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The influence of chitosan oligosaccharide on some hematological parameters in rats exposed to cadmium

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ABSTRACT

The aim of the study was to investigate the effects of chitosan oligosaccharide (COS) on some hematological parameters, and define the percentage of alpha-naphthyl acetate esterase (ANAE) positive lymphocytes in rats that had been exposed to cadmium (Cd). Thirty-two (n = 32) male Wistar albino rats were randomly divided into four groups as the control (C), chitosan oligosaccharide (COS), cadmium (Cd), and Cd + COS (CdCOS) groups. Blood samples were collected to assess erythrocytes (RBC), leukocytes (WBC), hemoglobin levels (HGB), hematocrit values (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelets (PLT), and alpha-naphthyl acetate esterase (ANAE) positive lymphocytes. The number of WBCs significantly increased in the Cd group compared to the C, CdCOS, and COS groups ($P<0.05$). Although the lymphocyte count decreased significantly in the Cd group ($P<0.05$), it was ameliorated by COS treatment in the CdCOS group compared to the Cd group ($P<0.05$). Also, the percentage of peripheral blood ANAE positive lymphocytes decreased significantly in the CdCOS and Cd groups ($P<0.05$). COS exhibits a partially protective effect on some hematological characteristics, apart from the percentage of ANAE positive lymphocyte in cases of chronic Cd exposure.

Key words: alpha-naphthyl acetate esterase; cadmium; chitosan oligosaccharides; hematology; rats; toxicity

Introduction

Heavy metals, such as cadmium (Cd), arsenic (As), chromium (Cr), lead (Pb), and mercury (Hg), are naturally occurring elements that have a high density and atomic weight when compared to water (TCHOUNWOU et al., 2012**)**. Use of these metals in industrial, agricultural, medical, and technological areas leads to their wide distribution in the environment (WHO, 2010). One of the common environmental pollutants is Cd which also occurs due to industrial activities including mining, smelting, and manufacturing of batteries, pigments, stabilizers, and alloys (BERNHOFT, 2013). Although the main routes of exposure to Cd are via ingestion of contaminated food (vegetables, potatoes, grains and seeds, liver and kidney, and crustaceans and mollusks), water or inhalation (air pollution or smoke), skin absorption is rare (SATARUG et al., 2003; ATSDR, 2008). As is well-known, acute or chronic exposure to Cd induces lipid peroxidation (LPO) by stimulation of the occurring superoxide anions, and oxidative stress by increasing free radical production in cells (ANDJELKOVIC et al.,

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2019). Furthermore, it initiates various pathological conditions in human and animal tissues, depending on certain factors including the dose, route of exposure, age, gender, genetics, and nutritional status (TCHOUNWOU et al., 2012; AMAMOUA et al., 2015). Cd accumulation mainly occurs in the kidneys and liver, but also in other important organs and systems (cardiovascular system, brain, lung, bones, immune/hemopoietic system, endocrine, and reproductive system) in the body (SATARUG et al., 2003).

In recent years, it has been suggested that the deleterious effects of Cd can be lessened by using certain substances such as chelating agents, antioxidants, probiotics, and vitamins (EL-BOSHY et al., 2015; DJURASEVIC et al., 2017**)**. Chitin (β-1,4-glucosidic linkage of 2-acetylamino-D-glucose) is one of the most abundant biopolymers found mainly in all crustaceans and insect shells, as well as in certain fungi, algae, squid, oysters, krill, clams, shellfish, and yeast throughout the world (PHILLIPS, 2017). Chitosan oligosaccharide (COS) is a natural N-deacetylated derivative of chitin (KEAN and THANOU, 2010), prepared from chitosan (THADATHIL and VELAPPAN, 2014). Low molecular weight, the higher degree of deacetylation and polymerization, its lower viscosity, and the complete water solubility of COS assists its various biological activities (NAVEED et al., 2019). COS exhibits anti-microbial, anti-inflammatory, anti-diabetic, antioxidant, antihypertensive, hypocholesterolemic, calcium absorption, and hemostatic effects (YOUSEF et al., 2012; NAVEED et al., 2019). Moreover, COS is a potent oxygen-free radical scavenger and has the ability to bind to divalent cations, including Cd (KIM et al., 2016; NAVEED et al., 2019). WARDANI and SUDJARWO (2018) reported that chitosan nanoparticles show immunostimulatory activity and have therapeutic potential for some immune depressed conditions. In addition, GUO et al. (2018) also showed the effect of COS on some hematological parameters in humans.

