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Protective effects of chitosan and chitosan oligosaccharide against oxidative damage in liver tissue of rats with fluorine poisoning

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Abstract: Fluorine toxicity has negative effects on soft tissue besides skeletal and dental tissues. In the present study, we have investigated the protective effect of chitosan (CS) and chitosan oligosaccharide (COS) on liver tissue of fluorine-intoxicated rats taking the antioxidant characteristics of chitosan and its derivatives into consideration. In this study, 42 male Wistar albino rats were randomly selected to determine the control and experimental fluorosis groups. Our study lasted for 12 weeks. As a consequence of the study, MDA significantly increased in the liver tissue of NaF group while some antioxidant values significantly decreased. It was detected that serum AST and LDH levels increased significantly while ALB and TP values significantly decreased in NaF group. The degenerations were identified in the liver histopathology of all fluoride-treated groups. We have concluded according to the results that chitosan oligosaccharide can be more effective compared with chitosan.

Keywords: Fluorosis, chitosan, oxidative stress, liver, antioxidant.

INTRODUCTION

Several forms of fluoride is widely present in nature as well as its compounds are commonly used. Fluorine is not found in free state in nature. Fluorine in waters is in ionic state. Therefore, it can easily pass through intestinal mucosa and may affect metabolic events. Low doses of fluoride have an important prophylactic effect by inhibiting dental caries. At higher doses, it causes dental and skeletal fluorosis (Hassan and Yousef, 2009, Saber, Mansour *et al.*, 2020). Fluorosis can also adversely affect soft tissues such as liver and kidney (Saber, Mansour *et al.*, 2020). Fluorosis causes oxidative stress in the liver and kidney affecting liver function tests and blood lipid profile significantly (Hassan and Yousef, 2009, Rahmani and Rezaei, 2020). Chitosan is a cationic polysaccharide derived by N-deacetylation of chitin as the second most common polymer in the skeleton of crustaceans after cellulose in nature. Chitosan can be easily modified depending on the presence of many hydroxyl and amino groups in its structure (Yan *et al.*, 2006). Different chitosan derivatives with specific activities have been reported (Baumann and Faust, 2001). Chitosan has characteristics such as non-toxicity and degradability making it an attractive biopolymer for researchers (Yan *et al.*, 2006, Zhang, Xing *et al.*, 2020). Thanks to their antioxidant properties, chitosan and chitosan oligosaccharides have been found to show significant positive effects on liver and kidney tissues as well as their positive impacts on lipid profile and blood biochemical parameters (Ramasamy *et al.*, 2014, Yan *et al.*, 2006, Zhang *et al.*, 2019). In this study, we have aimed to investigate the effects of chitosan and chitosan oligosaccharide on blood parameters, liver tissue oxidative stress parameters and liver histopathology in fluorine-intoxicated rats.

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MATERIALS AND METHODS

The present study was carried out using 42 male Wistar albino rats obtained from Van Yuzuncu Yıl University Experimental Animal Unit due to the approval of Van Yuzuncu Yıl University Animal Experiments Local Ethics Committee (Dated 29.03.2018 and No: 2018/03). The rats were housed in cages to feed with rat pellet feed in the rooms exposed to 12 hours of darkness followed by 12 hours of light at a temperature of 22±2°C during the experiment.

Experimental procedure

Before starting the 12-week study, 42 male Wistar albino rats were divided to 6 experimental and control groups including 7 rats in each group after measurement of their weights. Normal drinking water was given in the control group. Fluroidated drinking water with 100 ppm sodium fluoride was given to the fluorosis group (NaF) (Zhang, Zhou *et al.*, 2014). Fluoridated drinking water containing 100 ppm sodium fluoride was given to the fluorosis and chitosan group (NaF-CS) in addition to oral administration of 250mg/kg/day chitosan (Pan, Yang *et al.*, 2016). Fluoridated drinking water containing 100 ppm sodium fluoride was given to the fluorosis and chitosan oligosaccharide group (NaF-COS) in addition to administration of 250mg/kg/day (Zong, Yu *et al.*, 2012) chitosan oligosaccharide via gastric gavage. A single dose of 250mg/kg/day chitosan was administered orally in the chitosan group (CS). A single dose of 250mg/kg/day

chitosan oligosaccharide was administered via gastric gavage in the Chitosan oligosaccharide group (COS).

