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The Effect of Chitosan and Chitosan Oligosaccharide on Serum Mineral and Vitamin Levels in Rats with Experimental Fluorosis

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ABSTRACT Fluorine toxicity occurs in different parts of the world. It is caused by the increase in the fluorine level in the waters, especially due to the dissolution of volcanic rocks over time. Water with high fluorine content can be taken with plant and animal irrigations, and foods obtained from plants and animals, or it can be taken directly with drinking water. In this study, the effects of chitosan (CS) and chitosan oligosaccharide (COS) on some serum trace minerals and vitamin levels in experimental fluorosis rats were investigated. Six groups were formed as control, fluorosis, fluorosis CS and COS groups, healthy CS and COS groups. CS and COS were administered orally for 28 days at a dose of 250 mg/kg. At the end of the study, ketamine anesthesia was administered and the heart was directly cannulated. Trace minerals (Cu, Zn, Mn, Fe, Se) and vitamins (retinol, α-tocopherol, D_3) were analyzed. Cu, Zn, Se and Mn levels were found to be decreased in the F group compared to the control group. A significant increase was found in the Zn level in the F+CS group and in the Cu, Zn and Se levels in the F+CS group compared to the values in the F group. Serum retinol and α -tocopherol levels were decreased in the F, F+CS, F+COS and CS groups. In conclusion, our findings showed that there was a decrease in serum Zn, Cu, Se, retinol and α-tocopherol levels in rats with fluorosis, and COS was more effective than CS against the decrease in mineral and vitamin levels.

Keywords: *Fluorosis, Serum, Sodium fluoride, Trace elements, Vitamin.*

ÖZ

Deneysel Florozis Oluşturulan Ratlarda Serum Mineral ve Vitamin Seviyeleri Üzerine Kitosan ile Kitosan Oligosakkaritin Etkisi

Flor toksikasyonu dünyanın farklı yerlerinde meydana gelir. Özellikle volkanik kayaçların zamanla çözünmesi nedeniyle sulardaki flor seviyesinin artmasından kaynaklanır. Flor içeriği yüksek olan sular ile bitkilerin sulanması, hayvanların bu suları tüketmesi sonrası, bitki ve hayvanlardan elde edilen besinlerle veya doğrudan içme suyuyla alınabilir. Bu çalışmada, deneysel florozisli ratlarda kitosan (CS) ve kitosan oligosakkaritin (COS) bazı serum eser mineralleri ve vitamin düzeyleri üzerine etkileri araştırıldı. Kontrol, florozis, florozis CS ve COS grupları, sağlıklı CS ve COS grupları olmak üzere altı grup oluşturulmuştur. CS ve COS, 250 mg/kg'lık bir dozda, 28 gün boyunca oral yoldan uygulandı. Çalışmanın sonunda ketamin+ksilazin anestezisi uygulandı ve kalp direkt kanüle edildi. Serumda eser mineraller (Cu, Zn, Mn, Fe, Se) ve vitaminler (retinol, α-tokoferol, D_3) analiz edildi. Florozis grubunda (F), kontrol grubuna göre Cu, Zn, Se ve Mn seviyelerinde azalma olduğu görüldü. F+CS grubunda Zn seviyesinde ve F+CS grubunda Cu, Zn ve Se seviyelerinde F grubuna göre anlamlı bir artış tespit edildi. F, F+CS, F+COS ve CS gruplarında serum retinol ve α -tokoferol seviyeleri azaldı. Sonuç olarak, bulgularımız florozisli ratlarda serum Zn, Cu, Se, retinol ve α-tokoferol düzeylerinde azalma olduğunu, mineral ve vitamin düzeylerindeki azalmaya karşı COS'un CS'den daha etkili olduğunu gösterdi.

Anahtar Kelimeler: *Eser elementler, Florozis, Serum, Sodyum florür, Vitamin.*

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INTRODUCTION

Since fluorine has a high electronegative property, it is found in nature in the form of salt (fluoride) by combining with other elements. These salts are solids such as sodium fluoride (NaF) and calcium fluoride (CaF_2). Fluorides found in natural drinking water are the biggest source of fluorine taken into the body. The highest possible amount of fluoride in water is stated by the World Health Organization as 1.5 mg/L. Consumption of drinking water with fluorine above these values for a long time causes fluorine toxicity called fluorosis (Varol and Varol 2010; Cetin et al. 2020). In fluorosis, oxidative stress is induced by reactive oxygen species (ROS), which directly react with biomolecules, damaging lipids, proteins and DNA, causing cell and/or organ damage (Yur et al. 2013).

