

The Effects of Chitosan Oligosaccharide (COS) Treatment on Oxidative Stress and Its Relation with Intestinal Microflora in Rats Exposed To Cadmium

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ABSTRACT

The aim of the study was to investigate the effects of chitosan oligosaccharide (COS) treatment on oxidative stress and its relation with intestinal microflora in rats exposed to chronic cadmium toxicity. Animals were randomly divided into four groups as control (C; n=8), cadmium (Cd; n=8), chitosan oligosaccharide (COS; n=8), cadmium+chitosan oligosaccharide (Cd+COS; n = 8). After, cadmium chloride (CdCl₂) (2mg /kg/ day) was orally administered to Cd and Cd+COS groups three times a week for 4 weeks. Chitosan oligosaccharide (200 mg/kg/day) was also orally administered to COS and Cd+COS groups five times a week for 4 weeks. After completion of the experiment, serum TAS, TOS levels, plasma ALT, AST, GGT, T.pro, Alb, Bil, Creat and BUN values were measured. *Enterobacteriaceae*, *Lactococcus* spp. and *Lactobacillus* spp. counts were also detected. Serum TOS values were detected extremely higher in Cd group animals when compared COS group (p <0,05). In the small intestine of the Cd group animals, Cd administration caused a 0.66 log decrease in the *Lactococcus* spp. count. In conclusion, it was found that the antimicrobial effect of both compounds decreased as a result of COS-Cd chelating in Cd + COS group.

Keywords: Cadmium, chitosan oligosaccharides, microflora, oxidative stress, rat

Kitosan Oligosakkarit (COS) Tedavisinin Oksidatif Stres Üzerine Etkileri ve Kadmiyuma Maruz Kalan Sıçanlarda Bağırsak Mikroflorası ile İlişkisi

ÖZ

Bu çalışmanın amacı, kronik olarak Cd'ya maruz kalan ratlarda kitosan oligosakkarit'in (COS) oksidatif stres ve bağırsak mikroflorası üzerine etkilerinin araştırılmasıdır. Hayvanlar rastgele olacak şekilde; kontrol (C; n=8), kadmiyum (Cd; n=8), kitosan oligosakkarit (COS; n=8) ve kadmiyum+kitosan oligosakkarit (Cd+COS; n=8) gruplarına ayrıldı. Daha sonra, kadmiyum klorid (CdCl₂) (2mg/kg/day) Cd ve Cd+COS gruplarındaki hayvanlara haftada 3 kez 4 hafta boyunca oral yoldan verildi. Kitosan oligosakkarit de (200 mg/kg/day) COS ve Cd+COS grubundaki hayvanlara haftada 5 kez 4 hafta boyunca oral olarak uygulandı. Deneme sonunda, serum TAS, TOS seviyeleri, plasma ALT, AST, GGT, T.pro, Alb, Bil, Creat ve BUN değerleri ölçüldü. *Enterobacteriaceae*, *Lactococcus* spp. ve *Lactobacillus* spp. sayılarında belirlendi. Serum TOS seviyeleri Cd grubundaki hayvanlarda COS grubundakilere oranla önemli derecede yüksek bulundu (p <0,05). Cd grubundaki hayvanların ince bağırsaklarında, kronik Cd uygulaması *Lactococcus* spp. sayısında 0.66 log'lık bir düşüşe sebep oldu. Sonuç olarak, her iki bileşiğin antimikrobiyel etkinliği şelat oluşumuna bağlı olarak (COS-Cd) Cd+COS grubundaki hayvanlarda azalma gösterdi.