During the experiment; daily water consumption, body weight gain and feed consumption of the rats were recorded periodically. In the last week of the procedure, the rats were placed in metabolic cages and their 24-hour urine was collected in polyethylene tubes to detect fluoride (Zhang, Zhou *et al.*, 2014).

At the final stage of the experiment, the rats were placed in the dorsoventral position on a table after administration of 75 mg/kg ketamine (i.p), the heart was cannulated directly and blood samples were taken into vacuum tubes. The serum was removed by centrifugation of blood in tubes without anticoagulant at 3000 rpm for 5 minutes. The blood serum samples were analyzed for the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), triglyceride, cholesterol, total protein and albumin using a commercial kit with an automated-analyzer.

The liver of the rats sacrificed due to haemorrhage after cannulation of the heart under ketamine anaesthesia was removed. Some of the liver samples were placed in 10% formaldehyde and separated for histopathological analysis.

Preparation of tissue homogenate and biochemical analysis

A 5 ml of phosphate buffer (pH=7.4) was added onto an approximately 0.5g of liver tissue and homogenized using a homogenizer. It was assured to keep the cold chain at all stages of the experiment. The homogenates were centrifuged at 10000 xg and for 15 minutes +4ºC (Samarghandian, Azimi-Nezhad *et al.*, 2015). Using the obtained supernatants; Glutathione (GSH) analysis (Beutler, 1963), malondialdehyde (MDA) analysis (Dubovskiy, Martemyanov *et al.*, 2008), enzyme activity analysis of catalase (CAT) (Lartillot, Kedziora *et al.*, 1988), superoxide dismutase (SOD) (Sun, Oberley *et al.*, 1988), and glutathione peroxidase (GSH-Px) (Lawrence and Burk, 2012), spectrophotometric analysis for the calculation of advanced oxidation protein products (AOPP) (Hanasand, Omdal *et al.*, 2012) and cellular thiol fractions such as total thiol (T-SH), non-protein thiol groups (NP-SH) and protein thiol groups (P-SH) (Sedlak and Lindsay, 1968) and analysis of 8-hydroxy-2'deoxyguanosine (8-OHdG) using commercial kit (Cat.no: YLA0061RA) on ELISA device (BioTek, ELx808, Winooski, USA) were carried out.

Histopathological examination

At the final stage of the experiment, liver tissues reserved for histopathological examination were embedded in paraffin blocks after routine histological procedures. The sections of 4µm thickness taken from each block were stained with hematoxylin-eosin and examined under a light microscope with digital camera (DM 6000, Leica Microsystems, Wetzlar, Germany). The sections were examined under the light microscope to evaluate according to the lesions and their pictures were taken (Oguz, 2015).

STATISTICAL ANALYSIS

One-Way Analysis of Variance (ANOVA) was used for comparison between the group in terms of mean values while Duncan's test was applied for multiple comparisons between the groups. A p value of 0.05 was accepted as the statistically significance level in the calculations. All analyzes were done using SPSS (20.0) package program.

RESULTS

It was determined that increases in total body weight measurements in all the groups treated with sodium fluoride was significantly lower compared with the control group at the end of the experiment ($p<0.05$) whereas no statistically significant difference was found between initial weight measurements. A statistically significant decrease was found in the sodium fluoride applied groups regarding food efficiency ratio (FER) value compared with the control group. It was observed that FER value in the NaF-COS group was lower than that in the NaF group ($p<0.05$) (table 1).

An approximity was detected between the urine fluoride levels of control, CH and COS groups ($p>0.05$). No statistically significant difference was determined among the urine fluoride levels of the fluoride-treated groups whereas urine fluroide levels of those groups was statistically significantly higher than the control group ($p<0.05$) (fig. 1).

The level of MDA as an oxidative stress marker in the liver tissue of the NaF group was found significantly increased compared with the control group ($p<0.05$). The serum level of GSH and enzymatic activity level of SOD and CAT were found significantly lower in all the groups treated with sodium fluoride compared with the control group ($p<0.05$). No statistically significant change was found among the study groups regarding AOBP, P-SH and 8-OHdG levels ($p>0.05$). The level of MDA that increased in the NaF group was found significantly decreased in the NaF-COS group whereas the decrease in the level of MDA was not statistically significant in the NaF-CS group. Although, there was a decrease in GSH level of the groups that were treated with chitosan and chitosan oligosaccharide among the sodium fluoride applied groups, no statistically significant difference was determined between those groups and NaF group. No

statistically significant change was identified between the healthy rats treated with chitosan and chitosan oligosaccharide in terms of tested biochemical parameters ($p<0.05$) (table 2).

