

## RESEARCH ARTICLE

# Protective Effects of Chitosan and Chitosan Oligosaccharide on Sodium Fluoride-Induced Testicular Damage in Male Rats: A Stereological and Histopathological Study

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## Abstract

The aim of the present study was to investigate the potential protective effects of Chitosan (C) and Chitosan Oligosaccharide (COS) on sodium fluoride (NaF) induced testicular damage. Forty-two male Wistar rats were separated into six groups (n=7). The Control group was given drinking water without Fluoride. NaF group was given NaF (100 mg/L) in their drinking water. NaF+C group was given NaF (100 mg/L) and C (250 mg/kg/day). NaF+COS group was given NaF (100 mg/L) and COS (250 mg/kg/day). C group was given C (250 mg/kg/day). COS group was given COS (250 mg/kg/day). Rats were given C and COS by gastric gavage. As a result, the total number of spermatogonia, Leydig cells, the total volume of the testis, the total volume of germinal epithelium, the total volume of seminiferous tubules, the total length of seminiferous tubules, and the height of germinal epithelium significantly decreased in the NaF group compared to the control group (P<0.05). These parameters significantly increased in the NaF + C and NaF + COS groups compared to the NaF group (P<0.05). Also, GSH and CAT significantly decreased, while MDA significantly increased in the NaF group. C and COS alleviate these changes. These findings indicate that NaF can cause testis damage. C and COS can have cytoprotective effects against testis damage.

**Keywords:** Chitosan, Chitosan oligosaccharide, Rat, Sodium fluoride, Stereology, Testis

## Kitosan ve Kitosan Oligosakkaridin Erkek Sıçanlarda Sodyum Florür Kaynaklı Testis Hasarı Üzerindeki Koruyucu Etkileri: Stereolojik ve Histopatolojik Bir Çalışma

### Öz

Bu çalışmanın amacı, Kitosan (K) ve Kitosan Oligosakkarit (KOS) sodyum florür (NaF) kaynaklı testis hasarı üzerindeki potansiyel koruyucu etkilerini araştırmaktır. Kırk iki erkek Wistar sıçanı altı gruba ayrıldı (n=7). Kontrol grubuna florsuz içme suyu verildi. NaF grubuna içme suyunda NaF (100 mg/L) verildi. NaF+K grubuna NaF (100 mg/L) ve K (250 mg/kg/gün) verildi. NaF + KOS grubuna NaF (100 mg/L) ve KOS (250 mg/kg/gün) verildi. K grubuna K (250 mg/kg/gün) verildi. KOS grubuna KOS verildi (250 mg/kg/gün). Sıçanlara gastrik gavaj ile K ve KOS verildi. NaF grubunda, toplam spermatogonyum sayısı, Leydig hücresi sayısı, toplam testis hacmi, toplam germinal epitel hacmi, toplam seminifer tübül hacmi, seminifer tübüllerin toplam uzunluğu ve germinal epitel yüksekliği kontrol grubuna göre anlamlı olarak azalmıştır (P<0.05). Bu parametreler NaF + K ve NaF + KOS gruplarında NaF grubu ile karşılaştırıldığında istatistiksel açıdan önemli (P<0.05) biçimde artmıştır. Ayrıca NaF grubunda GSH ve CAT önemli derecede (P<0.05) azalırken, MDA önemli biçimde artmıştır. K ve KOS bu değişiklikleri hafiflettiği gözlenmiştir. Bu bulgular, verilen dozdaki NaF'nin testis hasarına neden olabileceğini, K ve KOS'un sitoprotektif etkilerinin olabileceğini göstermektedir.

**Anahtar sözcükler:** Kitosan, Kitosan oligosakkarit, Sıçan, Sodyum florür, Stereoloji, Testis

## INTRODUCTION

Fluoride is an essential molecule for the growth and development of living. It is found naturally in soil, water,

and nutrients<sup>[1]</sup>. The recommended concentration of fluoride must be between 0.5-1.5 mgL<sup>-1</sup> in drinking water according to the guideline of The World Health Organization<sup>[2]</sup>. However, excessive and prolonged consumption causes

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fluorosis, which is defined as fluoride toxicity in organisms [3]. Fluorosis can damage soft tissues such as brain [4], testis [1], duodenum [5], liver, and kidney [6]. In recent studies, special attention has been drawn to the side effects of fluorosis on the male reproductive system [7]. Although fluorosis has been reported to reduce the effectiveness of the antioxidant enzyme system and cause oxidative damage by inducing the formation of free oxygen species, its mechanism of cellular damage on the male reproductive system is not fully disclosed.

