eosin-Nigrozin dye (1.67 g eosin, 10 g nigrosine, 2.9 g sodium citrate, 100 ml distilled water) were dropped onto this mixture with the help of a dropper and mixed with the help of a coverslip and homogenized. Then, thin smears will be drawn from this mixture, and it will be dried in a very short time. After drying, the smears were examined at 400 magnification of the light microscope. A total of 200 spermatozoa were examined in a smear and the rate of abnormal spermatozoa was expressed as a percentage (Turk et. al. 2007).

Statistical Analysis

The results calculated from the experiments were statistically analyzed using the analysis of variance in the SPSS 21 package program. Differences between groups were determined by One-Way ANOVA Post Hoc LSD test and One-way ANOVA Post Hoc Games-Howell Tests. Tunnel apoptosis results were evaluated using the Kruskal Wallis test. For the reliability of the statistics, at least three repeated measurements were made.

Results

Weight Levels of Experimental Animals

The final live body weights of the rats before decapitation are presented in Table 1 When the results obtained were evaluated, it was determined that there was a numerical increase in body weight in the RJ group given alone compared to the Control group, but there was no statistical difference. It was found that there was a statistically significant decrease in body weights in the 50 mg/kg F and 100 mg/kg F groups administered alone compared to the control group. Although there was a decrease in body weights in the fluoride + royal jelly groups according to the control, it was determined that this decrease was less than the fluoride groups applied alone.

Malondialdehyde (MDA) Levels in Testicular Tissue and Plasma

The results of the testicular tissue MDA levels of the experimental groups show that the highest MDA levels occurred in the 100 mg/kg F, 50 mg/kg F, RJ + 50 mg/kg F and RJ + 100 mg/kg F, groups, respectively, compared to the control group. The MDA level of the RJ group was found to be close to the control group. In addition, the highest MDA value in testicular plasma levels occurred in the 100 mg/kg F group. This was followed by 50 mg/kg F, RJ + 50 mg/kg F, RJ + 100 mg/kg F groups. Compared to the control group, the lowest MDA level was determined in the RJ group, and it was found to be close to the control group.

When the MDA levels in testicular tissue and plasma were examined, it was determined that the MDA values of the Control and RJ groups were close and there was no statistical difference. In addition, it was determined that the MDA level decreased and statistically significant differences occurred in the combined RJ + Fluoride groups compared to the 50 mg/kg F and 100 mg/kg F groups applied alone. The results of testicular tissue and plasma MDA levels of the experimental groups are presented in Table 1.

Catalase Enzyme Activity of Testicular Tissue

In the experimental groups, the highest catalase activity was observed in the RJ group, which was close to the control group. Catalase activity was found to be higher in the Fluoride + Royal Jelly groups compared to the 50 mg/kg F and 100 mg/kg F groups applied alone. According to the control group, the highest catalase enzyme activity levels were respectively, RJ > RJ + 50 mg/kg F > RJ + 100 mg/kg F > 50 mg/kg F > 100 mg/kg F. The results of the testicular tissue catalase enzyme activity levels of the experimental groups are presented in Table 1.

Testicular Tissue Glutathione (GSH) Levels

According to the values presented in the Table and Fig., it was determined that the lowest GSH value was observed in the 100 mg/kg F group, the closest and highest GSH value to the control group was found in the RJ group. GSH levels were found to be lower in the 50 mg/kg F and 100 mg/kg F groups compared to the other groups. The results of the testicular tissue testicular tissue glutathione (GSH) Levels of the experimental groups are presented in Table 1.

Expression Grades of Proteins (TNF-α, Caspase-3, Bcl-2, Nrf-2, NF-κB, COX-2, IL1-α)

When the caspase-3 and Nrf-2 protein expression levels were examined, it was determined that the highest value among the groups was observed in the RJ group when compared to the control group. In addition, the lowest values were found in the 50 mg/kg F and 100 mg/kg F groups. It was determined that caspase-3 and Nrf-2 protein expression levels in the Royal Jelly + fluoride groups were lower than the control. On the other hand, caspase-3 and Nrf-2 protein expression levels in the Royal Jelly + Fluoride groups were higher than the fluoride groups administered alone (50 mg/kg F and 100 mg/kg F) (Fig. 1b, d).

Bcl-2, NF-κB, TNF-α, IL1-α and COX-2 protein expression levels were highest in the 100 mg/kg F group

Table 1 **a–f**: Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA Post Hoc LSD and Games-howell Test

Groups	Body weight (End) (g)	Testis Tissue MDA Levels (nmol/g)	Testicular Plasma MDA Level (nmol/ml)	Testicular Tissue Catalase Activity (U/mg protein)	Testis Tissue GSH Levels (μmol/mg protein)
Control	391 ± 4.00^{a}	8.81 ± 0.02^{e}	6.82 ± 0.02^{e}	75.76 ± 2.00^{a}	534.41 ± 2.00^{a}
RJ	399 ± 4.00^{a}	8.56 ± 0.02^{e}	6.45 ± 0.02^{e}	78.52 ± 2.00^{a}	550.58 ± 2.00^{a}
50 mg/kg F	255 ± 2.00^{e}	14.35 ± 0.01^{b}	11.53 ± 0.01^{b}	44.41 ± 3.00^{d}	401.47 ± 2.00^{d}
100 mg/kg F	200 ± 2.00^{f}	16.06 ± 0.02^{a}	13.82 ± 0.02^{a}	$37.05 \pm 3{,}00^{e}$	360.23 ± 2.00^{e}
RJ + 50 mg/kg F	325 ± 3.00^{c}	11.15 ± 0.01^{d}	9.29 ± 0.01^{d}	63.52 ± 3.00^{b}	460.94 ± 2.00^{b}
RJ + 100 mg/kg F	298 ± 2.00^{d}	12.90 ± 0.02^{c}	10.62 ± 0.02^{c}	51.47 ± 3.00^{c}	430.64 ± 2.00^{c}

among the groups. Bcl-2, NF-κB, TNF-α, IL1-α expression levels were found to be the lowest in the RJ group. It was determined that the expression level of COX-2 protein in the RJ group was close to the control group and there was no statistical difference. Bcl-2, NF-κB, TNF-α, IL1-α and COX-2 protein expression levels were higher in Royal Jelly + fluoride groups compared to the control group. On the other hand, when compared with fluoride groups (50 mg/kg F and 100 mg/kg F) applied alone, it was determined to have lower values compared to these groups (Fig. 1a, c, e, f, g).