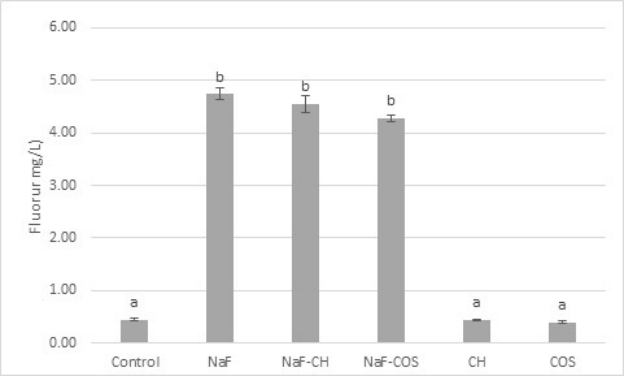

Fig. 1: The urine fluoride level (mg/L) of the experimental groups, the different letters on the columns indicate the statistical difference ($P<0.05$).

Serum albumin (ALB) and total protein (TP) levels were significantly lower in the NaF group whereas AST and LDH levels were significantly higher than the control group ($p<0.05$). In contrast with NaF group, a significant decrease was noted in AST and LDH values in the NaF-COS group. Besides that, a decrease was monitored in AST values in the NaF-CS group. It was determined that TP level in CS and COS groups indicated no difference whereas ALB level was found significantly decreased compared with the control group ($p<0.05$). No statistically significant difference among the study groups in terms of ALT values (table 3).

A significant increase was detected in cholesterol and LDL levels of the NaF group compared with the control group ($p<0.05$). It was observed that cholesterol level increased in the NaF group was found significantly decreased in the NaF-COS group ($p<0.05$) (table 4).

Hydrophilic degeneration, sporadic cell infiltration and necrosis were detected in the rat liver with fluorosis compared with the control group. The chitosan and chitosan oligosaccharide groups treated with sodium fluoride were found similar with the NaF group. Normal liver histology was encountered in the control group, chitosan and chitosan oligosaccharide groups (fig. 2).

DISCUSSION

The harmful effects of fluoride on the skeletal, dental and soft tissues have been demonstrated. Although there is abundant information related with the mechanism of bone and dental fluorosis, the mechanism of soft tissue fluorosis is not yet clear (Hassan and Yousef, 2009). However, it can be suggested considering the effects of fluoride that it induces free radicals (Bagmut, Kolisnyk *et al.*, 2018), inhibits protein metabolism and the activity of some antioxidant enzymes (Rahmani and Rezaei, 2020) such as glucose-6-phosphate dehydrogenase and alkaline phosphatase. It has been observed that some enzymes such as choline esterase disrupt their activity and negatively affect carbohydrate and lipid metabolisms (Reddy, Sailaja *et al.*, 2009). Particularly, it is known that fluoride negatively affects liver functions and causes oxidative stress in the liver tissue (Saber, Mansour *et al.*, 2020) and causes tissue degeneration as shown by histopathological examination (Niu, He *et al.*, 2018).

As shown in the studies, rats exposed to fluoride had a significant decrease in body weight followed by a significant reduction in food and water consumption. Fluoride administration in rats may be associated with atrophic gastritis and decreased gastrointestinal absorption. Hence, a decrease in appetite and consequently weight loss may occur. This may be attributed to the disruption of the antioxidant system (Miltonprabu and Thangapandiyan, 2015). In our study, a decrease was found in the body weight of rats treated with fluoride. It is also expected that consumption of chitosan and its derivatives combined with food may cause a higher decrease than the other groups because of their fat binding properties (Atalay and Erge, 2018) (table 1).

Chitosan is a biopolymer produced by deacetylation of chitin that is an important component of the exoskeletons of crustaceans such as shrimp, lobster, crab and many other invertebrates. Chitosan has been investigated to clarify its antioxidant, antienflammatory and antimicrobial activities and drug delivery potential (Agnihotri, Mallikarjuna *et al.*, 2004) as well as its effect on immunity. The physicochemical properties of chitosan and its derivatives determine molecular weights and degree of deacetylation. Low molecular weight chitosan and its derivatives are more useful thanks to their solubility and low viscosity (Laokuldilok, Potivas *et al.*, 2017, Liu, Chen *et al.*, 2020). It has been reported in the studies that chitosan (Ramasamy, Subhapradha *et al.*, 2014) and COS (Yan, Wanshun *et al.*, 2006) have important antioxidant effects on liver toxicity induced with carbon tetra chloride (CCl_4).