The use of herbal medicines for therapeutic purposes is increasing in the world. In some countries, herbal medicines are even more used than prescribed medicines [8]. Chitosan (C), a naturally abundant polysaccharide, has been the center of attention of scientists for more than 40 years due to its potential biomedical applications [9]. Chitosan is a natural substance used in medical applications for therapeutic purposes. It has the property of excellent biocompatibility, biodegradability, antioxidant, and accelerating wound healing [10]. Chitosan oligosaccharide (COS) is formed from the deacetylation and hydrolysis of chitin. COS has anti-inflammatory [11], anti-tumoral [12], and antioxidant [13,14] effects.

The testes have high amounts of antioxidants, and these antioxidants play important roles in the protection of testis against oxidative stress. But, if the injury is prolonged for a long time or is exposed to the severe toxic agent, the testes can become irreversibly damaged [15,16]. Therefore, in the present study, we aimed to examine whether C and COS have protective effects against the possible adverse effects induced by fluorosis on rat testes by stereological, histopathological, and biochemical methods.

## MATERIAL AND METHODS

### Chemicals

Chitosan (CAS number: 9012-76-4, Sigma-Aldrich), chitosan oligosaccharide (CAS number: 148411-57-8, Sigma-Aldrich), sodium fluoride (CAS number: 7681-49-4, Sigma-Aldrich), ketamine HCl (Ketalar 50 mg/mL, Inj., Pfizer, Istanbul, Turkey) and xylazine HCl (Alfazyne 2% Inj., Interhas A.S., Ankara, Turkey) were purchased from commercial companies.

### Animals and Experimental Design

The present study was approved by Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (decision number: 2019/09). In this study, a total of 42 adult male Wistar rats weighing 200-300 g were used, and the animals were purchased from the Van Yüzüncü Yil University Experimental Animals Research and Application Center. Rats were randomly divided into six groups (n=7). The Control group was given drinking water. NaF group received 100 mg/L NaF in their drinking water. NaF+C

group received NaF (100 mg/L) and C (250 mg/kg/day). NaF+COS group was exposed to NaF (100 mg/L) and also COS was given at 250 mg/kg/day. C group was given C (250 mg/kg/day), and COS group received COS (250 mg/kg/day). C and COS were given by gastric gavage. Rats were housed under standard conditions of temperature (25±2°C), relative humidity (50±10%), and 12 h light/12 h dark cycle. The animals were fed with a standard pellet diet (*ad libitum*). The experimental period continued for 12 weeks. At the end of the experiment, rats were sacrificed by ketamine overdose. The testis tissue was removed by an incision made from the scrotal region and fixed in 10% buffered formalin.

### Stereology

Isotropic identical random sections were obtained using the orientator method [17]. Thus, on average, 8-10 slabs were selected from each testis. The selected slabs were embedded in the same paraffin block. 10-15 consecutive sections were taken in 4-µm in thickness. The sections were stained with Hematoxylin-Eosin (H-E) and examined under a light microscope.

### Total Number of Cells

The physical dissector counting method was used to calculate the total number of spermatogonia, Sertoli, and Leydig cells. In the area limited by an unbiased counting frame, cells were calculated that existing in the reference section but not existing in the look-up section (*Fig. 1-A*). The following formula was used to calculate the cell number:  $N = N_v \times V_{ref}$ . Where  $N_v$  is the numerical density of the cell of interest (cells/unit volume) and  $V_{ref}$  is the total reference volume of the testis [15,18].