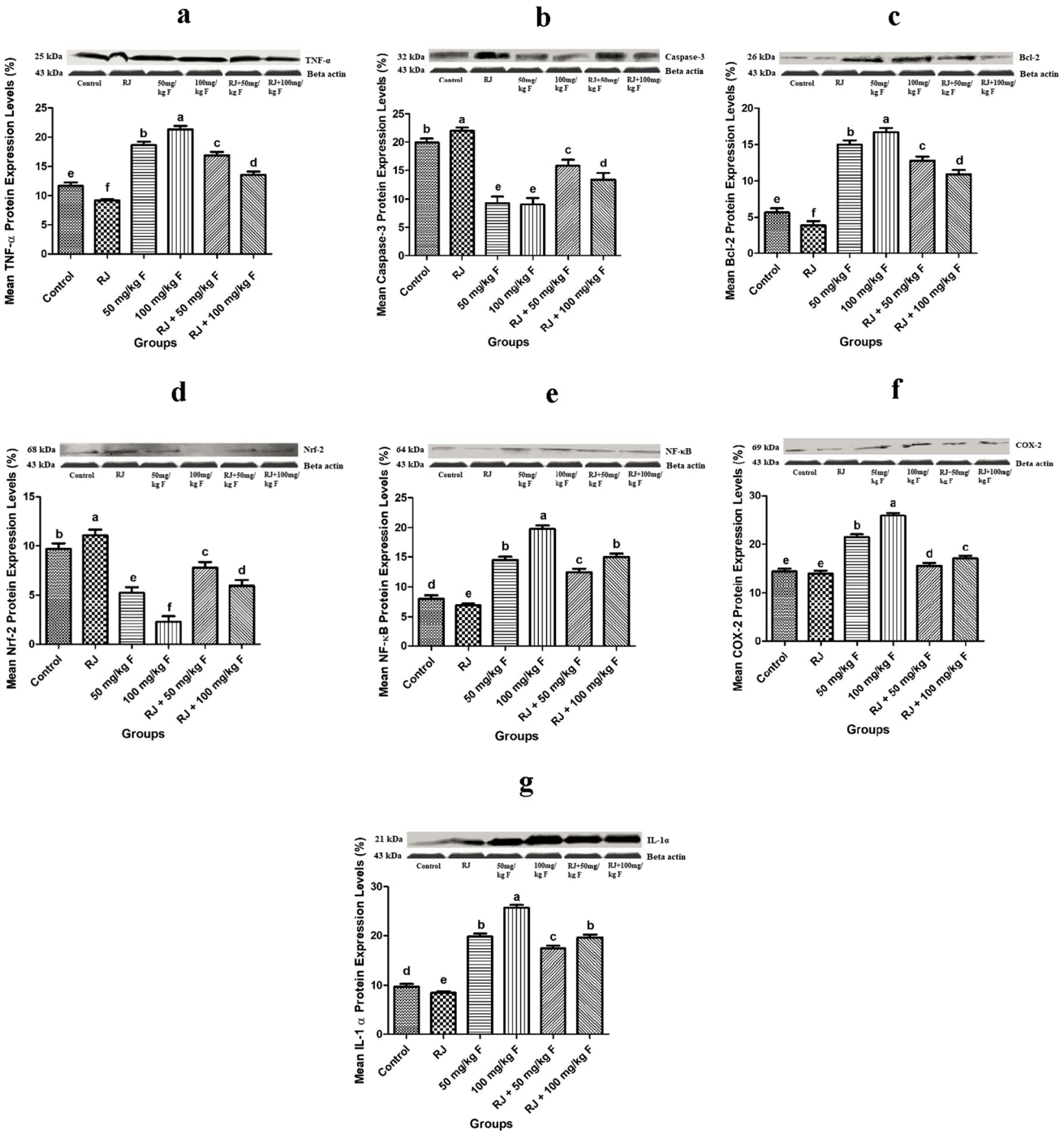


Fig. 1 Testis tissue western blotting mean protein expression results; **a**: TNF-α, **b**: Caspase-3, **c**: Bcl-2, d: Nrf-2, **e**: NF-κB, **f**: COX-2, **g**: IL-1α. **a**-**f**: different letters indicate statistically significant difference between groups. Values are statistically significant at $p < 0.05$ One-way analysis of variance (ANOVA)

Reproductive Organ Weights

Reproductive organ weights were calculated as Absolute and Relative and presented in Tables and Figs. When the Absolut and Relative Testis, Epididymis, Right cauda epididymis Seminal gland and Ventral prostate gland weight results were examined, the most weight reductions occurred in the 50 mg/kg F and 100 mg/kg F groups compared to the control group. Among the groups, the highest decrease was observed in the doses where fluoride was applied alone, and the least weight loss was determined in the RJ group. When the groups given fluoride and royal jelly were compared with the groups administered only fluoride, it was determined that there was a significant ($p < 0.05$) increase in testicular weight (Table 2 (a-b) and Fig. 2).

Spermatological Examination Findings

At the end of the experiment, the rats were decapitated, and spermatozoa were collected from the epididymis and evaluated in terms of density, motility and morphology. When the findings were examined in terms of sperm density and motility, although the motility rate and density increased numerically in the group treated with royal jelly alone, no statistically significant differences were found between the Control and RJ groups ($p < 0.05$). Compared to the control group, it was determined that the decreases in motility and density were the highest in the 50 mg/kg F and 100 mg/kg F groups. On the other hand, while the highest decrease among the groups was found in the doses in which fluoride was applied alone, it was determined that there were significant increases in the percentage of motility and density when the groups given fluoride and royal jelly were compared with the groups that were administered only fluoride (Fig. 3 and Table 3). When the findings of morphologically abnormal sperm ratio were examined, it was determined that there were significant increases in the head, tail and total abnormal sperm ratios in the 50 mg/kg F and 100 mg/kg F groups, which were treated with fluoride alone, according to the control group. Moreover, when the groups given fluoride and royal jelly were compared with the groups administered only fluoride, it was determined that there were significant decreases in the percentages of head, tail and total abnormal sperm ratio (Fig. 3 and Table 3).

Histopathological Findings

The damage levels in the testicular tissues of the groups were determined by applying the TUNNEL method to the testicular tissue. In the evaluation of staining, nuclei stained blue with hematoxylin were normal, while cells with brown nuclear staining were analyzed as damaged. The black arrow indicated in the visuals of the cells in the testicular tissue of the rats in the experimental groups indicates the TUNNEL positivity, in other words, the cell damage in the tissue. As a result of the examination of TUNNEL staining under

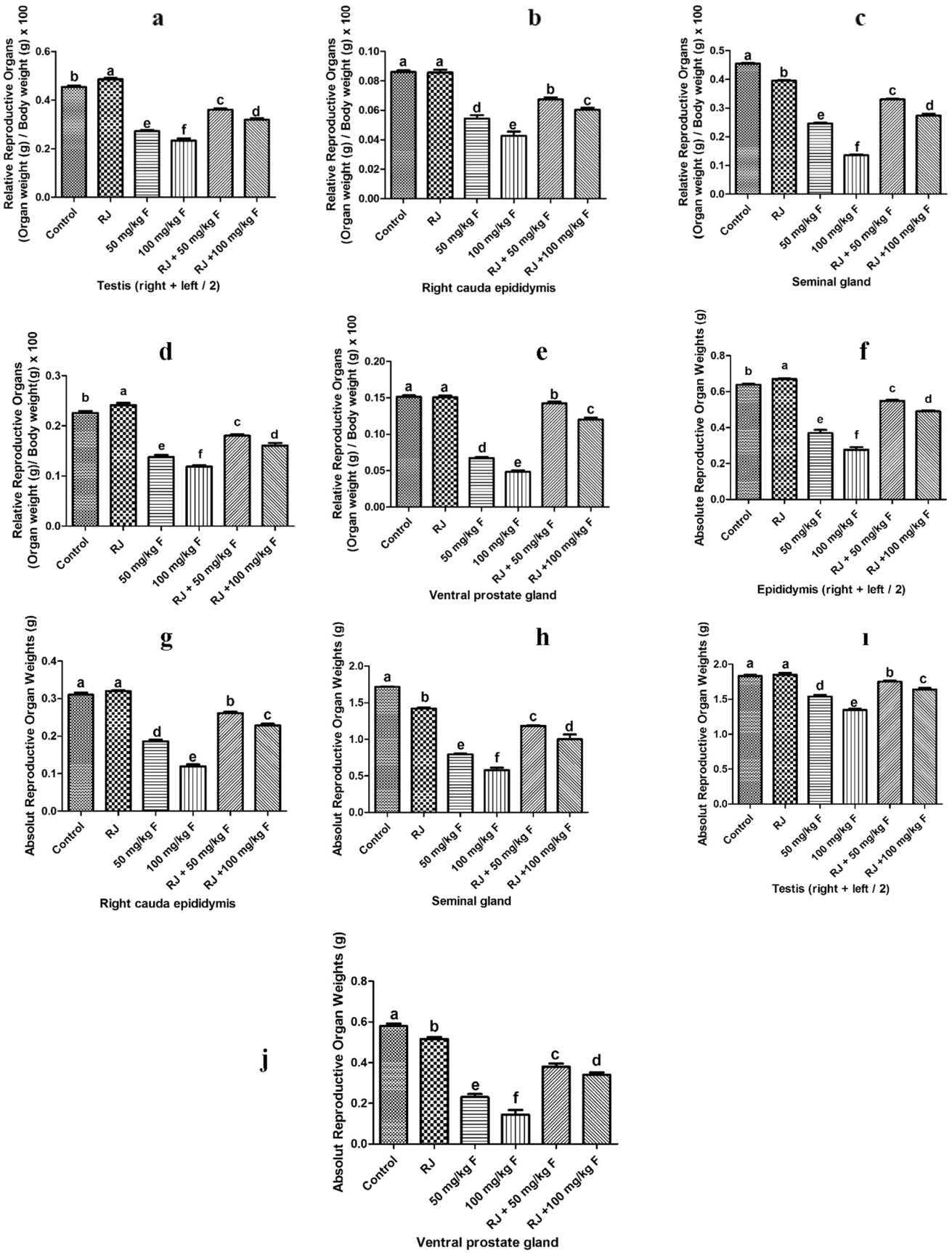
Table 2 **a**–**f**: Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA Post Hoc LSD Test