Anahtar Kelimeler: Kadmiyum, Kitosan oligosakkarit, mikroflora, oksidatif stres, rat

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INTRODUCTION

Cadmium (Cd), is a non-essential transition metal and considered to be an environmental pollutant, is naturally occurring element that has a high density and atomic weight when compared to water (Tchounwou et al. 2012, Gao et al. 2014). It is released into the environment by various human activities including mining, smelting, and manufacturing of batteries, pigments, stabilizers, and alloys (WHO 2010, Bernhoft 2013, WHO 2019). Cadmium is accumulating in catchments and soils under certain environmental conditions, thus increasing the risk of future exposure through food. The main routes of exposure to Cd are via ingestion of contaminated foods such as vegetables, potatoes, rice, wheat, green leafy grains and seeds, liver and kidney, and crustaceans and mollusks as well as contaminated water (IARC 1993, Paschal et al. 2000, Satarug et al. 2003, WHO 2007, ATSR 2008). It has been reported that acute or chronic exposed to Cd induces lipid peroxidation (LPO) (by stimulation of occurring superoxide anions) and oxidative stress (by increasing free radical production) in the cells (El-Demerdash et al. 2004, López et al. 2006). Moreover, it initiates various adverse effects in human and animals such as kidney dysfunction, liver injury and osteoporosis (Tchounwou et al. 2012, Satarug et al. 2011, Amamou et al. 2015). Cd accumulation is mainly occurred in the kidney and liver but also in brain, lung, bones, pancreas, placenta and testis in the body (Satarug et al. 2011, Amamou et al. 2015, Fowler 2009). In addition, Cd is a severe gastrointestinal irritant, which can leads to abdominal pain, burning sensation, nausea, vomiting, salivation when acute high dose ingested (Baselt and Cravey 1995, Hammett-Stabler 2000).

The gastrointestinal tract, is the interface between ingested nutrients and the body, plays an important role in maintaining of the health, food intake and regulating energy homeostasis (Zhang et al. 2014, Monteiro et al. 2017). In GIS, there are many bacterial populations whose have mutual relationship with intestinal epithelial cells that are known as symbiosis. Although *Enterobacteriaceae* are normal flora of the human intestinal system, they are common opportunistic pathogens can translocate across the mucosal barrier and lead to systemic infections if intestinal counts are extremely increased (Hsueh et al. 2010, Toh et al. 2012, Lai et al. 2016, Jean et al. 2016). On the other hand, lactic acid bacteria such as *Lactococcus* spp. and *Lactobacillus* spp also inhabit in the GIS that can produce lactic acid, acetic acid, formic acid and other acids to reduce intestinal pH. Besides, these microorganisms can secrete some antimicrobial molecules, such as ethanol, fatty acid, hydrogen peroxide and bacteriocins to defense against pathogenic bacteria in GIS (Ralitsa et al. 2015, Inglin et al. 2015). Although above mentioned bacteria

populations are mainly affected by the host's diet intake, the prevalence of bacteria in different parts of the GI tract appears to be depending on certain factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial cooperation, mucin secretion, nutrient availability and bacterial antagonism (Tannock 1983, Roberfroid et al. 2010, Amato et al. 2013). Imbalance among the intestinal epithelial cells, pathogen and/or commensal bacteria increases the rate of intestinal microbial disorders and sensitivity to external harmful compounds (Costello et al. 2012, Salim et al. 2014, Woodmansey 2007). Heavy metals also reach GI tract through ingestion of contaminated food and water. Although the toxicological effect of heavy metals on different body structures were detected, especially Cd, on GI microflora, is still remains unclear (Upreti et al. 2004, Inaba et al. 2005, Monachese et al. 2012).

Recently, it has been reported that harmful effects of Cd can be ameliorated by using some chelating agents, antioxidants, probiotics and vitamins (Pourmorad et al. 2006, Fang 2007, El-boshy et al. 2014, Djurasevic et al. 2017). One of them is chitosan oligosaccharide (COS) that is produced by chitosan/chitin via chemical hydrolysis or enzymatic degradation, known for its ability to bind to divalent cations such as Cd. As it known, it has an antioxidant, free radical consumer, antimicrobial, antifungal, anti-inflammatory, anti-diabetic and anti-obesity properties (Guan et al. 2016, Kim et al. 2016, Naveed et al. 2019). Therefore, our study has been designed to evaluate the influences of oral COS administration on oxidative stress, and its relation with intestinal microflora of the rats exposed to chronic Cd toxicity.