The hematopoietic system is very sensitive to many external compounds, such as drugs, toxins, and heavy metals. It has been found that Cd disrupts hematopoietic system functions (EL-BOSHY et al., 2015). The main influence of Cd on hematopoiesis was found as microcytic anemia in rats (KOCAK, 2004). After absorption into the body, Cd passes into the blood circulation, and binds with red blood cell (RBC) membranes and albumin. In the circulation, Cd increases the formation of metallothioneins and reactive oxygen species (ROS), that is, it leads to oxidative stress in RBC and T or B lymphocytes (KOLANJIAPPANA et al., 2002; MINETTI et al., 2008). Furthermore, chronic Cd toxication leads to neoplastic-like alterations in the morphology of lymphocytes and monocytes, as well as hemolysis, vacuolization, denuclearization, and dislocation of the nucleus in erythrocytes (SULJEVIC et al., 2019). It was also suggested that chronic exposure to Cd showed cytotoxic and genotoxic effects on peripheral blood and bone marrow cells (POPOVIC-BUBUJUK et al., 2013).

Alpha-naphthyl acetate esterase (ANAE) is a lysosomal enzyme of mononuclear leukocytes that is mainly used to differentiate T lymphocytes, B lymphocytes, and monocytes in humans, and also different animal species (MUELLER et al., 1975; DONMEZ et al., 2019). ANAE is reported to be acquired during the later stages of T lymphocyte maturation in the thymus. Similar to the other esterases, it has been reported that ANAE participates in the cytotoxic functions of active T lymphocytes, endocytosis, and degradation of the antigen within macrophages (MUELLER et al., 1975; SUR et al., 2005).

In the present study, we aimed to investigate the possible metal binding role of COS and its influence on some hematological characteristics, and ANAE positive lymphocytes that provide important information concerning the general condition of the hematopoietic system in chronic Cd intoxicated rats.

Materials and methods

Animals. In our study, we used male albino Wistar rats (n = 32; 3 weeks old; body weight ~ 200 ± 30 g) obtained from Balıkesir University Experimental Medicine Research and Application Center (BUEMRAC). We established four experimental groups as the untreated control (C; n = 8), Cd (n = 8), COS (n = 8) and Cd + COS

(CdCOS; n = 8) groups. The rats were housed in standard plastic rat cages at BUEMRAC at 23 ± 2 C^0 room temperature, 55 ± 10% relative humidity, and with 12 hours night /daylight periods during the experiment. The animals were provided access to standard rat feed and ~ 50 mL/day/rat fresh water ad libitum. All animal handling and procedures were approved by the Experimental Medicine Research and Application Center of Balıkesir University Experimental Animal Ethics Committee (2018/2-2). Cadmium chloride ($CdCl_2$) (2mg/kg/day) was administered orally by gavage to Cd and Cd+COS group animals, three times a week for 4 weeks (ALMENARA et al., 2013). On the other hand, COS (200 mg/kg/day) was also administered orally (by gavage) to the COS and Cd+COS groups five times a week for 4 weeks (KIM et al., 2016). After completion of the experiment (4 weeks later), blood samples were obtained by cardiac puncture under ketamine/xylazine (0.1 mL/100mg/body weight) anesthesia, and collected in heparinized sterile tubes. Then, the collected blood samples were transferred immediately to the lab under a cold chain.

Hematology. Leukocytes (WBC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), erythrocytes (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelets (PLT) and differential WBC counts were assessed in blood samples using a cell counter (Abacus Junior Vet -5, Vienna, Austria).

*Determination of the percentage of ANAE positive lymphocytes***.** From each heparinized blood sample, two blood smears were prepared and air-dried at room temperature (20 °C). To define ANAE activity, they were fixed in 50% glutaraldehyde: 50% acetone, pH 4.8, at −10 °C for 3 min. After fixation, they were air-dried at 20 °C and added to an incubation solution prepared by adding 80 mL buffered phosphate solution, pH 5.0, and 20 mg substrate (ANAE, N-8505; Sigma, Steinheim, Germany) dissolved in 0.8 mL acetone (Merck, Darmstadt, Germany) drop by drop to prevent crystallization of the substrate. Then, 4.8 mL hexazotized pararosanilin mixture, obtained by incubating 2.4 mL 4% sodium nitrite (S-3421; Merck) solution with 2.4 mL pararosanilin (P-3750; Merck) (1 g pararosanilin, 20 mL distilled water, 5 mL HCl concentrate) for 2 min, was added to the buffered phosphate solution containing α-naphthyl acetate. The final pH of the mixture was set to pH 5.8 with 1 N NaOH and the solution was filtered. After incubation for 2 h at 37 °C, the smears were rinsed three times in distilled water, and the nuclei were stained with 1% methyl green (Merck) in 0.1 M acetate buffer, pH 4.2, for 10 min (MAITI et al. 1990). In these smears, the cells with lymphocyte morphology and having 1 to 4 reddish-brown cytoplasmic staining as dots were scored as ANAE-positive lymphocyte (Fig. 1, arrow) (MUELLER et al., 1975; AYDIN et al., 2012), while other lymphocytes were considered negative. All specimens were examined under a light microscope (Leica DM 2500, Leica Microsystems GmbH, Wetzlar, Germany). In each ANAE stained specimen, 200 lymphocytes were counted and the percentage of ANAE positive lymphocytes was determined.