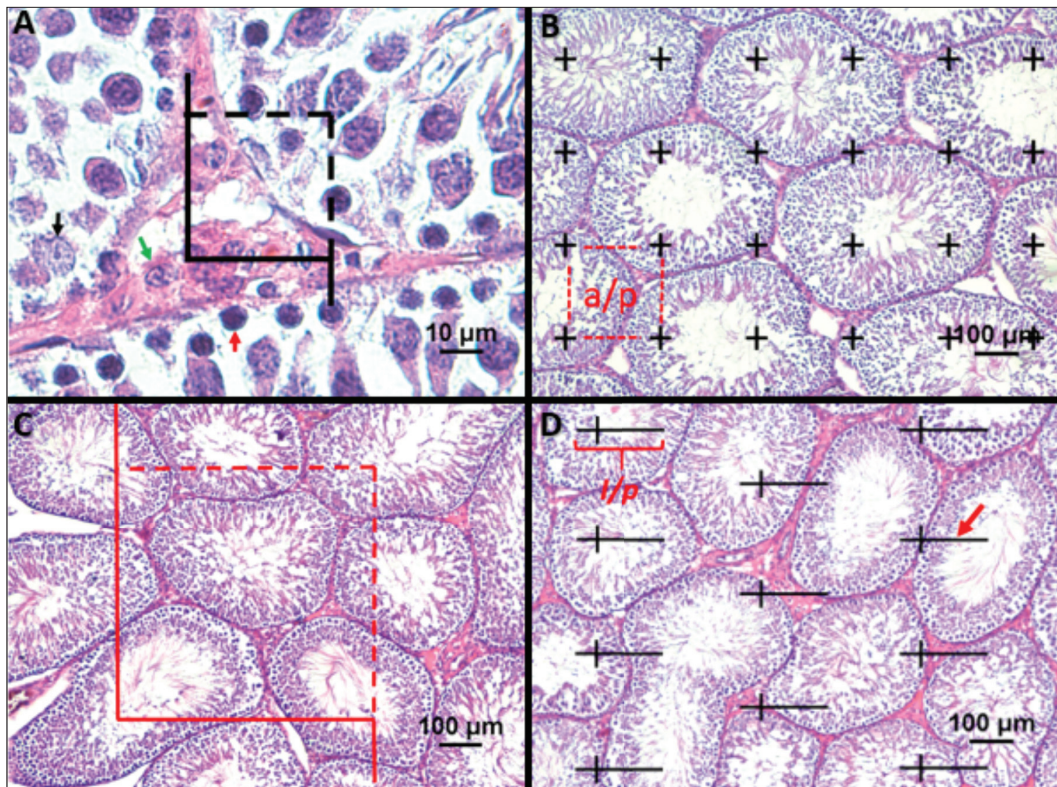
### Estimation of Total Volume

Cavalieri principle was followed to estimate the total volume of the testis, seminiferous tubule, germinal epithelium, and interstitial tissue. For this, the point grid was used (*Fig. 1-B*). The following formula was used to calculate the volume:  $V = \Sigma P \times a/p \times t$ . Where "V" is the volume of the structure, " $\Sigma P$ " is the total number of points hitting the structure, " $a/p$ " is the area covered by one point, and "t" is the section thickness [15].

After the histological tissue processing stages (Fixation, processing, sectioning and staining), the volume of tissue is usually shrunk. For estimation of testis final volume, the tissue shrinkage value must be determined. Thus, the degree of tissue shrinkage ( $d_{(shr)}$ ) is calculated by the following formula:

$$d_{(shr)} = 1 - (AA/AB)^{1.5}$$

Where  $d_{(shr)}$  is the degree of tissue shrinkage, AA is the area of the circular piece after histological processing stages, AB is the area of the circular piece before histological processing stages. Coefficient (3/2=1.5) is required to calculate



**Fig 1.** A. Application of physical disector counting method. An unbiased counting frame has been used. Black arrow: Sertoli cell, green arrow: Leydig cell, red arrow: Spermatogonia; B. The superimposed of a point grid on the image of section; C. The superimposed of an unbiased counting frame on the image of section; D. The superimposed of a grid of test lines on the image of the section

the tissue shrinkage degree in three dimensions (3) from two dimensional (2) areas. Accordingly, the final volume of the testis was calculated using the following formula:

$$V_{(\text{shrunken})} = V_{(\text{unshrunken})} \times [1 - d_{(\text{shr})}]$$

Where  $V_{(\text{shrunken})}$  is the volume after the histological processing steps,  $V_{(\text{unshrunken})}$  is the volume before the histological processing steps,  $d_{(\text{shr})}$  is the degree of tissue shrinkage [15].