MATERIALS and METHODS

Animals, Study Design and Experimental Procedure

Male albino Wistar rats ($n=32$; body weight $\sim 200 \pm 30$ g) were housed in standard plastic rat cages at 23 ± 2 °C room temperature, $55 \pm 10\%$ relative humidity and 12 hours night/day light period during the experiment. The animals had free access to drinking water and standard rat feed. All experimental procedures were approved by the Ethical Committee on Animal Experimentation of the University of Balikesir (2019/4-6). Before the experiment, animals were randomly divided into four groups as control (C; $n=8$), cadmium (Cd; $n=8$), chitosan oligosaccharide (COS; $n=8$), cadmium+chitosan oligosaccharide (Cd+COS; $n=8$). Then, animals in C group received standard rat feed and fresh drinking water ad libitum. Cadmium chloride (CdCl_2) (2mg / day) were orally administered to Cd and Cd+COS groups three times a week for 4 weeks. On the other hand, chitosan oligosaccharide (200 mg/kg/day) was also orally administered to COS and Cd+COS groups five times a week for 4 weeks. After completion of the

experiment (4 weeks later), rats were anesthetized by intraperitoneal injection of ketamine/xylazine (0.1 ml/100gm/body weight) and killed by cervical dislocation technique. Blood samples were collected via cardiac puncture and transferred into tubes. Plasma and serum were obtained from the blood samples by using a centrifuge (3000 rpm, 25 min, Heichrich, Germany). Obtained samples were stored at minus 80 °C in a refrigerator until analysis time. Besides, intestinal fluid content were aseptically collected from the small and large intestines of the each rats.

Determination of total antioxidant and oxidants levels

Serum total antioxidant status (TAS) and oxidant status (TOS) values were defined by ELISA (Thermoscientific Elisa Reader, USA) using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey), according to Erel's method that is automated and colorimetric (Erel 2004, Erel 2005).

Determination of some plasma enzyme levels

Plasma alanine amino transferase (ALT), aspartate amino transferase (AST), gamma glutamyl transferase (GGT), total protein (T.pro), albumine (Alb), bilirubin (Bil), creatinine (Creat) and blood urea nitrogen (BUN) values were measured by using automatic biochemical analyser (Architect C-8000, Abbott, USA) with commercial kits according to manufacturer instructions.

Microbiological analysis

During the necropsy, 1 g intestinal fluid content were aseptically collected from the small and large intestines of the each rats (separately with 3 replicates). Then, they were homogenized in the stomacher for 2 minutes with sterile 9 ml Maximum Recovery Diluent (MRD), serial dilutions were prepared from 10^{-1} to 10^{-6} . For determine to the *Enterobacteriaceae* count, 1 ml of the dilution was taken and cultured in Violet Red Glucose Bile (VRGB, Oxoid CM1082) Agar according to the double-plate technique. The plates were evaluated as *Enterobacteriaceae* because of the observing purple-pink colonies after aerobic incubation at 37 °C for 24 h (ISO 21528-2: 2017). On the other hand, 0.1 ml of the dilution was taken and cultured in the M17 (Oxoid CM0785) agar according to spread plate technique for despite to *Lactococcus* spp count. Then, plates were evaluated as *Lactococcus* spp depends on occurring yellow-cream colonies after anaerobic incubation at 30 ° C for 24 h (Lee et al.2010). For detection of *Lactobacillus* spp count, 0.1 ml of dilution was cultured on MRS (CM0361) agar. Plates were also evaluated as *Lactobacillus* spp. due to occurring of yellow-cream colonies after anaerobic incubation at 37 °C for 72 h (Bauer et al. 2002).

Statistical Analysis

Obtained datas were analyzed with using SPSS for Windows version 25.0, and levels were presented as means \pm SE. Differences among the groups were performed by analysis of variance (one-way-ANOVA) that is followed by Duncan's test.