Free radical formation and elimination occur in a dynamic equilibrium in the organism. If that balance is impaired in favor of free radicals, an oxidative stress state occurs (Al Syaad and Ibrahim, 2020, Koçak, Gökhan *et al.*, 2020). Oxidative stress may occur since fluoride disrupts the production of biomolecules that eliminate free radicals (GSH, GSH-Px, SOD and ascorbic) (Guo, Sun *et al.*, 2003, Meydan, Kizil *et al.*, 2020). Various results were obtained in different studies that evaluated oxidative stress in liver tissue after treatment with sodium floride. In a study, it was found that liver tissue MDA level did not change in rats treated with fluoride compared with the control group (Guo, Sun *et al.*, 2003). However, in contrast to that study, we have monitored in our study that

Table 1: Total body weight change and food efficiency ratio value over the 12-week trial period.

Group	Initial BW (g)	Final BW (g)	Total BW gain (g)	Food intake (g)	FER
Control	304.00±6.14[a]	376.86±5.11[a]	72.86±8.14[a]	20.18±0.48[d]	52.04±5.82[a]
NaF	304.29±4.29[a]	338.29±6.84[b]	34.00±4.68[b]	17.16±0.39[c]	28.34±3.90 [b,c]
NaF-CS	321.43±5.87[a]	344.29±9.23[b]	22.86±6.72b	22.20±0.49[b,c]	14.84±4.36[c,d]
NaF-COS	324.33±5.18[a]	339.67±5.05[b]	15.29±2.97[b]	27.12±0.72[a]	8.11±1.57[d]
CS	308.00±12.67[a]	375.29±5.57[a]	67.29±12.06[a]	20.92±0.36[c,d]	45.77±8.20[a]
COS	302.57±8.15[a]	370.86±5.45[a]	68.29±7.80[a]	23.10±0.33[b]	42.41±4.84[a,b]

Values are expressed as mean±SD (n=7). Means with different superscripts within a column indicate significant differences (p<0.05). FER, food efficiency ratio=(BW gainX100/food intake). Food intake: average feed consumed in each group (feed / number of rats consumed).

Table 2: The results regarding certain biochemical parameters related with use of chitosan and chitosan oligosaccharide in the liver tissue of rats intoxicated experimentally with fluoride

Group	Control	NaF	NaF-CS	NaF-COS	CS	COS
MDA µmol/g protein	302.75±15.35[c]	678.31±33.18[a]	602.06±48.54[a,b]	573.89±27.45[b]	339.10±21.43[c]	363.04±24.29[c]
GSH µmol /L	1.67±0.06[a]	1.33±0.12[c]	1.43±0.05[b,c]	1.46±0.02[b,c]	1.69±0.11[a]	1.55±0.16[a,b]
SOD U/mg	52.40±0.31[a]	47.60±1.85[b]	49.01±0.58[b]	47.75±1.57[b]	53.03±0.25[a]	52.13±0.23[a]
CAT UI/mg protein	15.71±0.51[a]	11.95±0.26b	10.85±0.46[b]	11.42±0.86[b]	15.54±0.75[a]	15.03±0.04[a]
GSH-Px ng/mg protein	79.14±3.27[a]	49.80±0.34[b]	52.47±7.86[b]	53.38±0.63[b]	81.24±1.01[a]	82.56±2.58[a]
AOBP mmolar	11.88±0.32	11.69±0.17	11.88±0.45	11.32±0.52	11.93±0.32	11.88±0.30
T-SH µmol/mg protein	9.26±0.01[a]	8.95±0.03[b]	8.92±0.01[b]	9.10±0.11[a,b]	9.26±0.09[a]	9.37±0.16[a]
NP-SH µmol/mg protein	1.14±0.07[a]	0.95±0.03[b]	0.93±0.03[b]	0.95±0.03[b]	1.10±0.02[a]	1.15±0.02[a]
P-SH µmol/mg protein	4.64±1.64	5.71±1.48	5.71±1.47	5.82±1.50	5.83±1.51	8.22±0.15
8-OhdG ng/mg protein	14.57±0.58	15.20±0.40	15.82±0.63	15.10±0.77	14.69±0.34	14.58±0.28

Table 3: Serum albumin, total protein and liver enzyme activity levels in rats treated with chitosan and chitosan oligosaccharide with experimental fluoride intoxication.