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and vitamins (vitamin C, vitamin E and β-carotene) have great importance in protection against this damage (Yur et al. 2013). The relationship between ROS formation, antioxidant defense systems and lipid peroxidation in fluorosis has been extensively investigated, but different results have been obtained (Reddy et al. 2003). In fluorosis, vitamins A, C and E help protect the organism from the harmful effects of oxidative radicals. At the same time, vitamin D plays an important role in improving fluoride-induced toxicity (Altug et al. 2013). Dietary antioxidants and consumption of antioxidant-rich foods have a beneficial effect on alleviating oxidative stress in fluorosis (Mesram et al. 2017; Bulduk et al. 2022). CS, which is used as a natural antioxidant, is obtained by deacetylating the biopolymer chitin. COS obtained by the reduction of CS or chitin have a strong antioxidant effect. In addition, COS and CS have biological activities such as antibacterial, antiinflammatory and immune-enhancing activities (Toz and Deger 2018). Antioxidant vitamins protect the oxidant-antioxidant balance by using ways such as clearing ROS, repairing tissue damage, and increasing antioxidant capacity. Trace elements take part in vital events such as serving as antioxidants in the body, being a cofactor of various enzymes, balancing membranes, and helping the functions of hormones (Cetin et al. 2020; Bulduk et al. 2022). In previous studies, changes in serum/tissue vitamin and mineral levels due to fluorosis were investigated (Bouaziz et al. 2007; Yasar and Yur 2008; Altug et al. 2013; Comba and Cinar 2016). However, no study was found on the effect of CS and COS on mineral and vitamin levels in fluorosis. Therefore, in this study, the effects of CS and COS on serum mineral (Cu, Zn, Mn, Fe, Se) and vitamin (retinol, α-tocopherol, vitamin D_3) levels in rats with experimental fluorosis were evaluated.

MATERIAL AND METHODS

Ethical approval was registered with the document dated 28.07.2020 and numbered 2020/07-03 of Van Yuzuncu Yil University Animal Experiments Local Ethics Committee.

Sodium fluoride (CAS Number: 7681-49-4), CS (CAS Number: 9012-76-4), COS (CAS Number: 148411-57-8) were obtained from Sigma-Aldrich. This study was carried out in Van Yuzuncu Yil University Experimental Animals Unit, using 42 male Wistar albino rats. During the experiment, rats were housed in rooms with 12 hours of darkness and 12 hours of light and a temperature of 22±2°C.

Experimental Procedure

The study was planned as 12 weeks. 42 male Wistar albino rats were divided into 6 groups, with 7 rats in each group. Wistar albino rats were eight to ten weeks old, weighing 150 to 200 g. The control group received normal drinking water. Drinking water of the fluorosis group (F) and fluorosis-induced experimental groups were given as 100 ppm sodium fluoride water (Zhang et al. 2014). Fluorosis and CS group (F-CS), CS group, fluorosis and COS group (F-COS) and COS groups were formed. CS (Pan et al. 2016) and COS (Zong et al. 2012) were administered orally at 250 mg/kg/day, the studies continued for an average of four weeks. At the end of the experiment, the rats were placed in the dorsoventral position after ketamine HCl (50 mg/kg) and xylazine (10 mg/kg) (i.p) administration. The hearts of the rats were directly cannulated and blood samples were taken into vacuum tubes. In tubes without anticoagulant, the blood was centrifuged at 3000 rpm for 5 minutes. After the serums were separated, they were kept at -18 °C temperature until analysis.

Biochemical Analysis

Fe and Mn analysis of the serum obtained from the blood in the study were measured using atomic absorption spectrometry (AAS, Thermo Scientific, IEC 3000 Series). Cu, Zn, Se analysis were performed using inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Scientific, ICAP3000 Series) and vitamin (retinol, α-tocopherol, D3) analyzes using high performance liquid chromatography (HPLC, Agilent 1100 Series) device.