#### **Estimation of Length Density and Total Length of Seminiferous Tubule**

The length density of seminiferous tubules was calculated using an unbiased counting frame (Fig. 1-C). The unbiased counting frame with prohibited and independent lines is put on the images. The profiles of the seminiferous tubules, which are either totally or partially inside the counting frame and do not contact prohibited lines and their extensions are counted [19]. The length density ( $L_v$ ) of seminiferous tubules is calculated using the following formula:

$$L_v = 2 \times \Sigma Q / (\Sigma F \times a/f)$$

" $\Sigma Q$ " is the total number of the profiles of seminiferous tubules counted per testis, " $\Sigma F$ " is the total number of frames and " $a/f$ " is the frame area. The total length of seminiferous tubules (L) is obtained by multiplying the length density ( $L_v$ ) value with the total volume value.

#### **Estimation of Height of Germinal Epithelium**

To estimate the height of germinal epithelium, primarily the surface density of epithelium must be estimated. The surface density was calculated using a grid of test lines. A grid of test lines was put on the images of the sections. Each test line is accommodated with one point (Fig. 1-D). The surface density of the epithelium ( $S_v$ ) was calculated using the following formula:

$$S_{V(\text{epithelium/ref})} = 2 \times \Sigma I / (\Sigma P \times l/p)$$

The height of germinal epithelium =  $V_{(\text{epithelium/ref})} / S_{V(\text{epithelium/ref})}$

" $\Sigma I$ " is the total number of intersection points of the luminal surface of the epithelium and the test lines, " $\Sigma P$ " is the total number of points hitting testis and " $l/p$ " is the length of a test line. The total surface area is calculated by multiplying the surface density by the total volume [19].

#### **Histopathological Observations**

Four  $\mu\text{m}$  thick sections taken from paraffin blocks of testis were stained with H-E and examined by light microscope (Nikon Y-IM 7551012, Japan). For histopathological evaluation, an average of 15-20 areas was evaluated by random sampling for each animal in the groups. The findings were semiquantitatively evaluated according to the number of lesions observed in the examined areas.

### Biochemical Analysis

The testis samples were homogenized in phosphate buffer (pH: 7.4). Homogenates were centrifuged at 10,000 G for 15 min at +4°C. The obtained supernatants were used for biochemical analysis. Analysis of GSH, MDA, and CAT was evaluated by spectrophotometric method.

### Statistical Analysis

Statistical analyses were accomplished using SPSS 21.0 software. Differences between groups were evaluated by the Kruskal-Wallis test followed by Mann-Whitney U test. P-value  $\leq 0.05$  was accepted as statistically significant. All data were expressed as means  $\pm$  standard deviations.

## RESULTS

### The Total Number of Spermatogonia, Sertoli, and Leydig Cells

The results indicated that there was a significant reduction in the total number of spermatogonia and Leydig cells in the NaF group compared to the control group ( $P < 0.05$ ). However, this reduction was prevented in the NaF+C and NaF+COS groups compared to the NaF group ( $P < 0.05$ ) (Table 1).

### The Total Testicular Volume

As shown in Table 2, when compared to the control group, the total volume of the testis, the total volume of germinal

epithelium, and the total volume of seminiferous tubule significantly decreased ( $P < 0.05$ ) in the NaF group. But, no significant change was observed in the total volume of interstitial tissue. Also, the total volume of the testis, the total volume of germinal epithelium, and the total volume of seminiferous tubule significantly increased in NaF+C and NaF+COS groups compared to NaF group ( $P < 0.05$ ) (Table 2).