a

Groups	Relative Reproductive Organs (Testis-right + left / 2)	Relative Reproductive Organs (Right cauda epididymis)	Relative Reproductive Organs (Seminal gland)	Relative Reproductive Organs Epididymis (right + left / 2)	Relative Reproductive Organs Ventral prostate gland
Control	0.454 ± 1.00^{a}	0.086 ± 1.00^{a}	0.455 ± 1.00^{a}	0.223 ± 1.00^{a}	0.151 ± 1.00^{a}
RJ	0.486 ± 1.00^{b}	0.082 ± 1.00^{a}	0.395 ± 1.00^{b}	0.235 ± 1.00^{b}	0.150 ± 1.00^{a}
50 mg/kg F	0.272 ± 1.00^{e}	0.055 ± 1.00^{d}	0.246 ± 1.00^{e}	0.138 ± 1.00^{e}	0.067 ± 1.00^{d}
100 mg/kg F	0.230 ± 1.00^{f}	0.042 ± 1.00^{e}	0.137 ± 1.00^{f}	0.118 ± 1.00^{f}	0.048 ± 1.00^{e}
RJ + 50 mg/kg F	0.360 ± 1.00^{c}	0.067 ± 1.00^{b}	0.330 ± 1.00^{c}	0.180 ± 1.00^{c}	0.142 ± 1.00^{b}
RJ + 100 mg/kg F	0.320 ± 1.00^{d}	0.060 ± 1.00^{c}	0.278 ± 1.00^{d}	0.160 ± 1.00^{d}	0.120 ± 1.00^{c}

b

Groups	Absolut Reproductive Organ Weights (g) Epididymis (right + left / 2)	Absolut Reproductive Organ Weights (g) Right cauda epididymis	Absolut Reproductive Organ Weights (g) Seminal gland	Absolut Reproductive Organ Weights (g) Testis (right + left / 2)	Absolut Reproductive Organ Weights (g) Ventral prostate gland
Control	0.640 ± 0.01^{a}	0.300 ± 0.02^{a}	1.720 ± 0.02^{a}	1.840 ± 0.02^{a}	0.580 ± 0.02^{a}
RJ	0.670 ± 0.02^{b}	0.320 ± 0.02^{a}	1.430 ± 0.01^{b}	1.850 ± 0.02^{a}	0.530 ± 0.02^{b}
50 mg/kg F	0.370 ± 0.04^{e}	0.180 ± 0.01d	0.780 ± 0.01^{e}	1.540 ± 0.03^{e}	0.240 ± 0.02^{e}
100 mg/kg F	0.280 ± 0.01^{f}	0.120 ± 0.02^{e}	0.610 ± 0.02^{f}	1.310 ± 0.07^{f}	0.180 ± 0.01^{f}
RJ + 50 mg/kg F	0.550 ± 0.03^{c}	0.260 ± 0.02^{b}	1.180 ± 0.01^{c}	1.780 ± 0.07^{b}	0.390 ± 0.02^{c}
RJ + 100 mg/kg F	$0.490 \pm 0,01^{d}$	0.230 ± 0.01^{c}	0.990 ± 0.01^{d}	1.600 ± 0.01^{c}	0.340 ± 0.01^{d}


a
Relative Reproductive Organs (Organ weight (g) / Body weight (g) x 100
Testis (right + left / 2)
b
Relative Reproductive Organs (Organ weight (g) / Body weight (g) x 100
Right cauda epididymis
c
Relative Reproductive Organs (Organ weight (g) / Body weight (g) x 100
Seminal gland
d
Relative Reproductive Organs (Organ weight (g)/ Body weight(g) x 100
e
Relative Reproductive Organs (Organ weight (g) / Body weight (g) x 100
Ventral prostate gland
f
Absolute Reproductive Organ Weights (g)
Epididymis (right + left / 2)
g
Absolut Reproductive Organ Weights (g)
Right cauda epididymis
h
Absolut Reproductive Organ Weights (g)
Seminal gland
ı
Absolut Reproductive Organ Weights (g)
Testis (right + left / 2)
j
Absolut Reproductive Organ Weights (g)
Ventral prostate gland
Control
RJ
50 mg/kg F
100 mg/kg F
RJ + 50 mg/kg F
RJ +100 mg/kg F

◂**Fig. 2** Relative and Absolut reproductive organ weights in rats; **a**: Testis (Right + left /2) relative organ, **b**: Right cauda epididymis relative organ, **c**: Seminal gland relative organ, **d**: Epididymis (Right + left /2) relative organ, **e**: Ventral prostate gland relative organ, **f**: Epididymis (Right + left /2) absolut organ, **g**: Right cauda epididymis absolut organ, **h**: Seminal gland absolut organ, **ı**: Testis (Right + left /2) absolut organ, **j**: Ventral prostate gland absolut organ. **a**-**f**: different letters indicate statistically significant difference between groups. Values are statistically significant at $p < 0.05$ One-way analysis of variance (ANOVA) Post *Hoc* LSD Test

light microscopy, it was observed that there was a significant increase in the TUNNEL positivity level when the 50 mg/kg F and 100 mg/kg F groups, in which fluoride was applied alone, were compared with the control group. No significant difference was observed between the control group and the RJ group. On the other hand, TUNNEL positivity levels were decreased in the groups in which fluoride and royal jelly were applied together compared to the 50 mg/kg F and 100 mg/kg F groups in which only fluoride was applied alone. Images are given in Fig. 4a-Fig. 4f. As a result of the examination of TUNNEL staining for the determination of apoptotic cells under light microscopy; TUNNEL positivity of the RJ (Royal jelly 100 mg/kg) (Fig. 4b) ($p = 0.886$) group when compared to the control group (Fig. 4a) TUNNEL positivity increased statistically significantly in the 50 mg/kg F (Fig. 4c) ($p = 0.0\ 02$) and 100 mg/kg F (Fig. 4d). ($p = 0.002$) groups compared to the control group. However, no statistically significant difference was observed between the 50 mg/kg F and 100 mg/kg F groups in terms of TUNNEL positivity ($p = 0.932$). Compared to the 50 mg/kg F group (Fig. 4c), TUNNEL positivity was observed to be statistically significantly decreased in the RJ + 50 mg/kg F (Fig. 4e) group ($p = 0.002$). Compared to the 100 mg/kg F group (Fig. 4d), TUNNEL positivity was observed to be statistically significantly decreased in the RJ + 100 mg/kg F group (Fig. 4f) ($p = 0.003$). Apoptotic index values are presented in Table 4. As a result of the examination of the staining with hematoxylin & eosin under light microscopy, it was observed that the testicular tissues of the Control (Fig. 5a) and RJ (Royal jelly 100 mg/kg, Fig. 5b) groups had a normal appearance. Significant vacuolization (black arrow) and germ cell shedding (black star) were observed in testicular tissues of the 50 mg/kg F (Fig. 5c) and 100 mg/kg F groups (Fig. 5d) compared to the control group (Fig. 5a) However, when the fluoride 50 mg/kg F group (Fig. 5c) was compared to the group in which fluoride was administered together with royal jelly, it was observed that germ cell shedding regressed in the RJ + 50 mg/kg F group (Fig. 5e) but vacuolization continued. When the group given 100 mg/kg F (Fig. 5d) and the group that received fluoride together with royal jelly was compared, it was observed that germ cell shedding regressed in the RJ + 100 mg/kg F group, but vacuolization continued (Fig. 5f).