Statistical Analysis

After the test for the data of the groups, it was seen that they had a normal distribution. One-way Analysis of Variance (ANOVA) was used to compare the data, and Duncan's test was used for multiple comparisons between groups. Data were presented as mean ± standard deviation (SD). A P value of 0.05 was accepted as the statistical significance level in the calculations. All analyzes were performed using the SPSS (23.0) package program.

RESULTS

Serum Cu, Zn, Mn and Se levels were decreased in the F group compared to the control group. Compared to the F group, there was a non- significant increase in Cu and Se levels in the F+CS group, but an important increase in the F+COS. The difference in Cu and Se levels was insignificant between the F+CS and F+COS groups. There was a significant increase in Cu level in the CS and COS groups compared to the control group. Serum Zn levels were found to be significantly decreased in Group F. When compared with the control and F groups, it was determined that the Zn level increased significantly in the CS, COS, F+CS and F+COS groups.

It was found that serum Mn levels were significantly decreased in the F and F+CS groups compared to the control group. It was determined that there was no difference in Mn levels between all CS and COS applied groups and F group. Retinol levels were found to be decreased in the F, CS, F+CS and F+COS groups compared to the control group. There was no significant difference between control and COS groups retinol levels. Compared to the control group, α-tocopherol levels were found to be significantly lower in all F- treated groups and in the CS group. There was an increase in α-tocopherol level in the F+COS group compared to the F group. It was determined that there was a similarity between the control group and the COS group in α-tocopherol levels. It was determined that there was no significant difference in serum Fe and vitamin D levels in all groups.

Table 1. Serum trace mineral and vitamin levels of control and experimental groups.

Parameter (ppm)	C (n=7)	F (n=7)	F+CS (n=7)	F+COS (n=7)	CS (n=7)	COS (n=7)
Cu	1.34±0.12^{b}	1.13±0.06^{d}	1.16±0.21^{c,d}	1.30±0.14^{b,c}	1.52±0.14^{a}	1.58±0.04^{a}
Zn	1.36±0.02^{b}	1.14±0.03^{c}	1.84±0.14^{a}	1.71±0.15^{a}	1,77±0.07^{a}	1.88±0.19 ^{a}
Mn	0.55±0.02 ^{a}	0.41±0.03^{b}	0.42±0.03 ^{b}	0.43±0.02^{a,b}	0.45±0.05^{a,b}	0.47±0.07^{a,b}
Fe	3.38±1.43	2.37±0.19	2.84±0.37	2.76±0.67	2.72±0.36	3.26±0.30
Se	1.11±0.07^{a}	0.91±0.02^{c}	0.99±0.03^{b,c}	1.05±0.04^{a,b}	1.07±0.11^{a}	1.12±0.03^{a}
Retinol	0.23±0.01^{a}	0.16±0.03^{b}	0.16±0.02^{b}	0.16±0.01b	0.16±0.02^{b}	0.20±0.03^{a}
α-Toc	2.20±0.08^{a}	0.24±0.08 ^{d}	0.78±0.56^{c,d}	1.00±0.34^{c}	1.20±0.30^{b,c}	1.72±0.63^{a,b}
Vit D	0.27±0.07	0.19±0.06	0.24±0.02	0.27±0.10	0.27±0.05	0.27±0.03

Values are expressed as Mean ± SD, different letters in the same line show statistical significance ($p<0.05$). C: control group, F: fluorosis group, F+CS: fluorosis and chitosan group, F+COS: fluorosis and chitosan oligosaccharide group, COS: chitosan oligosaccharide group, CS: chitosan group. α-Toc: α-tocopherol, Vit D: Vitamin D. One-Way Analysis of Variance (ANOVA) was used to compare the values of the groups, and Duncan's test was used for multiple comparisons between groups.