RESULTS and DISCUSSION

Serum TOS values were detected extremely higher in Cd group animals when compared COS group ($p < 0,05$). On the other hand, it was not found significant difference among C, Cd and Cd+COS groups according to TOS values, shown in Table 1. In addition, serum TAS values decreased due to Cd administration in Cd group animals compared to other groups ($p < 0,05$).

Plasma Bil and Creat levels were found the highest in Cd group compared to other groups ($p < 0,05$). Besides, COS administration did not lead to any changes in Cd+COS group according to Bil and Creat levels ($p > 0,05$). Conversely, plasma T.pro and Alb values were detected lower in Cd group compared to C, COS and Cd+COS ($p < 0,05$). In addition, plasma BUN levels were ameliorated due to COS administration in Cd+COS group ($p < 0,05$). Although plasma ALT, AST and GGT levels were detected higher in Cd group, the levels of the mentioned parameters decreased in COS group animals, statistically ($p < 0,05$), shown in Table 2.

The average *Enterobacteriaceae*, *Lactococcus* spp. and *Lactobacillus* spp. counts were detected as 4.34, 4.25 log cfu / g, 6.47; 7.09 log cfu / g; 8.37, 7.39 log cfu / g in both (small and large) intestines of the control group animals, respectively. On the other hand, *Enterobacteriaceae* counts were found similar in the control group with another experimental groups in both small and large intestines ($P > 0.05$). In the small intestine of the Cd group animals, Cd administration caused a 0.66 log decrease in the *Lactococcus* spp. count. In contrary, Cd+COS chelate lead to increase in the counts of *Lactococcus* spp. in small intestines of the rats ($p < 0.01$). There was a significant difference between the C group and the other experimental groups according to *Lactobacillus* spp. count in small intestines ($p < 0.01$). Besides, *Lactobacillus* spp. counts significantly decreased in Cd, COS and Cd+COS when compared to the C group. In terms of *Lactobacillus* spp, the highest decrease was observed in the small intestines of the COS group animals. In the large intestines of the rats, *Lactobacillus* spp. count significantly increased in COS and Cd+COS, however decreased in Cd group when compared to C ($p < 0.01$). The highest increase in the *Lactobacillus* spp. counts were observed as 0.54 log in the Cd+COS group, shown in Table 3.

Although Cd is a well-known environmental pollutant which induces severe organ and tissue damage in human and animals, effect of Cd on GI microflora and its relation with oxidative stress is still remains unclear (Satarug et al. 2011, Amamou et al. 2015, Fowler 2009).

In present study, exposed to chronic Cd toxicity increased (not statistically) the serum TOS levels, however significantly suppressed the serum TAS in Cd group animals. These results were consistent with previous studies (Karabulut-Bulan et al. 2008, Koçak and Akçil 2006, Kumaş et al. 2016). Either increased TOS nor decreased TAS levels were ameliorated with COS treatment in Cd+COS group when compared to Cd in our study. Similarly, the dose of chitosan more than 20 mg/kg/day was found effective on Cd-induced oxidative damage (SOD activity and MDA content) in the rat kidney by Zhou et al. (2013). Protective effects of COS and chitin on various metal and chemical compound induced oxidative stress were also determined by other researchers which was consistent with our results (Kim et al. 2005, Yan et al. 2006, Li et al. 2011, Toz and Değer 2018). It can be considered that the administration of COS reinforced the antioxidant defence system and also ameliorated the Cd induced oxidative stress in present study.