Statistical analysis. The statistical analysis was carried out using by analysis of variance (ANOVA) followed by Duncan's test using the SPSS 25.0 program (SPSS, Inc., Chicago, IL). Values for $P \leq 0.05$ were considered significant.

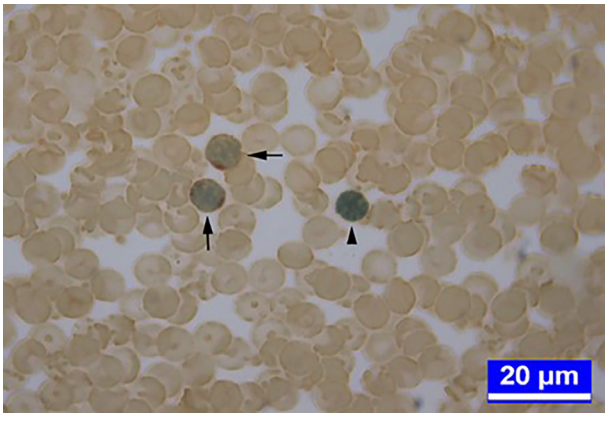

Fig. 1. ANAE demonstration of rat peripheral blood in the control group. Arrows: ANAE positive lymphocytes. Arrowhead: ANAE negative lymphocyte.

Results

Hematology. The results obtained from this study are presented in Tables 1 and 2. The number of WBC significantly increased in the Cd group compared to the C, CdCOS, and COS groups (P<0.05). RBC, HGB, HCT, MCH, and MCHC values did not change significantly in either the Cd or COS group, compared to the C group (P>0.05). On the other hand, significantly lower MCV values were detected in the Cd group than in the other three groups (P<0.05). These reductions was ameliorated by COS in the CdCOS group (P>0.05). In addition, COS treatment led to a significant increase in PLT values in the COS group compared to the C group (P<0.05). We found a significant decrease in the count (#) of lymphocytes in the Cd group (P<0.05). Although the count (#) of lymphocytes decreased significantly in the Cd group (P<0.05), it was ameliorated by COS treatment in the CdCOS group compared to the Cd group (P<0.05), as shown in Table 2. In addition, no significant change was detected in the groups in relation to the percentages of lymphocytes, monocytes, and neutrophils (P>0.05).

Percentage of ANAE positive lymphocytes. Although we found no significant difference between the COS and C groups, we detected a decrease in the percentage of ANAE positive lymphocytes in the Cd and CdCOS groups (P<0.05), as shown in Table 2.

Table 1. Effects of Cd and COS on some hematological characteristics

Characteristics	Cd (n=8)	COS (n=8)	CdCOS (n=8)	C (n=8)
WBC (10^9/L)	7.42±0.52^{a}	5.46±0.66^{ab}	5.47±0.81^{ab}	3.41±0.18^{b}
RBC (10^{12}/L)	8.82±0.37	7.82±0.88	8.61±0.74	7.24±0.27
HGB (g/dl)	15.03±0.75	13.53±0.70	14.60±1.25	12.60±0.27
MCH (pg)	16.70±0.50	17.32±0.52	17.03±0.53	17.47±0.41
MCV (fL)	44,38±0,51^{b}	47.18±0.28^{a}	45.78±0.81^{ab}	45.75±0.44^{ab}
MCHC (g/dl)	38.37±0.97	37.10±1.21	37.23±1.06	38.12±0.29
PLT (10^9/L)	726.75±27.92^{ab}	755.75±56.72^{a}	599.37±75.80^{b}	688.87±2.30^{ab}
HCT (%)	39.22±1.91	36.41±1.71	39.47±3.59	33.68±0.34

Groups: C, control; COS, chitosan oligosaccharide; Cd, cadmium; CdCOS, COS + Cd. a−b Means in the same row with different superscripts differ significantly (p < 0.05).