Group	ALT U/L	AST U/L	LDHU/L	ALB g/L	TP g/L
Control	34.00±1.85	131.57±2.98[b]	694.67±115.98[c]	31.33±0.64[a]	69.67±0.78[a]
NaF	39.00±4.76	160.00±8.01[a]	2220.66±146.07[a]	26.33±0.64[d]	57.00±0.55[c]
NaF-CS	34.67±1.39	117.68±3.78[b,c]	2150.67±239.77[a]	27.33±0.19[c,d]	58.00±0.01[c]
NaF-COS	36.43±0.72	111.33±3.97[c]	1560.65±112.43[b]	28.00±0.53[c]	63.35±0.94[b]
CS	36.33±1.76	83.67±1.46[d]	694.67±115.98[c]	29.65±0.36[b]	67.33±1.58[a]
COS	35.67±1.25	78.67±3.09[d]	687.33±85.25[c]	29.68±0.18[b]	67.67±0.18[a]

Different letters in the same column are statistically significant (P<0.05). ALT: alanine aminotransferase, AST: aspartate aminotransferase LDH: lactate dehydrogenase enzyme ALB: albumin, TP: total protein.

Table 4: Serum lipid profile in rats treated with chitosan and chitosan oligosaccharide with experimental fluoride intoxication.

Group	TC mg/dL	TG mg/dL	HDL mg/dL	LDL mg/dL	VLDL mg/dL
Control	64.67±1.71[b]	46.01±1.47	42.63±1.77	12.83±3.57[b,c]	9.20±0.29
NaF	73.33±0.94[a]	42.00±3.56	43.20±1.13	21.73±0.31[a]	8.40±0.71
NaF-CS	72.33±2.01[a]	38.67±1.55	45.00±0.78	19.60±1.84[a,b]	7.73±0.31
NaF-COS	64.00±1.31[b]	40.67±3.57	40.50±1.57	15.37±2.85[a,b,c]	8.13±0.71
CS	64.67±1.311[b]	45.00±2.55	41.83±4.43	13.83±1.05[a,b,c]	9.00±0.512
COS	61.33±3.79[b]	44.03±1.08	41.73±4.32	10.80±3.97[c]	8.80±0.22

Aynı kolonda farklı harf istatistiksel olarak anlamlıdır (P<0.05). TC: total kolesterol, TG: trigliserit, HDL: yuksek dansiteli lipoprotein kolesterol, LDL: dusuk dansiteli lipoprotein kolesterol, VLDL: cok dusuk dansiteli lipoprotein kolesterol

significant increases were found in the MDA levels of rat liver tissue treated with fluoride. This result is similar to the outcomes of many other studies (Hassan and Yousef, 2009, Nabavi, Nabavi *et al.*, 2013, Niu, He *et al.*, 2018, Rahmani and Rezaei, 2020, Zhang, Zhou *et al.*, 2014). It may be suggested that chitosan and its derivatives present their antioxidant properties by giving electrons to radicals depending on the presence of hydroxyl and amino groups in their atomic structure (Tomida, Fujii *et al.*, 2009). MDA is a product of the peroxidation of lipids resulting from the action of free radicals in the cell membrane (Hassan and Yousef, 2009, Yang, Chen *et al.*, 2020). Due to free-radical quenching effects of CS and COS (Laokuldilok, Potivas *et al.*, 2017), the decrease in MDA

level is a natural result due to reduction of free radicals resulting from fluoride treatment.