### The Total Length of Seminiferous Tubule

The data of the present study showed that the total length of seminiferous tubules significantly decreased in the NaF group compared to the control group ( $P < 0.05$ ). The total length of seminiferous tubule was significantly increased in NaF+C and NaF+COS groups compared to NaF group ( $P < 0.05$ ) (Table 2).

### Height of Germinal Epithelium

As the results in Table 2 showed that NaF caused a significant decrease in the height of germinal epithelium ( $P < 0.05$ ). However, the height of germinal epithelium significantly increased in NaF+C and NaF+COS groups compared to NaF group ( $P < 0.05$ ) (Table 2).

### Results of the Histopathological Evaluations

The control group displayed normal histological architecture. The number of germinal epithelial layers was between 5-9. The Spermatogenic cells and Sertoli cells were seen in the germinal epithelium. There were numerous morphologically mature sperm cells in the lumina of the seminiferous

**Table 1.** Effects of C and COS on the total testicular cell numbers ( $\times 10^6$ ) in fluoride-induced testicular damage

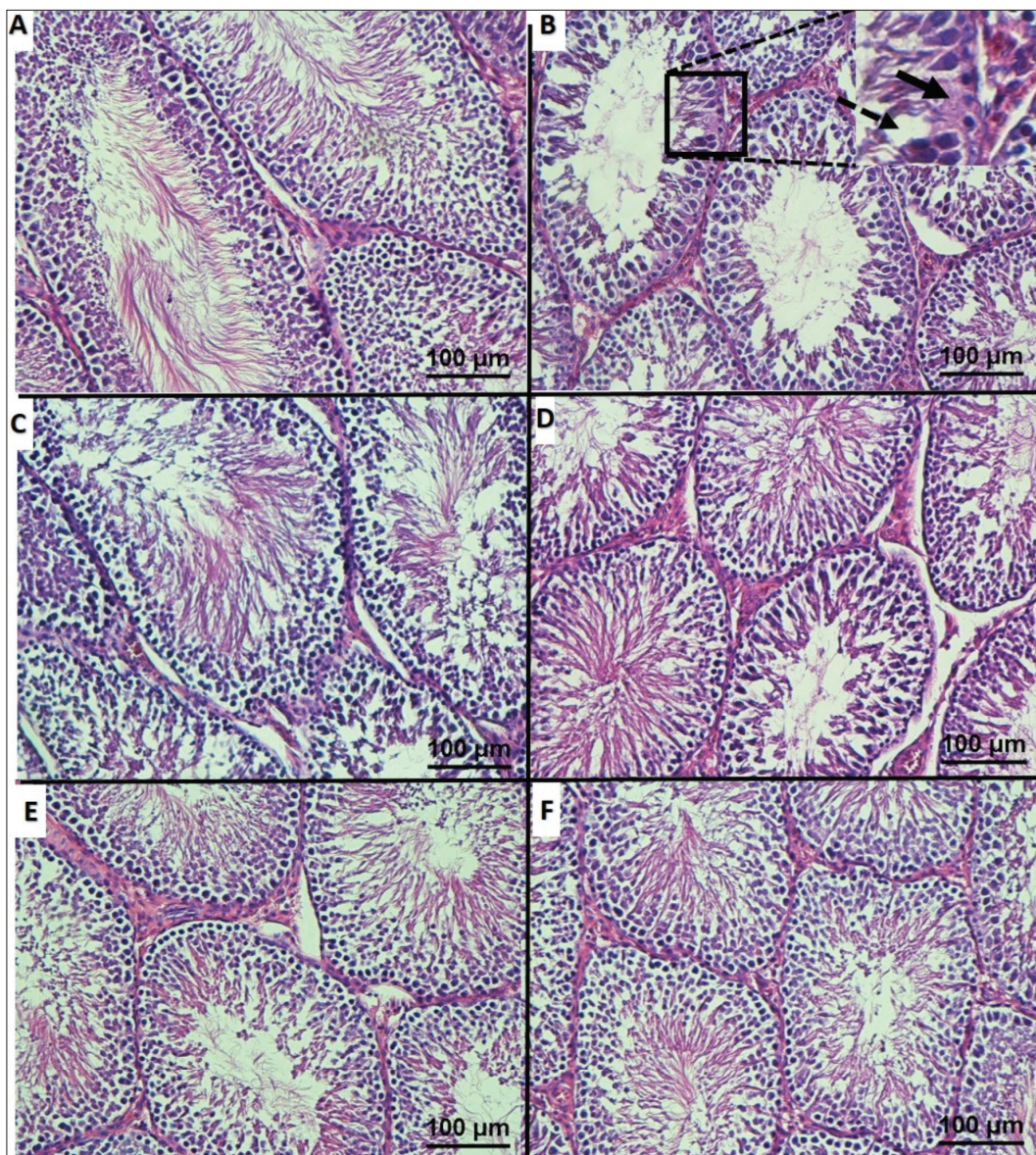
Groups	Spermatogonia	Sertoli Cell	Leydig Cell
Control	15013534.13 $\pm$ 693584.13 <sup>b</sup>	8021941.12 $\pm$ 479449.01	5987347.25 $\pm$ 722449.09 <sup>b</sup>
NaF	12800925.05 $\pm$ 697264.84 <sup>a</sup>	7336928.54 $\pm$ 668999.64	4437461.49 $\pm$ 775097.53 <sup>a</sup>
NaF+C	13854743.58 $\pm$ 592270.19 <sup>a,b</sup>	7232094.41 $\pm$ 734270.39	5411601.51 $\pm$ 390257.57 <sup>b</sup>
NaF+COS	14761358.87 $\pm$ 743880.32 <sup>b</sup>	7435793.68 $\pm$ 689778.15	5571682.93 $\pm$ 554982.46 <sup>b</sup>
C	14942930.47 $\pm$ 1025808.47 <sup>b</sup>	7761246.84 $\pm$ 100584.08	6013985.48 $\pm$ 859375.71 <sup>b</sup>
COS	15135822.09 $\pm$ 831371.61 <sup>b</sup>	8245787.11 $\pm$ 435083.26	5748381.14 $\pm$ 768643.24 <sup>b</sup>