Table 2. Effects of Cd and COS on differential WBC and ANAE positive lymphocyte percentages

Percentage/count	Cd (n=8)	COS (n=8)	CdCOS (n=8)	C (n=8)
Lymphocyte (10^9/L)	2.42±0.20^{c}	4.72±0.56^{a}	3.97±0.55^{b}	6.00±0.47^{a}
Monocyte (10^9/L)	0.82±0.13	1.01±0.08	1.00±0.18	0.70±0.00
Neutrophil (10^9/L)	0.36±0.08	0.41±0.07	0.49±0.11	0.25±0.01
Lymphocyte %	73.10±6.41	78.23±2.11	72.67±3.02	69.57±2.52
Monocyte %	16.52±2.59	13.92±1.18	17.62±1.88	20.67±1.50
Neutrophil %	10.37±3.94	5.91±0.90	9.70±1.51	8.51±0.90
ANAE positive lymphocyte %	37.11±2.15^{b}	52.77±1.76^{a}	43.39±2.59^{b}	48.90±2.90^{a}

Groups: C, control; COS, chitosan oligosaccharide; Cd, cadmium; CdCOS, COS + Cd. a−cMeans in the same row with different superscripts differ significantly (p < 0.05).

Discussion

It was reported that chronic Cd accumulation induces anemia through three mechanisms, as follows: accumulation of Cd in the kidney, liver and spleen, which leads to hemolysis due to deformity of the peripheral red blood cells (RBCs), iron deficiency through competing with duodenal iron absorption, and renal anemia derived from hypoproduction of erythropoietin (EPO) in humans and animals (HORIGUCHI et al., 2010; EL-BOSHY et al., 2015).

In the present study, exposure to oral Cd treatment for 4 weeks did not cause any significant changes in RBC, HGB, MCH, and MCHC values in the Cd group compared to the C group. These results were inconsistent with previous studies (OGNJANOVIĆ et al., 2003; NAZIMA et al., 2016). GLUHCHEVA et al. (2011) reported that chronic Cd accumulation led to a decrease in HGB values in mice. In addition, exposure to different doses of Cd for different durations has been reported to produce deleterious effects in rats (KARMAKAR et al., 2000; OGNJANOVIĆ et al., 2003). Although it is known that exposure to Cd causes microcytic hypochromic anemia, and contributes to a decreased HCT concentration (DEMIR et al., 2006; EL-BOSHY et al., 2015), these values were not affected by Cd treatment in the Cd group compared to other groups in our study. MCV values were defined as lower in Cd group than in the C, COS and CdCOS groups in the present study. HORIGUCHI et al. (2011) reported that injections of Cd for one month induced normochromic and normocytic anemia, which had been indicated by the detection of decreased RBC count, HGB, and HCT, with almost no changes in MCV and MCH values in the hemogram. Different results may have been obtained due to different dose or exposure time to Cd as well as animal species. RBC, HGB, HCT, MCH and MCHC values were similar in the C and COS groups, and these characteristics were not significantly changed in the CdCOS group compared to the Cd group (Table 1). In addition, the decreased MCV values of the experimental rats due to Cd treatment were ameliorated by COS administration in the CdCOS group animals. In our study, COS treatment led to an increase in PLT values in the COS group compared to the C group. Besides, no significant change was detected between the Cd and C groups in terms of PLT values. In a previous study, KIM et al. (2001) reported that administration of 500, 1000, and 2000 (mg/kg/day) doses of COS did not affect the hematological parameters (WBC, RBC, HGB, HCT, MCV, PLT, MPV, Lym, Mid and Gran) in rats. Similarly, COS (50 mL/day, during 5 days) treatment did not lead to significant differences in the values of PLT, MCV, MCH and MCHC in Hanwoo calves (ALAM et al., 2012). These different results may have been obtained due to the different doses or animal species. It may be suggested that COS has a partially protective effect against Cd intoxication.

The number of WBC significantly increased in the Cd group compared to the C, COS and CdCOS groups in our study. Although we found no significant change in the percentage of lymphocytes, monocytes and neutrophils, we observed a significant decrease of lymphocyte count in the Cd group compared to the C, COS and Cd + COS groups. Although the number of WBC increased while the count of lymphocytes decreased in the Cd group, these values were slightly ameliorated by oral COS administration in CdCOS group animals in our study. WARDANI and SUDJARWO (2018) reported that administration of chitosan nanoparticles was found to increase the total WBC count at doses of 300 and 600 mg/kg in rats. The difference in WBCs and lymphocyte (#) may be due to increased free radicals, decreased antioxidant activity, or suppression of nonspecific and specific immune responses (FAHIM et al., 2012). EL-BOSHY et al. (2015) also reported that interleukin-1 beta (IL-1β), TNF-α, IL-6, IL-10, and the number of peripheral neutrophils increased, while interferon-gamma and the number of lymphocytes decreased due to Cd treatment. Cd toxicity also suppresses lymphocyte proliferation and natural killer cell activity (CIFONE et al., 1990). In addition, Cd toxicity during the prenatal period causes abnormal thymocyte development by disturbing T lymphocyte production during the postnatal period (HOLÁSKOVÁ et al., 2012). The anti-inflammatory, anti-oxidant, and radical