DISCUSSION AND CONCLUSION

Although fluorine is considered an important trace element considering its role in stabilizing teeth and bones, it is known that high fluoride intake (>1 ppm) causes toxic effects. Fluoride toxicity is known to affect teeth and bones. However, it has harmful effects on the liver and kidneys as well as other tissues and organs (Tkachenko et al. 2021; Çetin et al. 2019). Studies by many researchers have shown that oxygen radical formation and lipid peroxidation are among the harmful effects of chronic fluorosis (Chlubek and Poland 2003; Yur et al. 2013; Komuroglu et al. 2022). It has been reported that the use of antioxidants and antioxidant-rich foods can act as an antidote in the treatment of fluorosis in the prevention of oxidative stress-induced lipid peroxidation (Samal et al. 2016; Mesram et al. 2017). Chitin is a biopolymer found in the skeleton of insects such as crab shrimp and in the fungi. CS and COS, known as prebiotics, are obtained by enzymatic or chemical hydrolysis of chitin. CS and its derivatives have been the subject of research due to their abundance in nature, non-toxicity and antioxidant activities. It has been stated that CS and CS derivatives show antioxidant properties due to active hydroxyl and amine groups. It has been reported that CS and its derivatives, those with low molecular weight and the broken forms of intermolecular hydrogen bonds, will have more antioxidant properties (Xie et al. 2001). Trace minerals are elements that are required at the micro level as part of daily diets. Trace elements are essential for metabolic functions. Fe for CAT, Se for GSH-Px, Cu, Zn and Mn elements for SOD act as cofactors. In the case of a decrease in these elements, deterioration in the antioxidant mechanism is observed (Ersoy et al. 2011; Tkachenko et al. 2021). Trace element concentration in body fluids and tissues varies depending on disease state, gastrointestinal tract absorption, food intake, age and gender (Underwood 2012).

Vitamins are organic compounds that the body needs in trace amounts in order to maintain growth, development and health in living organisms. Vitamins show these effects, which are important for the organism, in the form of direct or indirect participation in many biochemical and physiological events in metabolism. In addition, it is reported that vitamins play a role in the execution of functions such as prophylaxis of diseases, treatment of acute diseases, protection of cancer and coronary diseases (Champe et al. 2005). There are some studies on vitamin levels and trace element in experimental fluorosis. In this study, the effects of CS and COS on serum trace mineral (Cu, Zn, Se, Mn, Fe) and vitamin (retinol, α-tocopherol, D_3) levels in experimentally fluorosis induced rats were investigated. It has been determined that there are changes in serum mineral levels in human (Tkachenko et al. 2021; Ersoy et al. 2011; Pei-zhong, Zhong-jie, and Tao 2002; Meral et al. 2004; Chen et al. 2002; Meral et al. 2004; Ersoy et al. 2011; Tkachenko et al. 2021) and animal (Kahl et al. 1973; Donald et al. 1979; Kessabi et al. 1983; Maraşlı et al. 1995; Singh and Swarup 1999; Tao et al. 2005; Tao et al. 2006; Kant et al. 2009; Altug et al. 2013) studies in fluorosis. Different results have been reported regarding mineral levels in natural or experimental fluorosis studies in animal species. In this study, decreased serum Cu levels in rats with experimental fluorosis were similar to those reported in sheep (Maraşlı et al. 1995), goats (Altug et al. 2013), cattle (Singh and Swarup 1999; Czerny et al. 2000; Samal et al. 2016), pigs (Tao et al. 2005) and humans (Chen et al. 2002; Meral et al. 2004; Ersoy et al. 2011; Tkachenko et al. 2021) with fluorosis. In our study, a significant decrease was found in the Zn level as well as in the Cu level. This result is inconsistent with some studies stating that the difference in Zn levels between the control and fluorosis groups were similar (Altug et al. 2013; Maraslı et al. 1995), but it is consistent with the results other than these studies. Cu plays a vital role in lipid metabolism in humans and animals (Samal et al. 2016). Plasma Cu and cholesterol concentrations are opposite to each other. In our previous research with the animals used in this study, we found that the serum cholesterol level increased significantly (Özdek et al. 2020). As Czerny et al. (2000) stated, this increase in cholesterol level may have caused a decrease in Cu level. The fact that the change in Fe level was not statistically significant in our study is consistent with the study result of Tkachenko et al. (2021). However, it does not match data from studies that found significant decreases (Altug et al. 2013; Samal et al. 2016) and rises (Chen et al. 2002) in Fe levels. Fluorine increases the absorption and utilization of Fe (Tao et al. 2005). A study of rats treated with fluoride showed a reduction in blood Fe with a concomitant increase in bone marrow and liver Fe uptake (Kahl et al. 1973). Also, Tao et al. (2005) found decreased Fe content in serum, spleen, liver, kidney and bone of rabbits treated with fluoride. It has been suggested that excessive fluoride