Values are expressed as means  $\pm$  SD. <sup>a</sup>  $P < 0.05$ , compared with the control group, <sup>b</sup>  $P < 0.05$ , compared with the NaF group

**Table 2.** Effects of C and COS on the total volume ( $\text{mm}^3$ ) of testes, the total length of seminiferous tubules (m), and the height of germinal epithelium ( $\mu\text{m}$ ) in testis damage induced by fluoride

Groups	The Total Volume of Testis	The Total Volume of Interstitial Tissue	The Total Volume of Germinal Epithelium	The Total Volume of Seminiferous Tubules	The Total Length of Seminiferous Tubules	The Height of Germinal Epithelium
Control	1063.33 $\pm$ 98.74 <sup>b</sup>	194.33 $\pm$ 15.35	508.50 $\pm$ 55.02 <sup>b</sup>	846.00 $\pm$ 43.89 <sup>b</sup>	10.28 $\pm$ 0.59 <sup>b</sup>	69.17 $\pm$ 3.31 <sup>b</sup>
NaF	816.16 $\pm$ 64.26 <sup>a</sup>	179.50 $\pm$ 13.72	393.50 $\pm$ 49.39 <sup>a</sup>	644.17 $\pm$ 59.15 <sup>a</sup>	8.71 $\pm$ 0.66 <sup>a</sup>	51.50 $\pm$ 1.87 <sup>a</sup>
NaF + C	930.50 $\pm$ 56.31 <sup>a,b</sup>	186.67 $\pm$ 14.84	450.17 $\pm$ 31.33 <sup>a,b</sup>	715.01 $\pm$ 27.19 <sup>a,b</sup>	9.37 $\pm$ 0.42 <sup>a,b</sup>	60.33 $\pm$ 2.15 <sup>a,b</sup>
NaF + COS	981.00 $\pm$ 77.11 <sup>b</sup>	185.71 $\pm$ 16.03	469.29 $\pm$ 43.16 <sup>b</sup>	773.71 $\pm$ 65.67 <sup>a,b</sup>	9.51 $\pm$ 0.48 <sup>a,b</sup>	63.00 $\pm$ 2.16 <sup>a,b</sup>
C	1119.00 $\pm$ 60.85 <sup>a,b</sup>	192.67 $\pm$ 20.60	493.33 $\pm$ 33.17 <sup>b</sup>	804.00 $\pm$ 58.51 <sup>b</sup>	10.01 $\pm$ 0.65 <sup>b</sup>	71.67 $\pm$ 2.52 <sup>b</sup>
COS	1148.67 $\pm$ 77.67 <sup>b</sup>	202.00 $\pm$ 15.53	529.00 $\pm$ 32.23 <sup>b</sup>	873.01 $\pm$ 47.00 <sup>b</sup>	10.49 $\pm$ 1.15 <sup>b</sup>	72.33 $\pm$ 1.53 <sup>b</sup>