In our study, oral Cd treatment (low dose, 2mg/kg) led to increase of plasma ALT, AST, GGT enzyme levels (an important indicators of liver functions) in Cd group animals. Besides, an important markers of kidney functions are BUN and Creat levels also negative effected by Cd treatment in present study. These findings were corresponding with previous studies (Koçak and Akçil 2006, Lakshmi et al. 2012, Renugadevi and Milton 2010). Although plasma ALT, AST, GGT and BUN levels were improved by using COS in experimental groups, it couldn't affect to the plasma Creat levels in our study, interestingly. It was also reported that high dose chitosan diet ameliorated the Cd induced increased AST levels but did not lead to significance alterations in plasma ALT, BUN and Creat levels (Kim et al. 2016). In addition, T.pro and Alb values also negative effected by Cd toxicity in Cd group animals. It was consistent with Hussein et al. (2009) and Oyinloye et al. (2016). Increased liver and kidney enzyme levels, and reduction of T.pro and Alb values confirm the tissue damage due to chronic Cd toxicity in present study. There was limited information about the effects of COS on T.pro and Alb levels of Cd induced toxication in rats. Bil levels also increased in Cd group animals but did not effected from COS administration in present study. Hamden et al. (2009), Ibiem et al. (2013) and Markiewicz-Górka et al. (2011) also defined similar results in Cd treated rats according to Bil values. It may be explained that COS can be partially ameliorated the Cd induced tissue damages in the liver and kidney.

In the small intestinal microflora of the rats, neither Cd nor COS didn't cause any significant changes in the counts of *Enterobacteriaceae* in present study. Conversely, *Escherichia coli* and *Klebsiella* spp., which are the members of *Enterobacteriaceae* group, counts decreased due to Cd (high doses) treatments in the small intestine of the mouse in a previous study (Fazeli et al. 2011). As it known, COS has positive effects on host gut health and intestinal microbial community (Zhang et al. 2014), however Cd+COS treatment not affected the *Enterobacteriaceae* count in our study. It can be explained by either antimicrobial effect of both compounds decreased as a result of COS+Cd chelating, both compounds were rapidly absorbed without showing their antimicrobial effects or the doses were insufficient to demonstrate known effects. In addition, Cd treatment did not lead to changes in *Enterobacteriaceae* count in large intestine in Cd group compared to C group. It has been suggested that *E. coli* and *Klebsiella* spp. counts reduced in the large intestine of the mice due to Cd in a previous study which was not corresponding with present study (Fazeli et al. 2011). Although it has been enounced that COS influences GI flora, and thus improving intestinal health, it was not found a significant change in *Enterobacteriaceae* counts in the large intestine of the Cd+COS group animals. It can be occurred due to different dose, time of exposure to Cd and/or animal species.

A significant decrease was found in the count of *Lactococcus* spp. in small intestine of Cd group animals in present study. It was also suggested that gram-positive basilcus and enterococcus microorganisms were more sensitive to Cd toxicity than gram-negative *E. coli* and *Klebsiella* spp. (Fazeli et al. 2011). On the other hand, Cd+COS treatment increased the *Lactococcus* spp count in large intestine of the rats. These results can be explained by the fact that total bacterial rates of microflora varied with decreased count of *Enterobacteriaceae* due to Cd+Mel administration or the high pH in the small intestine.

Lactobacillus spp. count was found lower in Cd group than C, Mel and Cd+Mel groups in small intestine microflora of the rats in our study. It has been reported by Fazeli et al. (Fazeli et al. 2011). that *Lactobacillus* spp. count decreased due to different high doses of Cd treatment in small intestines of the mice which was corresponding with our results. Although decreased *Lactobacillus* spp. counts were detected by Fazeli et al. (2011) in large intestines depend on the different doses of Cd, it increased due to Mel and Cd+Mel treatments in large intestine of the rats except C group in our study. It was also reported that Mel treatment increased the *Lactobacillus* spp. counts in large intestines of colitic mice which was consistent with present study (Wang et al. 2019). It can be also expressed that Cd+Mel treatment may be reduced *Enterobacteriaceae* count and lead to

increase *Lactobacillus* spp./*Lactococcus* spp. rates, and/or activated the antioxidant system that can be

confirmed by an increase in serum TAS levels in our study.

Table 1. Serum TAS and TOS levels in different experimental groups.