Discussion

Reproductive health problems can occur as a result of the increased exposure to environmental pollutants and chemicals. When the pathogenesis of male infertility is evaluated, it is closely related to defective spermatogenesis due to environmental factors, congenital abnormalities, defective sperm due to immunological and neurogenic factors or damage to the testicular tissue have an important place. Many studies show that the quality of human sperm decreases due to environmental pollutants and chemicals, and deformations in the testicular tissue occur [37, 38]. Fluoride is a highly active environmental element and is commonly found in the environment as inorganic or organic compounds due to its great reactivity. Fluoride (F) is found in natural form in soil, water and food in varying amounts. It is also widely used as an additive in toothpaste, mouthwash and drinking water to prevent tooth decay. High fluoride consumption can cause an increase in body. In many studies, it is evident that excess fluoride damages both bones and teeth as well as other tissues and systems such as the nervous, immune, respiratory, digestive and reproductive systems. Human health and the environment is threatened by the ever-increasing levels of fluoride in food, water and air. Excessive fluoride exposure is shown to cause various pathological changes in soft tissues, namely reproductive tissues, especially the male reproductive system [39–43]. Various etiological factors have been defined in male infertility. Among these factors are nutrition-related (nutritional) factors. Good nutrition also plays a beneficial role in the continued production of sperm cells. Royal jelly is a mixture of monosaccharides, fatty acids, protein, minerals, vitamins [44], traces of some minerals and enzymes, which are very important for nutrition and healing. Therefore, it is shown as a valuable source of antioxidants. Royal jelly has been shown to inhibit lipid peroxidation both in vitro and in vivo, possessing antioxidant properties that may play an important role in its effects on the aging process and stress reactions in rats. Some studies have reported the positive effects of royal jelly on reproductive potential, fertility health and infertility [45–48]. In this study, results showing testicular damage due to fluoride exposure and the protective effect of royal jelly against this damage were obtained by using histopathological, biochemical and molecular biological methods. Based on the analysis of the body and reproductive organ weights as a whole, it can be said that the body weight and reproductive organ weights are negatively affected by fluoride. Because fluoride has effects on weight, weight loss can be considered as a physical indicator of a fluoride-induced pathology or damage underlying the weight loss. The results obtained with body and reproductive organ weight measurements are presented in Table 2 (a-b) and Fig. 2, 3. On the basis of the results, fluoride can

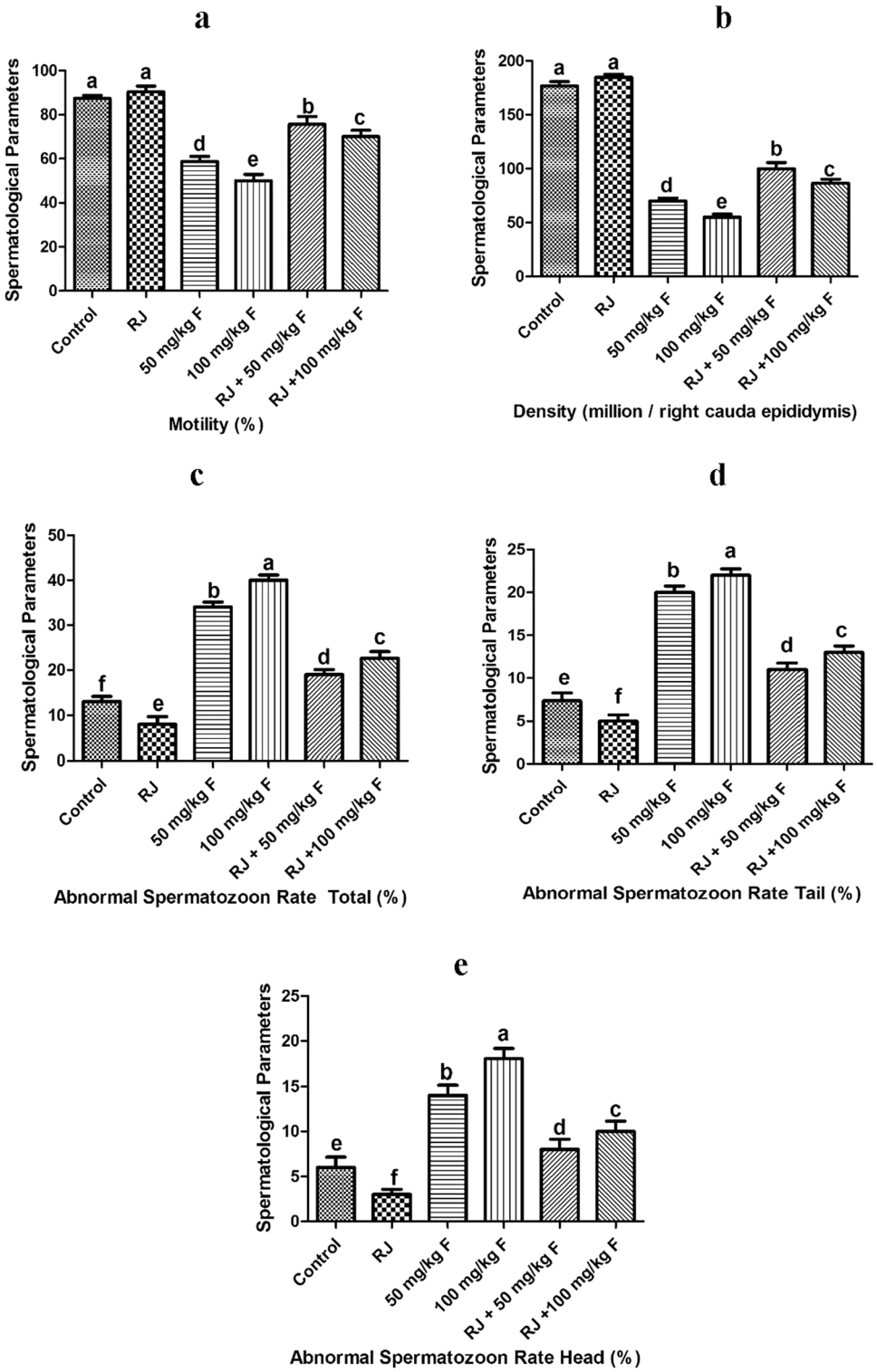


Fig. 3 Spermatological examination findings; **a**: Motility (%), **b**: Density (million / right cauda epididymis), **c**: Abnormal Spermatozoon Rate Total (%), **d**: Abnormal Spermatozoon Rate Tail (%), **e**: Abnormal Spermatozoon Rate Head (%). **a**-**f**: different letters indicate statistically significant difference between groups. Values are statistically significant at $p < 0.05$ One-way analysis of variance (ANOVA) Post *Hoc* LSD Test

be found to have a negative effect and royal jelly has a positive effect in terms of body weight parameter. There are some study results in the literature that support this idea. In a study, significant decreases in the relative weights of prostate, epididymis and testis of rats given fluoride were determined. In the same study, it was also reported that the relative weights approached the control level following the co-administration of vitamin-E or calcium + Vitamin-E [49]. In another study, fluoride-induced biochemical changes in the reproductive organs of male mice were investigated, and in this study, a decrease in body weights after fluoride administration was observed [50].