Values are expressed as means  $\pm$  SD. <sup>a</sup> Significant differences as compared with the control group at  $P < 0.05$ , <sup>b</sup> Significant differences as compared with the NaF group at  $P < 0.05$



**Fig 2.** Photomicrographs of testicular sections. Control (A), a normal histological structure of testis. NaF group (B), atrophy and necrosis in seminiferous tubule (dashed arrow: atrophy, arrow: necrotic cell), a decrease of the number of germinal epithelial layers. NaF+C (C) and NaF+COS (D) groups were restored according to the NaF group. C (E) and COS (F) groups were similar to the control

tubules. Leydig cells are located in the interstitial regions (Fig. 2-A). In the NaF group; severe atrophy, degenerate and necrotic cells were observed in the germinal epithelium. The germinal epithelial layers were between 3-6. There were very few mature sperm cells in the tubular lumina (Fig 2-B). Less atrophy and degeneration were observed in the germinal epithelium of the NaF+C (Fig. 2-C) and NaF+COS (Fig. 2-D) groups. The histological structure of testes in the C (Fig. 2-E) and COS (Fig. 2-F) groups was similar to the control.

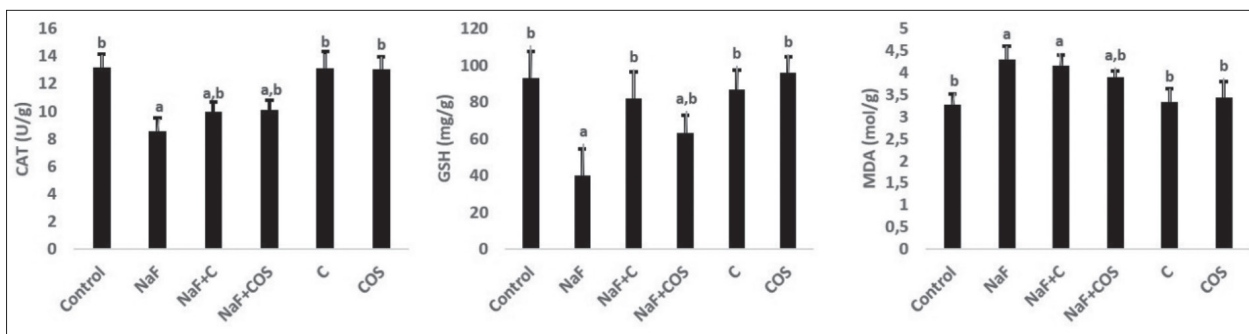
### Biochemical Analysis

The level of MDA, a marker of oxidative stress, was significantly higher in the NaF group compared to the control group. In the NaF+COS group, a significant decrease

in MDA level was observed compared to the NaF group ( $P < 0.05$ ). Furthermore, the levels of GSH and CAT significantly decreased in the NaF group compared to the control group. However, compared to the NaF group, the levels of GSH and CAT were significantly higher in the NaF+C and NaF+COS groups ( $P < 0.05$ ). In C and COS groups, the levels of MDA, GSH, and CAT were similar to the control group (Fig. 3).