Parameters	Groups			
	C (n=8)	COS (n=8)	Cd (n=8)	Cd+COS (n=8)
TOS	26,50±5,94 ^a	2,97±1,08 ^b	69,65±29,95 ^a	32,45±11,54 ^a
TAS	1,73±0,03 ^a	1,81±0,00 ^a	0,73±0,01 ^b	1,73±0,02 ^a

a,b,c; The differences between average values indicated by different letters in the same row of the same parameters are important ($p < 0.05$).

Table 2. The average biochemical parameters in different experimental groups ($X \pm SEM$).

Parameters	Groups			
	C (n=8)	COS (n=8)	Cd (n=8)	Cd+COS (n=8)
Bil (g/L)	1.02±0.00 ^b	1.03±0.02 ^b	1.35± 0.07 ^a	1.08±0.01 ^b
ALT (U/L)	37.82±1,94 ^c	37.65±1.19 ^c	81.01±5.18 ^a	72.80±4.72 ^b
AST (U/L)	60.16±4,03 ^c	62.16±5.17 ^c	147.0±16.82 ^a	108.16±9.88 ^b
GGT (U/L)	1.16±0,11 ^c	1.15±0.12 ^c	2.50±0.23 ^a	1.40±0.24 ^b
Alb (g/dL)	3.51±0,11 ^a	3.51±0.14 ^a	2.84±0.21 ^b	3.53±0.90 ^a
BUN (mg/dl)	18.70±1.21 ^c	16.11±1.35 ^d	40.71±2.25 ^a	30.26±2.24 ^b
Creat (mg/L)	5.04 ± 1.02 ^b	5.06 ± 0.99 ^b	7.41 ± 0.88 ^a	4.99 ± 1.02 ^b
T.Pro (g/dL)	5.57±0,13 ^a	5.20±0.13 ^a	4.56±0.15 ^b	5.12±0.19 ^a

a,b,c,d; The differences between average values indicated by different letters in the same row of the same parameters are important ($p < 0.05$).

Table 3. *Enterobacteriaceae*, *Lactococcus* spp. and *Lactobacillus* spp. counts in different experimental groups.

Groups		<i>Enterobacteriaceae</i>	<i>Lactococcus</i> spp	<i>Lactobacillus</i> spp.
Control	small	4,3438±0,15 ^a	6,4771±0.00 ^{ab}	8,3764±0.80 ^{abc}
	large	4,2580±0.00 ^a	7,0922±0.58 ^{ab}	7,6931±0.29 ^{bcd}
COS	small	4,1664±0.23 ^a	6,8368±0.54 ^{ab}	7,1238±0.75 ^d
	large	4,1838±0.28 ^a	7,5131±0.24 ^{ab}	8,6993±0.97 ^{ab}
Cd	small	4,6819±0.24 ^a	5,8458±0.29 ^b	8,1228±0.33 ^{abcd}
	large	5,3764±0.2 ^a	6,3280±0.67 ^{ab}	7,3920±0.38 ^{cd}
Cd+COS	small	4,1230±0.9 ^a	7,6723±0.99 ^{ab}	7,2177±0.76 ^{cd}
	large	4,4269±0.01 ^a	8,1476±0.60 ^a	8,9155±0.29 ^a

a,b,c,d; The differences between average values indicated by different letters in the same line of the same parameters are important ($p < 0.05$).

CONCLUSION

It is known that lactic acid bacteria and probiotics should be taken with food at a level of at least 7 log / gr in order to have beneficial effects in humans. Taking this information into account, both *Lactococcus* spp. and *Lactobacillus* spp. counts decreased as 0.63

and 0.77 log in the Cd group compared to the control group, respectively. This decrease in lactic acid bacteria may also be related with serum TAS and TOS values in Cd group animals. In addition, it was found that the antimicrobial effect of both compounds decreased as a result of COS-Cd chelating in Cd + COS group. This situation can be

observed in terms of serum TAS and TOS levels in our study.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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