intake impairs Mn metabolism (Singh 1982). It has been shown that the amount of serum Mn is decreased in pigs (Tao et al. 2005), humans (Meral et al. 2004) and goats (Altug et al. 2013) and increase in cattle (Singh and Swarup 1999). Tkachenko et al. (2021) found that the decrease in Mn level in humans was not significant.

In this study, in agreement with the literature mentioned above, the amount of serum Mn was significantly reduced in rats with fluorosis (Altug et al. 2013; Tao et al. 2005; Meral et al. 2004). Decreased serum Mn level in this study, as suggested in the previous study (Li, Tan, and Zhang 1990), may be due to the formation of insoluble structures in the digestive system due to the decrease in absorption of Mn in the body. There is little literature on the effects of fluoride on serum Se levels. In our study, we found that serum Se amount were decreased in rats with fluorosis. Similarly, Tkachenko et al. (2021) found that there was a decrease in serum Se amounts at children with endemic fluorosis. Se is a necessary cofactor for the activity of several enzymes involved in the antioxidant system. Se is an essential component of GSH-Px. Se increases fluoride excretion in rats with fluorosis and is involved in the regulation and recovery of free radicals and lipid metabolism disorders. Se can help reduce high fluoride levels. Hence, it can protect the skeleton against floride toxicity. The curative effect of Se is related to its role in various physiological functions, in addition to its biologically active antioxidant role. Reddi et al. (2009) showed that Se can have a positive effect against fluoride exposure. Studies in children with chronic fluorosis have reported that decreases in blood GSH-Px activity are due to low levels of Se (Tkachenko et al. 2021; Tkachenko and Skaletska 2009). Fluorine, a highly electronegative halogen, has high affinity for the electropositive element. In our study, the reduction of fluorine by forming complexes with trace minerals such as Cu, Zn and Mn in the gastrointestinal tract or the protective roles of these minerals from oxidative stress by formation of free radicals may be due to the decrease in serum levels of these minerals. In our previous research, it was revealed that fluorosis caused deterioration in antioxidant defense by increasing oxidative stress in the liver of rats (Ozdek et al. 2021). The decrease in blood Cu, Zn and Mn levels may have been effective in the decrease in SOD, the decrease in Fe level CAT and the decrease in Se level may have been effective in the decrease in GSH-Px enzyme activity. Vitamins play an important role in maintaining the oxidant antioxidant balance. Studies on vitamin levels regarding fluoride exposure are both scarce and conflicting. Apart from its protective effect on lipid peroxidation, vitamin E also has regulatory effects on humoral and cell-mediated immunity (Altıner et al. 2017). High α-tocopherol levels in serum have been detected in guinea pigs (Vatassery, et al. 1980) and goats (Altug et al. 2013) in chronic fluorosis. In the study conducted at sheep with fluorosis, no significant change was found in vitamin E levels (Yasar and Yur 2008). In this study, a significant decrease was observed in serum α-tocopherol levels in rats with fluorosis, unlike the literature above.