Table 3 **a–f**: Differences between groups with different letters are statistically significant ($p < 0.05$). One-way ANOVA Post Hoc LSD Test

Groups	Spermatological Parameters Motility (%)	Spermatological Parameters Density (million / right cauda epididymis)	Spermatological Parameters Abnormal Spermatozoon Rate Total (%)	Spermatological Parameters Abnormal Spermatozoon Rate Tail (%)	Spermatological Parameters Abnormal Spermatozoon Rate Head (%)
Control	87 ± 1.00^{a}	176 ± 1.00^{a}	13 ± 1.00^{e}	7 ± 2.00^{e}	6 ± 2.00^{e}
RJ	90 ± 1.00^{a}	184 ± 1.00^{a}	8 ± 1.00^{f}	5 ± 2.00^{f}	3 ± 1.00^{f}
50 mg/kg F	58 ± 1.00^{d}	70 ± 1.00^{d}	34 ± 1.00^{b}	20 ± 1.00^{b}	14 ± 1.00^{b}
100 mg/kg F	50 ± 1.00^{e}	55 ± 1.00^{e}	40 ± 1.00^{a}	22 ± 1.00^{a}	18 ± 1.00^{a}
RJ + 50 mg/kg F	82 ± 1.00^{b}	100 ± 1.00^{b}	19 ± 2.00^{d}	11 ± 2.00^{d}	8 ± 1.00^{c}
RJ + 100 mg/kg F	70 ± 1.00^{c}	88 ± 1.00^{c}	23 ± 1.00^{c}	13 ± 1.00^{c}	10 ± 1.00^{d}

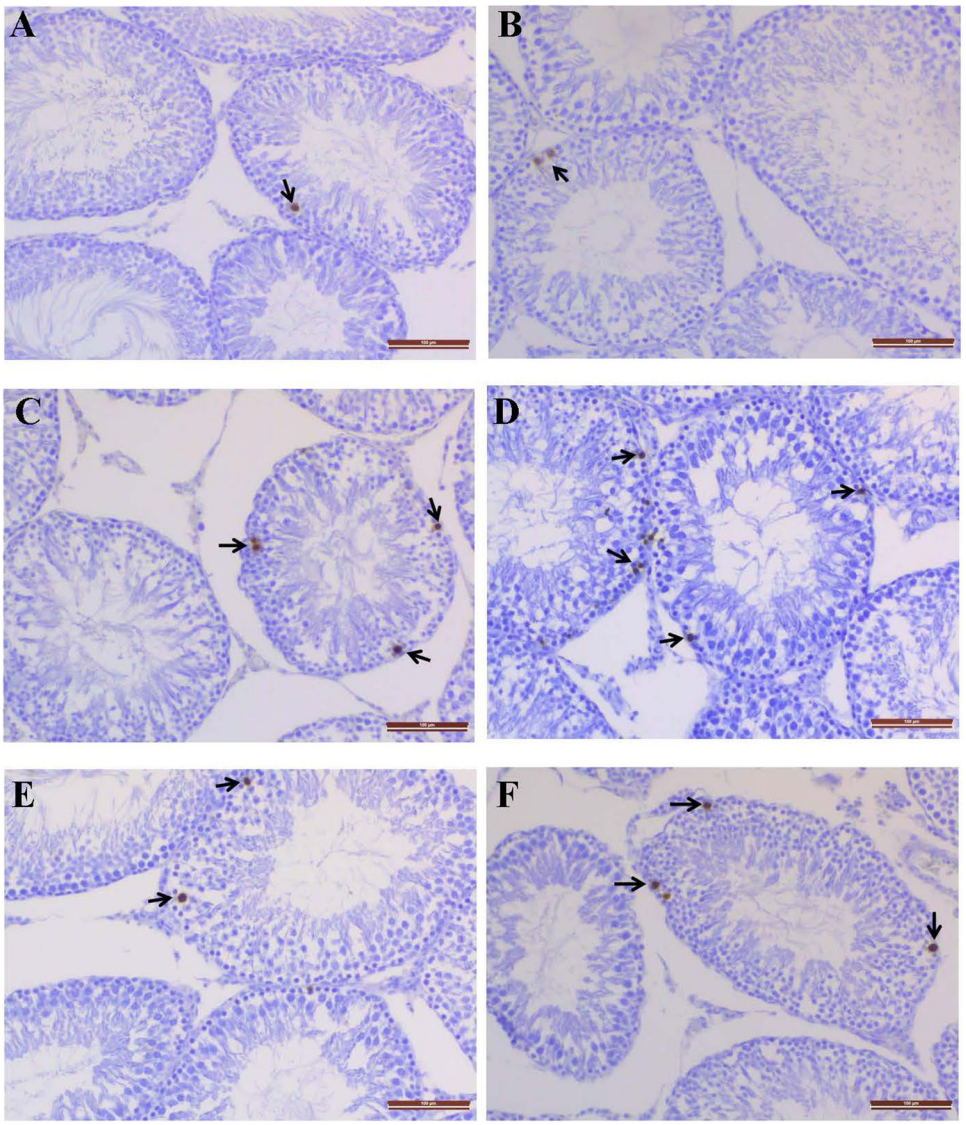


Fig. 4 Testis tissue TUNEL results; **A**: Control, **B**: RJ, **C**: 50 mg/kg **F**, **D**:100 mg/kg **F**, **E**: RJ + 50 mg/kg **F**, **F**: RJ + 100 mg/kg **F**

According to the measurements obtained in our study, the addition of royal jelly to the fluoride groups reduced the weight loss caused by the application of fluoride alone. It can be evaluated that royal jelly supplementation improves weight loss caused by fluoride and that royal jelly can provide protection against fluoride toxicity. As a matter of fact,

Table 4 Apoptotic index determined by TUNEL method

Groups	Apoptotic index median (min–max)
Control	1.00 (1.00–3.00)
RJ	1.00 (1.00–2.00)
50 mg/kg F	5.50 (3.00–7.00)[a]
100 mg/kg F	5.00 (4.00–8.00)[a]
RJ + 50 mg/kg F	1.00 (1.00–3.00)[b,c]
RJ + 100 mg/kg F	1.50 (1.00–2.00)[b,c]

Values are given as median (min–max)

[a]Compared to the control group

[b]Compared with the 50 mg/kg F group

[c]Compared with the 100 mg/kg F group ($p < 0.05$)

there are some studies supporting our comments in the literature. In a study, testes/body weight ratio was found to be significantly lower in rats treated with a chemotherapeutic agent, while this testicle/body weight ratio was returned to the control group with royal jelly administration [51]. In another study, while royal jelly provided a significant increase in testicular weight with its healing effect despite the decrease in testicular weight caused by hydrogen peroxide, it was shown that there were no statistically significant differences in epididymis, prostate and body weights in the same study [52]. In our study, these studies in the literature, which are compatible with the results we obtained, support the data we found. The data of tissue MDA and plasma MDA values obtained in testicular tissue are presented in Table 1. When the results obtained in testicular tissue and plasma were evaluated, it was evident that fluoride had an effect on increasing MDA level by inducing oxidative stress in testicular tissue and plasma. As a result of the experiment, royal jelly was determined to decrease the MDA levels increased by fluoride. According to the testicular tissue and plasma MDA results obtained in our study, we can say that fluoride has a pro-oxidant effect and royal jelly has an antioxidative effect in testicular tissue. When we compared the results we obtained with the results of some studies conducted with fluoride in the literature, it was seen that there were results confirming our own findings. In a study [53], fluoride applied for 90 days in rat testis tissue was determined to cause significant increases in oxidative stress by causing high malondialdehyde levels even at the lowest dose of 1 mg. In a different study in which 15 mg/L fluoride application was made in drinking water alone, it was determined that fluoride application alone caused an increase in oxidative stress indices in the testicles and epididymis of rats by measuring MDA levels. In another study [49], fluoride given 20 mg/L alone was found to increase the MDA level 50% more than the control group. These effects of royal jelly, which is shown to have a lowering effect on MDA increased by fluoride in testicular tissue, are consistent with the findings of previous studies investigated in testicular tissue. For example, a study has shown significant increase in MDA of diabetic rats compared to the control group, and a partial decrease in testicular tissue MDA levels in the diabetic group caused by royal jelly administration [54]. In another study, the protective potential of royal jelly against cadmium-induced infertility in male rats was investigated, and it was shown that the MDA levels increased by cadmium decreased with royal jelly [55]. When our testicular tissue GSH findings are evaluated, it can be stated that fluoride reduces the GSH levels, while royal jelly alleviates the harmful effects of fluoride by improving the formation of reduced glutathione (GSH). The idea that fluoride is an effective oxidative stress inducer by increasing the MDA level and decreasing the GSH level has been strengthened.