scavenging effects of COS lead to the triggering of immune responses. In addition, the direct in vivo and in vitro immuno-stimulating activities of COS have also been described in previous studies (ZHANG et al., 2014; WARDANI and SUDJARWO, 2018). Different types of lymphocytes have different roles in inflammation and tumor suppression. COS enhances the proliferation and phagocytosis of neutral red phagocytosis by RAW264.7 macrophages. Moreover, it produces an anti-inflammatory response by stimulating the release of NO and TNF-α by macrophages (ZHANG et al., 2014). JIANG et al. (2019) suggested that treatment of mice with COS (200 mg/kg) promoted TNF-α, IL-2, Fas, and Fas-L production in serum.

ANAE, a lymphocyte lysosomal enzyme acquired from medullary thymocytes in the thymus during the embryonic stage, has been demonstrated in mature and immunocompetent T lymphocyte activity in peripheral blood. ANAE is specific for mature T lymphocytes (Fig. 1) (Cell-mediated immunity), but not B lymphocytes, which function in the humoral immunity component of the adaptive immune system by secreting antibodies (DONMEZ et al., 2007). We found that the percentage of ANAE positive lymphocytes in peripheral blood changed significantly in the Cd group compared to the C group. Similarly, DONMEZ et al. (2019) reported that acute Cd treatment led to a decrease in ANAE positive lymphocytes in peripheral blood in rats. SIMSEK et al. (2009) also reported that Cd and lead administration reduced the percentage of peripheral blood ANAE positive lymphocytes that was consistent with our results. On the other hand, oral COS administration did not significantly affect the ANAE positive lymphocytes in the CdCOS group when compared to Cd. It may be observed that a dose of 200 mg/kg of COS failed to improve metal binding properties in Cd exposed rats.

Conclusions

COS exhibited partially positive effects on some hematological characteristics except the percentage of ANAE positive lymphocytes following chronic Cd toxicity. COS appears to exhibit slightly protective properties for the hematopoietic system. These findings provide useful information and suggestions for use of COS in metal intoxication and other related research fields, such as the hematopoietic system.

Declaration of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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KISADERE, I., M. F. AYDM, H. H. DÖNMEZ: Utjecaj oligosaharida hitozana na određene hematološke pokazatelje u štakora izloženih kadmiju. Vet. arhiv 92, 87-95, 2022.

SAŽETAK

Cilj ovog rada bio je istražiti učinak oligosaharida hitozana (COS) na određene hematološke pokazatelje odnosno postotak limfocita pozitivnih na alfa-naftil-acetat-esterazu (ANAE) u štakora izloženih kadmiju (Cd). Mužjaci (n = 32) pasmine Wistar albino, slučajnim odabirom podijeljeni su u četiri skupine: kontrolnu (C), skupinu s oligosaharidom hitozanom (COS), skupinu s kadmijem (Cd) i skupinu s kadmijem i oligosaharidom hitozanom (CdCOS). Prikupljeni su uzorci krvi kako bi se odredili eritrociti (RBC), leukociti (WBC), hemoglobin (HGB), hematokrit (HCT), prosječni hemoglobin u eritrocitima (MCH) i njegova koncentracija (MCHC), prosječan obujam eritrocita (MCV), trombociti (PLT), te limfociti pozitivni na alfa-naftil-acetat-esterazu (ANAE). Broj leukocita znakovito je porastao u skupini Cd u usporedbi s kontrolnom skupinom te skupinama C, CdCOS, i COS ($P < 0,05$). Iako je broj limfocita znakovito smanjen u skupini Cd ($P < 0,05$), to je ublaženo primjenom COS-a u skupini CdCOS u usporedbi sa skupinom Cd ($P < 0,05$). Također, postotak limfocita iz periferne krvi pozitivnih na ANAE znakovito je smanjen u skupinama CdCOS i Cd ($P < 0,05$). COS je, u slučaju kronične izloženosti štakora kadmiju, pokazao djelomice zaštitni učinak na određene hematološke značajke, osim na postotak limfocita pozitivnih na ANAE.

Ključne riječi: alfa-naftil-acetat-esteraza; kadmij; oligosaharid hitozan; hematologija; štakori; toksičnost