Lipid peroxidation and oxidative stress are effective in the pathogenesis of fluorosis. This decrease in serum α-tocopherol level may be related to the increase in oxidative stress and lipid peroxidation. In this study, it was determined that the amount of retinol in the serum decreased significantly in rats with fluorosis. It has been stated that serum β-carotene and vitamin A levels are low in sheep with fluorosis, which is due to oxidative stress caused by inadequate food intake and high fluorine levels (Yasar and Yur 2008). In a study conducted in animals with fluorosis, it was stated that the level of vitamin A changed due to reasons such as feeding season, amount of feeding and food supplementation, while the level of retinol did not change. Vitamin D provides the mineral (Ca, P) balance in the body and is an important biomolecule for muscle, bone health and immune system. Studies conducted in goats with fluorosis (Altug et al. 2013) and pigs with experimental fluorosis (Andersen et al. 1986) have determined that serum vitamin D is at a level that facilitates the absorption of Ca absorption during digestion. As in the literature above, the differences in serum vitamin D levels in fluorosis were insignificant in this study. The physicochemical properties, molecular weights and deacetylation degrees of CS and its derivatives determine. CS and its derivatives with low viscosity and good solubility were preferred more in the studies. Their molecular weights are low (Laokuldilok et al. 2017). Derived from CS by chemical and enzymatic hydrolysis, the COS form is more soluble than the main component. COS reduces the level of cholesterol and the number of pathogenic bacteria in the intestinal tract. It strengthens the immune system and has anticarcinogenic, antioxidant and antidiabetic effects (Leblebicier and Aydogan 2018). Studies of liver toxicity with carbon tetra chloride (CCl_4) have shown that CS (Ramasamy et al. 2014) and COS (Yan et al. 2006) have significant antioxidant effects. In the study in which lead poisoning was created, it was determined that the amount of protein oxidation in the blood increased, the amount of reduced glutathione and total thiol decreased. However, with the application of CS and its derivatives, it was determined that lead was removed from the blood and there were different degrees of improvement in biochemical parameters. As a result, it has been stated that CS and its derivatives are beneficial for mitigating lead-induced oxidation damage in vivo (Wang et al. 2016). In a study on lead poisoning, it was stated that CS was effective in removing lead from the circulation and therefore strengthened the antioxidant defense system in erythrocytes and prevented oxidative damage (Toz and Deger 2018). In this study, it was shown that the amount of serum Cu and Zn increased in the groups as a result of the application of CS and COS, but the changes in Se, Fe and Mn levels were not statistically significant. Leblebicier and Aydogan (2018) support our result by detecting an increase in plasma Cu amounts and an insignificant increase in Zn levels in chicks treated with COS. This supports the hypothesis that prebiotics positively affect mineral absorption. Because indigestible carbohydrates facilitate the transfer of water to the large intestine, prebiotics can increase mineral absorption in the cecum, thus increasing the volume of fluid in which the minerals can dissolve. It also increases the absorption of ionized minerals by reducing cecum pH and fermentation (Roberfroid 2000). In our study, it was determined that the Zn levels in the F+CS group, and the Cu, Zn and Se levels in the F+COS group increased significantly compared to the F group, and there was no significant difference in the Fe and Mn levels. In a study, it was found that although chitosans with different molecular weights and degrees of deacetylation did not have a significant effect on Fe, Zn and Cu levels in the liver, spleen, heart and kidneys of mice, medium molecular weight CS significantly increased Fe, Zn and Cu levels in the liver (Zeng et al. 2008). In a study in which chitin and CS were applied, it was stated that both chitin and CS were increased to 10% and 20% levels in the diet, and Fe absorption was suppressed in animals consuming 10% CS. In addition, it was determined that there was a positive balance for P, Ca, Mg, Zn and Cu. It has been stated that the inability of rats to use Fe in the diet and the body's loss of Fe is due to the inflammatory state of the small intestine

caused by CS and, to a lesser extent, chitin (Gordon and Williford 1983). The significant and/or insignificant decreases in the levels of fat-soluble vitamins in the groups treated with CS and COS may be due to the inhibitory effects of these substances on lipid absorption in the intestines. It has been stated that COS has an undesirable effect by causing a significant decrease in plasma vitamin E level (Koide 1998). It has been reported that the long-term addition of CS to the diet in rats with polyhypovitaminosis may cause fat-soluble vitamin deficiencies (Vrzhesinskaia et al. 2011). In conclusion, our findings showed that there was a significant decrease in serum Cu, Zn, Se, retinol and α-tocopherol levels in rats with fluorosis, and COS was more effective than CS against the decrease in mineral and vitamin levels.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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Ethical approval was registered with the document dated 28.07.2020 and numbered 2020/07-03 of Van Yuzuncu Yil University Animal Experiments Local Ethics Committee.

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AUTHOR CONTRIBUTIONS

Idea / Concept: YD, UÖ
Supervision / Consultancy: YD
Data Collection and / or Processing: YD, UÖ
Analysis and / or Interpretation: İHY, UÖ
Writing the Article: YD, UÖ
Critical Review: YD, UÖ, İHY

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