There are studies where it has been found out that GSH levels decrease with fluoride toxicity [56]. In a study, it was found that low and high dose fluoride applications in testicular tissue significantly reduced GSH levels [57]. Another study (100 mg/kg F) showed that administration of sodium fluoride significantly reduced GSH levels [58]. The effect of fluoride on GSH values in our study is consistent with the results of these studies in the literature. With royal jelly supplementation, an increase in GSH approaching control levels was achieved. These results can be considered as an indication that royal jelly has an effective antioxidant activity that increases the reduced glutathione (GSH) level. Our results are consistent with the studies in the literature showing that royal jelly increases the GSH level. In a study, it was reported that royal jelly feeding significantly inhibited lipid peroxidase (LPO) concentration in testicular tissue of hamsters. In addition, it has been reported that royal jelly protects organs from free radical-induced cellular damage, and acts as an RJ antioxidant due to its free radical scavenging function [59]. In a different study, it was shown that royal jelly administration improved cadmium-induced genotoxicity and oxidative stress in mice by increasing glutathione (GSH) levels [60]. We tried to determine the effect of fluoride exposure on antioxidant enzyme levels in testicular tissue of rats with catalase activity. The data we obtained are presented in Table 1. As a result of the study, the decreases in the 50 mg/kg F and 100 mg/kg F groups, which were applied only to fluoride, were determined to be almost half compared to the control group. This reveals that the toxicity of fluoride exposure is very effective. In some of the studies in the literature, it has been reported that fluoride inhibits the activity of antioxidant enzymes such as catalase. In a study, the protective effect of melatonin on fluoride-induced oxidative stress and testicular dysfunction in rats was analysed. The applied fluoride has been reported to decrease the testicular catalase activity values compared to the control,

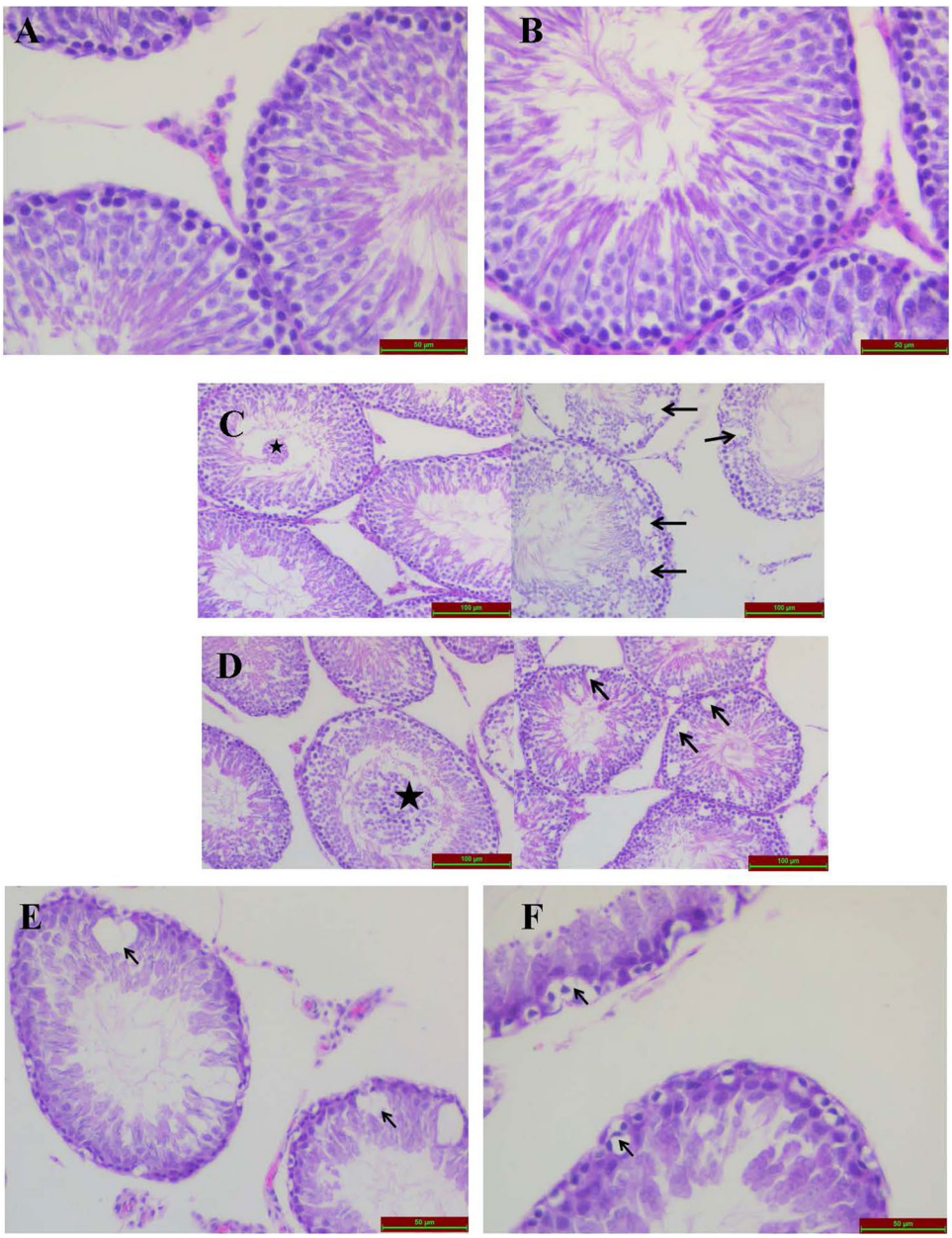


Fig. 5 Testis tissue histopathological results; **A**: Control, **B**: RJ, **C**: 50 mg/kg **F**, **D**:100 mg/kg **F**, **E**: RJ + 50 mg/kg **F**, **F**: RJ + 100 mg/kg **F**

and this decrease was found to be higher depending on the dose [55]. In another study, the protective effects of chitosan and chitosan oligosaccharide on sodium fluoride-induced testicular damage in male rats were investigated, and it was reported that GSH level and catalase activity were significantly decreased in the NaF group, while MDA was significantly increased [61]. These studies on testicular tissue and the results obtained in our own study confirm or support each other. Upon analysing the results we obtained in catalase activity measurements in terms of royal jelly, CAT activity was determined in the group given royal jelly, which is close to the control group. This situation can also be considered as an indication that royal jelly supplement can be a reliable nutritional supplement in a healthy structure. In addition, it can be said that royal jelly protects the tissue against the effect of fluoride toxicity by increasing CAT activity, depending on the increase in catalase in the groups in which royal jelly and fluoride are given together. In previous studies in the literature, royal jelly has been confirmed to have an increasing effect on catalase activity. A study has found out that nicotine decreased catalase levels in testicular tissue, and royal jelly application significantly increased it [62]. In another study, it was demonstrated that royal jelly applied in the testicular tissue against a chemotherapeutic drug has a chemo protective effect by increasing the CAT level [63].

In the light of all these studies in the literature and the data we found in our thesis study, we can say that royal

jelly has an anti-oxidative effect on testicular tissue, which is a soft tissue, and this is done by increasing the activity of an antioxidant enzyme, catalase. When we evaluate the GSH, MDA and CAT results as a whole, we can think that fluoride-induced free radical formation is induced in the testicles. And in order to overcome this situation, we can argue that royal jelly application is beneficial as a therapeutic nutritional supplement. Decreased sperm motility and sperm count, which are important factors in male infertility, and increased sperm anomaly levels were evident in fluoride applied groups. These negative effects of fluoride on sperm parameters reveal the necessity of evaluating fluoride as a factor that may cause male infertility. It was determined that there were significant increases in sperm motility percentage and sperm amount in the groups with royal jelly added fluoride when only fluoride applied groups were compared. It has been found to improve to levels close to control. Upon analysis of the findings of morphologically abnormal sperm ratio, it was determined that there were significant decreases in the groups treated with royal jelly together with fluoride. The results of sperm parameters in our study are presented in Fig. 3 and Table 3. The fact that the destructive effect of fluoride, especially in the doses applied alone, can be corrected with a curative effect in this way, has clearly demonstrated that royal jelly has a highly curative activity against fluoride exposure on sperm parameters. This result showed that royal jelly can play a protective (prophylactic) role. Prophylactic dose is very important in prophylactic treatment. In this respect, we suggest that more studies on dosage should be conducted on royal jelly. Consistent with in vitro and human research in the literature, more than 60 animal studies (including rats, mice, roosters, and rabbits) have found that fluoride adversely affects the male reproductive system. These studies found effects such as decreased testosterone levels, decreased sperm motility, changes in sperm morphology, decreased sperm count, increased oxidative stress, and decreased reproductive capacity [64]. In our study, the effects of fluoride groups on sperm parameters confirm or support the results of this study in the literature. There are various studies in the literature reporting that royal jelly provides partial recovery against the negative effects of different active ingredients on sperm. In a study, it was shown that the application of royal jelly against nicotine, which reduces sperm motility and vitality, which is one of the sperm parameters, and increases the number of sperm with DNA damage, has a protective effect against these negative conditions [62]. In Streptozotocin (STZ)-induced diabetic rats, Streptozotocin has been shown to reduce sperm count, motility, viability and testicular weight, on the other hand, it has been shown that parameters such as testicular weight, sperm count, motility, and viability improved and the MDA levels of testicular tissue decreased in diabetic rats treated with royal jelly [65]. Upon examining the results of the supplements (ascorbic acid, vit C, vit D, vit E. Zinc) used in the literature, we can argue that the royal jelly we used in our study should be as important as the nutritional supplements in medical treatments in infertility. Cell nuclei stained with Harris hematoxylin were considered normal in TUNNEL staining analysis, and those with brown nuclear staining were considered apoptotic. The black arrow indicated in the cells in histological images indicates TUNNEL positivity, in other words, cell damage in the tissue. The images of the results of the TUNNEL method in our study are presented in Fig. 4a-Fig. 4f.