## DISCUSSION

Infertility affects about 15% of couples who want to have children. About half of these cases are of male origin. Oxidative stress induced by toxic substances is considered one of the most important causes of male infertility. Oxidative



**Fig 3.** Effect of C and COS on the activity of CAT and GSH and content of MDA in NaF-induced testis damage. Values are expressed as means  $\pm$  SD. <sup>a</sup> Significant differences as compared with the control group at  $P < 0.05$ , <sup>b</sup> Significant differences as compared with the NaF group at  $P < 0.05$

stress is a pathological condition associated with cellular damage caused by free oxygen species (ROS). ROS can cause cell death by disrupting DNA, lipids, proteins, and enzymes. Thus, it may cause significant deteriorations in the semen parameters associated with male infertility [19].

Fluoride, which is found in many sources on earth, plays an important role in bone development, growth and remodeling. However, taking it too much can cause fluorosis [1]. In the previous studies, it was reported that fluorosis has harmful effects on the male reproductive system [7]. In our study, exposure to NaF increased MDA content and significantly decreased GSH, CAT activity which is an indicator of enhanced oxidative stress in the rats. A significant decrease in the stereological parameters of the testes is an indicator of the toxic effect of fluoride on the testis [20].

Spermatogonia are the precursor cells of sperm cells which are mature male reproductive cells. Spermatogonia are easily affected by toxic substances. In a previous study, it was reported that fluoride increased the number of apoptotic spermatogonia [21]. Also, fluoride decreased sperm count and sperm viability [7]. In the present study performed using stereological methods, it was estimated that the decrease in the total number of spermatogonia as a result of cell death is caused by the cytotoxic effect of NaF (by inducing oxidative stress). The significant decrease in total seminiferous tubule volume, total seminiferous tubule length, total seminiferous tubule germinal epithelium height, and volume may occur due to the decrease in the number of spermatogonia. These quantitative data support each other. It is estimated that these decreased parameters may also result in a decrease in the number of mature sperm cells, thus causing male infertility [7,21].

Leydig cells secrete testosterone which is the male sex hormone playing very important androgenic effects in the maturation of male sexual organs and the development of secondary sex characters. Testosterone is also a necessary hormone for normal sperm production. It has been reported that fluorosis reduces nerve growth factor (NGF) expression, which stimulates sperm motility, induces Leydig

cell differentiation and proliferation, and testosterone production [8]. Also, it was demonstrated that NaF caused a significant decrease in levels of testosterone in rats [22] and mice Leydig cells. In our study, the decreased total number of Leydig cells caused by fluoride may result in decreased testosterone level, thereby spermatogenesis may be impaired [7,23].

Chitosan has been reported to have a protective effect against reproductive toxicity [22]. In the present study, chitosan exhibited antioxidant properties by preventing the decrease in CAT and GSH levels caused by fluorine. In addition, it was observed that chitosan alleviated the decrease in spermatogonia and Leydig cell numbers, total testicular volume, the total volume of the seminiferous tubule, the total volume of germinal epithelium, seminiferous tubule length, and germinal epithelium height.

The previous studies have shown that COS exhibited anti-oxidative effects in pancreatic  $\beta$  cells [24] and endothelial cells [25]. In the present study, COS reversed the increase of MDA content, a decrease of GSH and CAT activity induced by NaF. Thus, it was concluded that COS exhibited an antioxidative effect. We found that COS prevents the decrease in mean spermatogonia and Leydig cell numbers induced by the cytotoxic effect of fluorosis. Also, COS protected the total volume of the testis, the total volume of interstitial tissue, total volume and the total length of seminiferous tubules, and height of germinal epithelium from cytotoxic effects of fluorosis.

As a result, NaF caused a decrease in the number of spermatogonia, Leydig cells, and testis volume. Also, NaF was observed to cause oxidative stress in rat testis. Thus, the results of our study indicated that NaF may cause testis damage. C and COS may have a protective effect against the toxic effects of NaF on the testis.

#### STATEMENT OF AUTHOR CONTRIBUTIONS

FA planned the study, performed experiments, commented on the data, edited the manuscript, analyzed the data, approved the final version of the manuscript to be

responsible for all of the study. UÖ performed the biochemical analyses and interpreted them, approved the final version of the manuscript, and agreed to be responsible for all of the study.

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## CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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