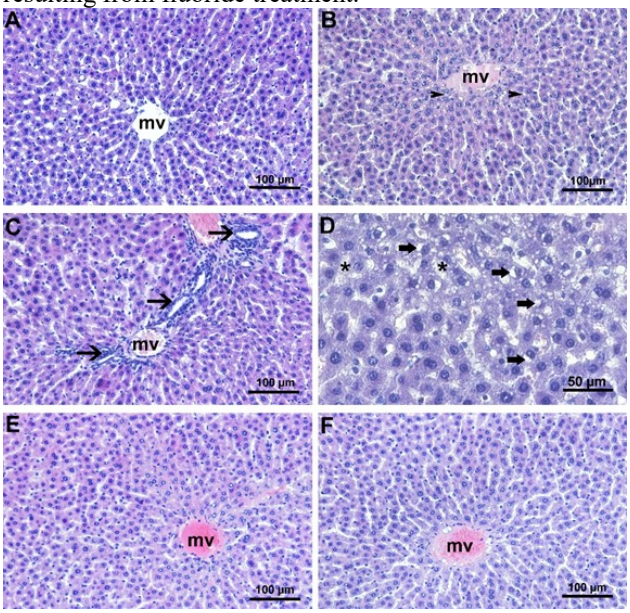

Fig. 2: Histological examination of the liver tissue of animals in the experimental groups (H&E). A) Control group mv; central vein, bar 100µm. B) Fluorosis group, arrowheads; hydrophilic degeneration in some liver cells. bar 100µm. C) Fluorosis-chitosan group, arrows; cell infiltration. bar 100µm. D) Fluorosis- chitosan oligosaccharide group, arrows; hydrophilic degeneration, *necrosis, bar 50µm. E) Chitosan (mv; central vein, bar 100µm.) And F) Chitosan oligosaccharide group (mv; central vein, bar 100µm).

The studies have reported that treatment with fluoride decreases the level of GSH and the activities of antioxidant enzymes such as SOD, CAT and GSH-Px (Hassan and Yousef, 2009, Nabavi, Nabavi *et al.*, 2013, Rahmani and Rezaei, 2020, Saber, Mansour *et al.*, 2020). We have obtained similar results in also our study. In another study, no significant change was determined in the level of MDA and enzymatic activity of CAT and SOD due to oxidative stress induced by hydrogen peroxide after treatment with COS (Lan, Chang *et al.*, 2020). There was no significant change in the level of GSH and enzymatic activity of SOD, CAT and GSH-Px in the group treated with chitosan and chitosan oligosaccharide combined with fluoride compared to the NaF group. It is known that the activity of antioxidant enzyme SOD is the form that requires copper, zinc and manganese while iron and selenium are minerals required for the activity of antioxidant enzymes CAT and GSH-Px, respectively (Karabulut and Gulay, 2016). Chitosan and its derivatives may be the factors needed in reducing the absorption of cofactors beside SOD, CAT and GSH-Px enzymes by binding metals in their structures thanks to the amino and hydroxyl groups found in their composition (Wang and Chen, 2014) and thereby decreasing the activity of these enzymes in the metabolism. Regarding the levels of thiol groups as the antioxidant markers (Sedlak and Lindsay, 1968) (table 4), protein thiol groups (P-SH) levels indicated approximity in all groups while total thiol groups (T-SH) and non-protein thiol groups (NP -SH) levels were detected to be low in all groups treated with fluoride. Therefore, it may be considered that treatment with CS and COS showed no positive effect on thiol groups in the fluoridated rats.

It has been reported that chitosan oligosaccharide decreases AST and ALT levels in the process of experimental liver damage induced by carbon tetrachloride and this may be related with the inhibition capability of COS on lipid peroxidation (Yan, Wanshun *et al.*, 2006). In another study, increases AST and decreased ALB and TP values were encountered while liver damage occurred in rats treated with carbon tetrachloride (Qureshi, Imran *et al.*, 2020). In the present study, significant increases were determined in the activity of AST and LDH enzymes as a consequence of fluoride-induced liver oxidative stress and a significant decrease was found in only AST levels in the group that received CS although significant decreases were encountered in those values after treatment of COS (table 3). That difference can be explained by the fact that COS inhibits cell lipid peroxidation more effectively than CS (Laokuldilok, Potivas *et al.*, 2017).

It has been stated that chitosan and chitosan oligosaccharide may have a regulatory effect on lipid profile. It has been found in a study which has evaluated the effects of CS and COS in the rats fed with high-fat diet that increased triglyceride, total cholesterol, HDL and LDL levels decreased significantly in these rats (Chiu, Feng *et al.*, 2017). In the present study, LDL cholesterol level that increased due to administration of fluoride decreased by treatment of CS and COS however this decrease approximated to control group and was not statistically significant (table 4).

CONCLUSION

In this study, the MDA content increased and the activity of antioxidant enzymes decreased in the liver of the rats exposed to fluoride. After occurrence of oxidative stress, degeneration was observed in the liver tissue. There was no significant improvement in the histopathology of the rat livers after treatment of CS and COS. However, it can be stated that these substances have positive effects in case of fluoride intoxicity considering reduction of the increased levels of MDA, decreased AST levels and also their positive impact on lipid profile.

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