When the images of the histological findings were examined, significant increases were observed in TUNNEL positive cells in terms of testicular germ cell apoptosis and atrophy of the seminiferous tubule epithelium at the 50 mg/kg F and 100 mg/kg F doses that were only administered with fluoride. It was determined that the application of royal jelly together with the 50 mg/kg F and 100 mg/kg F groups reduced the cell and tubule degenerations seen in these groups. According to the apoptotic index results determined by the TUNNEL method (Table 4), the apoptotic index value in the testis tissue was determined the most in the 50 mg/kg F and 100 mg/kg F groups. Compared with the control group; It was determined that the apoptotic index value was similar in the RJ group, but at a lower level than the fluoridated groups. In Royal Jelly + Fluoride groups, it was determined that the apoptotic index decreased and improved compared to the groups that were treated with fluoride alone (50 mg/kg F and 100 mg/kg F). As a result of histological examination of testicular tissue under light microscopy by staining with hematoxylin & eosin (Fig. 5); Significant vacuolization and germ cell shedding were observed in the testicular tissues of the 50 mg/kg F and 100 mg/kg F groups compared to the control group. On the other hand, when Royal Jelly + Fluoride groups were compared with the fluoride applied groups (50 mg/kg F and 100 mg/kg F), it was observed that germ cell shedding regressed in the Royal Jelly + Fluoride groups, but vacuolization continued. As a result of histological evaluations made with both the TUNNEL method and microscopic imaging, it has been clearly demonstrated that fluoride causes damage to the testicular tissue. In addition, royal jelly was found to protect and heal against fluoride-induced testicular damage. In studies in the literature, histological findings have reported that fluoride causes testicular toxicity. It is seen that the effects that occur at different intensities vary depending on the fluoride application dose, duration and the examined tissue difference. In general, the results in the literature are consistent with the findings in our study. In a study in which the negative effects of chronic exposure to fluoride on spermatogenesis and testicular histopathology in rats were shown, it was determined that chronic fluoride toxicity caused thinning of the tubular wall of the rats' testicles, atrophy and edema

in the intertubular spaces. There was also a decrease in the number of spermatocytes and spermatozoa [66]. The results we obtained in our own study support this connection established by the researchers. There are also studies in the literature showing that royal jelly is curative against the damage caused by testicular damage. In many different studies with the application of royal jelly, it was found that the application of RJ to diabetes mellitus rats improved the kidney histopathology, and the curative effect of the royal jelly against the liver damage of fumonisin. In addition, royal jelly administration has been found to be a potential preventive agent against cisplatin-induced hepatic toxicity [21, 23, 67, 68]. In our study, the negative effects of fluoride on sperm parameters were shown, and it is thought that these effects may be related to the damage caused by fluoride in the testicular tissue. However, much more work is needed to fully understand the molecular mechanisms underlying the damage caused by fluoride in sperm and testis tissue.

High release of Bcl-2, one of the antiapoptotic proteins, is considered as an indicator of undesirable disease or damage states. Therefore, the increase in Bcl-2 expression shows the importance and strong effect of fluoride toxicity. Bands of Bcl-2 protein expression are given in Fig. 1c. Protein secretion was indicated to increase in fluoride groups. Although fluoride has a high inducing effect on Bcl-2 protein secretion, it can be interpreted as an improvement by reducing the high Bcl-2 level caused by fluoride. In addition, this situation can be evaluated as an indicator of the antioxidant effect of royal jelly. As a matter of fact, it has been reported in the literature that Bcl-2 level may increase in damage to germ cells in testicular tissue [69]. In addition, there are studies in the literature showing that Bcl-2 levels increase as an indicator of cell damage due to fluoride effect in different tissues [70, 71]. In these studies, it was reported that apoptosis was suppressed by increasing Bcl-2 level and decreasing Bax level with the effect of fluoride in heart and muscle tissues. Based on the data we have obtained, we can consider the increase in the level of Bcl-2, an antiapoptotic protein, in the testicular tissue as an indication that the damaged cells are not taken under control by a mechanism such as apoptosis. When we analyse the caspase-3 protein expression levels, the decrease in the level of caspase activation at the doses of 50 mg/kg F and 100 mg/kg F that only applied fluoride is a data supporting this. Caspase-3 plays an active role in the functioning of the apoptosis mechanism. Caspase-3 levels are also increased in apoptosis [72]. The bands of caspase-3 protein expression are given in Fig. 1b. We can say that fluoride causes damaged cell formation by inducing Bcl-2 and suppressing caspase-3. Therefore, the decrease in Caspase-3 level and the increase in Bcl-2 level can be considered as an indication that the apoptosis mechanism is insufficient and the survival of the damaged cell continues. It can be expressed that the cell damage in fluoride applied groups is out of control without being affected by apoptosis too much, with the increase in anti-apoptotic Bcl-2 level and the decrease in Caspase-3. Inadequate apoptosis, like excessive apoptosis, is an uncontrolled inappropriate apoptosis and can cause various diseases [73]. On the other hand, the data we obtained has indicated that royal jelly reduces this damaging effect caused by fluoride. We think that the increase in Caspase-3 activity and the decrease in Bcl-2 level are related to this curative effect of royal jelly. The decrease in Bcl-2 and the increase in Caspase 3 in the groups given royal jelly and fluoride together, compared to the groups given only fluoride, led to this opinion. According to these results, it can be stated that royal jelly prevents the development of fluoride-induced testicular damage in rats by suppressing Bcl-2 and regulating the caspase pathway. In the literature, royal jelly has been shown to have a protective effect against the damage caused by fluoride in different tissues [70, 71]. In a study, it has been shown that proteins isolated from royal jelly increase the formation of apoptosis by increasing caspase 3 by decreasing Bcl-2 in hepotoxicity caused by CCl_4 and in hepatocellular carcinoma cells, thus reducing tissue damage by destroying cancerous cells [74]. Based on this study, we can say that in our results, apoptosis was promoted, since Bcl-2 decreased and Caspase3 increased in Royal Jelly + fluoride groups, and the damage caused by insufficient apoptosis mechanism was ameliorated. It is also supported by the histological, spermatological and other experimental results in our study that the damage decreased in the groups to which royal jelly was added. In some studies, it has been reported that some free radicals may cause overproduction of pro-inflammatory cytokines such as interleukin-1α (IL-1α), TNF-α [20, 75, 76]. Therefore, in our study, TNF-α and IL-1α protein expression levels, which indicate inflammatory markers that may be related to the formation of oxidative stress, were investigated. Bands of TNF-α, and IL-1α protein expression levels are presented in Fig. 1a, 1g. TNF-α is a cell signaling cytokine involved in systemic inflammation by inducing the acute phase reaction. When we examined the TNF-α results in our study, it was found that the TNF-α level was excessively increased in the 50 mg/kg F and 100 mg/kg F groups, which were only administered fluoride, compared to the control group. In addition, similar results were obtained for IL-1α protein expression levels. When both results are evaluated together, it can be suggested that TNF-α and IL-1α protein expressions increase in response to fluoride toxicity. TNF-α and IL-1 synthesis also increase in cases of infection or inflammation. It has been reported that TNF-α is also increased in response to IL-1α in some diseases [77, 78].

When the results we found were evaluated with the studies in the literature, it was seen that similar findings were also shown in various studies [79–82]. In a study, it was determined that fluoride caused an increase in IL-1α synthesis in

rats exposed to fluoride for 30 days [83]. In another study, chronic fluoride exposure was found to increase the expression of TNF-α, IL-1β in rat seminiferous tubules [84]. In another study, TNF-α and IL-1α levels were investigated in pathological conditions in the testis. As a result of the study, it was revealed that IL-1α and TNF-α signaling pathways are stimulated and their expression increased in pathological conditions such as testicular torsion (distortion of testicular rotation around itself) and testicular autoimmune orchitis (testicular inflammation) [85]. TNF-α and IL-1α proinflammatory cytokines have been reported to play important roles in normal testicular homeostasis and their expression is increased in testicular dysfunctions [81]. Understanding the roles of proinflammatory cytokines in the testis and testicular pathologies will help develop therapies to protect against the negative effects of testicular inflammation and provide insight into male infertility. Our results showed that expressions of TNF-α and IL-1α levels were induced in testicular tissue in response to fluoride toxicity. Decrease in TNF-α and IL-1α expressions compared to 50 mg/kg F and 100 mg/kg F groups due to the improvement in testicular tissue with royal jelly application was reported. In our study, we can say that we obtained findings consistent with the results of studies investigating protein expressions with royal jelly in the literature. In different studies in the literature, it has been determined that royal jelly has a protective effect against damage caused by cadmium, and that the levels of TNF-α and IL-1β, which are increased by cadmium in this protective activity, decrease with the application of royal jelly together with cadmium in studies performed on testicular tissues [86]. In another study, royal jelly was shown to reduce TNF-α and IL-1β levels, which were increased in ethylene glycol-induced renal inflammation in rats [87]. When we evaluate the results in our study and the results in the literature, we can suggest that royal jelly has a corrective activity on tissue damage, while inhibiting the increase in the expression of TNF-α and interleukin-1 protein, which are proinflammatory cytokines, and thus has a potential protective activity by partially improving it with treatment. NF-κB is a transcription factor whose activity can be induced as a response to oxidative stress or damage due to oxidative stress. Various studies have reported that the activation of NF-κB increases in the case of oxidative stress, thereby releasing a large number of inflammatory cytokines [88–91]. In this respect, NF-κB can be said to be so important that it can initiate various signaling pathways in immunity. Bands of NF-κB protein expression levels are presented in Fig. 1e. According to our findings, NF-κB protein expression level increased significantly with fluoride application compared to the control group. On the basis of this result, we can say that fluoride creates a severe oxidative stress in testicular tissue and up-regulation of NF-κB is triggered by oxidative stress activation and its expression level increases. On the other hand, we found that royal jelly attenuated NF-κB activation induced by fluoride-induced testicular damage or oxidative stress. This can be explained as royal jelly reduces oxidative stress and down-regulates NF-κB activation. We can also say that royal jelly thus has an anti-inflammatory feature. On the other hand, we found that royal jelly attenuated NF-κB activation induced by fluoride-induced testicular damage or oxidative stress. This can be explained as royal jelly reduces oxidative stress and down-regulates NF-κB activation. We can also say that royal jelly thus has an anti-inflammatory feature. In another study, it was revealed that fluoride increased the expression of NF-κB in testicular tissue [92].

In another study, sodium fluoride application was shown to activate the NF-κB signaling pathway, and in this activation, the expression of anti-inflammatory cytokines decreases in mice and causes renal inflammation in the kidney tissue. In addition, in this study, the activation of the NF-κB signaling pathway was characterized by increased protein expression levels of TNF-α, interleukin-1, and COX-2 in fluoridated groups [93]. It has been reported in various studies that TNF-α can stimulate NF-κB signaling pathways, and that increases in TNF-α, IL-1-α, IL-1β, and NF-κB expression under pathological conditions are associated with the progression of the disease [94–97]. Therefore, according to studies in the literature, we can suggest that TNF-α, IL-1α and COX-2 levels increase under pathological conditions as a result of toxicity and oxidative stress caused by fluoride, and that this increase activates the NF-κB signaling pathway and increases NF-κB release. We can say that the mechanism of action of fluoride in the formation of testicular damage causes the induction of NF-κB. However, the toxicity mechanism of fluoride has not been clarified yet, and there is a need for more detailed research on this subject. Studies on the effect of royal jelly on this NF-κB protein expression are available in the literature. In a study with royal jelly, administration of royal jelly was shown to reduce cadmium-induced nephrotoxicity in mice [98]. In this study, the increases in TNF-α and IL-1-β decreased with royal jelly application. It has been shown that this decrease is caused by a strong inhibitory effect on IL-1β and TNF-α expression by suppressing the NF-κB activity of royal jelly. From this point of view, according to the results of our study, we can argue that the expressions of TNF-α, IL-1α and NF-κB are induced due to the toxicity of fluoride, and that these increases in proteins regress with the application of royal jelly, and thus the severity of toxicity decreases. Bands of COX-2 protein expression levels are presented in Fig. 1f. It has been determined that fluoride increases COX-2 expression due to its toxicity in testicular tissue, and COX-2 levels in fluoride groups of royal jelly supplement are reduced to levels close to control in a very good way. These findings are consistent with the results of various studies in the literature.

COX enzymes have been reported to produce a biological effect in the pathogenesis. Significant increased levels of COX-2 levels in inflamed tissues have also been reported [99]. Recent studies have focused on the effect of COX on the regulation of testicular function and male fertility [100, 101].. When we examined the Nrf-2 protein expression levels obtained, it was determined that the Nrf-2 levels of the groups administered fluoride alone were considerably lower than the control group. A significant increase in Nrf-2 values with the application of royal jelly together with fluoride was observed. On the basis of these results, we have shown that royal jelly provides protection against the negative effect of fluoride toxicity on Nrf-2 transcriptional activity. Based on these data, we can state that royal jelly has a protective and curative effect against the toxic effects of fluoride in the testicular tissue.

Conclusion

As a result, we suggest that we should avoid a use that may create an overdose when using fluoride products. In summary, attention should be paid to the amount of fluoride in the water we drink, the foods we take, and the fluoride-containing products we use in cosmetics, dental and oral health. These products should be consumed consciously and carefully, especially when used by children. More research is needed to reveal the tissue damage fluoride can cause in humans. While people are exposed to fluoride throughout their lives, it is not correct to calculate fluorine intake by considering only the fluorine concentrations in drinking water. Therefore, we recommend that more research be conducted to determine the fluoride intake levels from commonly used fluoride products and to determine their effects. Since there are many different types of royal jelly, it is important to check the contents of royal jelly products. Although royal jelly seems to be a safe and effective nutritional supplement, more in vitro and in vivo studies are needed to introduce royal jelly into routine applications in treatment and to better understand its medicinal potential. Therefore, we recommend that more extensive studies be conducted with different types of royal jelly.

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Author's Contributions AA: Wrote the article, review-editing, investigation, methodology, formal analysis, GP: wrote the article, laboratuary analysis, reading article, GT: Methodology, formal analysis, TK: histopathological analysis, formal analysis, MKB: laboratuary analysis, formal analysis, OG: laboratuary analysis, formal analysis, SB: laboratuary analysis, formal analysis, AEP: reading article, SDC: laboratuary analysis, formal analysis.

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Data Availability Statement All data of present study was inserted in this manuscript.

Declarations

Ethical Approval All rats used for this study were obtained by Firat University Experimental Animals Research Center (FUDAM, ethical committee number of this study: 02/09/2020, 2020/12).

Informed Consent The authors declare consent to participate in this study.

Consent for Publication The authors declare consent to publish this study.

Conflict of Interest No potential conflict of interest was reported by the authors.

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