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RESEARCH ARTICLE

Effects of clinoptilolite on heavy metal levels in milk, proinflammatory cytokine responses (IL-1β and IL-6) and oxidative stress in dairy cows

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ABSTRACT

The effects of clinoptilolite on milk copper (Cu), lead (Pb), zinc (Zn), cadmium (Cd) and iron (Fe) concentrations, proinflammatory cytokine responses, oxidative stress status, whole blood cell counts and liver and kidney functions were investigated in dairy cows exhibiting no signs of any kind of toxicity. Clinoptilolite was added to the feed at a dose of 200 mg kg^{-1} body weight in the clinoptilolite-treated group ($n = 14$), but was not added to the feed in the control group ($n = 7$). In the milk samples ($n = 21$) collected before the experiment, the Cu, Pb, Zn, Cd and Fe values were 0.021 ± 0.020, 0.104 ± 0.01, 3.42 ± 0.32, <0.000, 0.56 ± 0.34 ppm, respectively. At the end of the experiment (30th day), among the elements measured in milk samples collected from the clinoptilolite-treated group, only the Pb value (0.076 ± 0.01) was lower than the 0-day value of the clinoptilolite-treated group (0.104 ± 0.01) and the 30th-day value of the control group (0.105 ± 0.01) was found to be statistically lower. Changes determined at the end of clinoptilolite application in serum superoxide dismutase (SOD), malondialdehyde (MDA), albumin, glucose, urea and urine creatinine/urine total protein (uCr/uTP) values, which were interpreted as the effect of lead exposure before the trial, were evaluated as the positive effect of clinoptilolite. It was concluded that the addition of clinoptilolite to the feed in dairy cows caused a significant decrease in the amount of Pb in milk, and positive changes in the parameters related to oxidative stress in serum and in parameters related to renal function.

KEYWORDS

clinoptilolite, IL-1β, IL-6, milk, MDA, Pb, SOD

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INTRODUCTION

Pollution caused by heavy metals, which are inorganic pollutants, in water, soil, and the atmosphere is becoming an increasing problem worldwide. Heavy metal pollution is the result of rapid growth in the agricultural and metal industries, improper disposal of waste,

increased use of fertilisers and pesticides, and increased vehicle emissions. Toxic heavy metals, such as lead (Pb) and cadmium (Cd), enter the body of an animal mainly through grasses or feed grown on contaminated soil or watered with contaminated water, and partially through the inhalation of polluted air. Their levels in milk have resulted in significant health problems (Hjortenkrans et al., 2008; Khan et al., 2008; Bilgücü et al., 2016; Durkalec et al., 2018; Briffa et al., 2020).

The levels of iron (Fe), copper (Cu), and zinc (Zn) in milk samples vary according to the nutritional status of animals and environmental factors (Pilarczyk et al., 2013; Bilgücü et al., 2016; Khachlouf et al., 2018).

The Ministry of Agriculture and Rural Affairs' Communiqué on Maximum Limits of Contaminants (Official Gazette, Saturday, May 17, 2008, Issue 26879) and 'Communiqué on Unwanted Substances in Feeds' (Official Gazette, Saturday, April 19, 2014, Issue 28977) list the maximum acceptable amounts for heavy metals and mineral substances. European Commission regulations were prepared with the recommendations of the European Food Safety Authority (EFSA). The EFSA states that Cd and Pb are naturally occurring chemical compounds. The organisation also mentions that environmental pollution increases their exposure and that the accumulation of these elements in the body can lead to harmful effects over time. This regulation is reported in the European Commission's Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (European Commission, 2006).

Pb and Cd cause disorders in the cardiovascular system, kidneys, nervous system, blood, skeletal system, and liver. These heavy metals cause acute toxicity when they enter the body in large, one-time amounts, and subacute or chronic toxicity when they enter it in small amounts. Deficiencies/excesses of essential body elements result in metabolic dysfunction and pathophysiological changes in the organs (Pilarczyk et al., 2013; Abdou and Hassan, 2014; Andjelkovic et al., 2019).

It is stated that oxidative stress is an important toxicity mechanism in Pb and Cd intoxication (Paithankar et al., 2021). In cases where serum Pb and Cd concentrations are high, the following occur: a significant decrease in superoxide dismutase (SOD) and a significant increase in malondialdehyde (MDA), haemolysis due to oxidative stress in patients, and effective results of antioxidants in the treatment of patients; Pb and Cd are therefore considered signs of oxidative degradation in the body (El-Neweshy and El-Sayed, 2011; Abdou and Hassan, 2014; Dhaliwal and Sushma, 2016).

In rats exposed to Pb, an increase in proinflammatory cytokines (IL-1β and IL-6), haemolysis due to lead-induced oxidative stress and anaemia occur (Dyatlov and Lawrence, 2002; Aslani et al., 2012; Ray, 2016; Chibowska et al., 2020).

Depending on the intensity and duration of Cd and/or Pb exposure in animals, changes in the blood, liver and kidney-related parameters, metabolites, proinflammatory cytokines, and oxidative stress parameters were reported as indicative of heavy metal toxicity (El-Neweshy and El-Sayed, 2011; Aslani et al., 2012; Abdou and Hassan, 2014). An increase in serum triglyceride levels and decreases in high-density lipoprotein (HDL) and cholesterol occur as an indication that lipids are used in the gluconeogenesis to produce energy in animals under heavy metal stress (Abdou and Hassan, 2014; Bekus et al., 2016).

Clinoptilolite, a zeolite material, is a substance with extraordinary ion exchange and adsorption properties; it is useful in binding and/or eliminating various substances in the rumen, abomasum and intestine. When clinoptilolite is administered with feed, it protects intestinal microbiota homeostasis, improves antioxidant and endogenous anti-inflammatory activities and, thus, increases the overall well-being of the animal. Due to its indirect systemic detoxification effect, clinoptilolite reduces or prevents the toxic effects exerted in the organs by substances known to be harmful to the body. It has been stated that the observed local immunomodulatory effects of clinoptilolite may have a systemic effect on the entire immune status (Papaioannou et al., 2005; Pavelic et al., 2018; Mastinu et al., 2019).

In this study, after evaluating the presence and levels of Cu, Pb, Zn, Cd and Fe in the milk of dairy cows and the effects of this status on serum IL-1β, IL-6, SOD, MDA and several other blood parameters, the aim was to investigate the effects of clinoptilolite given at a dose of 200 mg kg^{-1} body weight in the feed for 30 days on the levels of heavy metals and other blood parameters in milk.

MATERIALS AND METHODS

Approval for this study was obtained from the Kırıkkale University Animal Experiments Local Ethics Committee (decision No. 2019-12-54). In the study, 21 Holstein cows, aged 3–5 years and kept in the same farm with a body condition score (BCS) value of 3.25–3.75 and a daily milk yield of approximately 35–40 L/cow, were used. The cows did not show clinical signs suggestive of any toxicity. Approximately 200 mg kg^{-1} body weight of clinoptilolite was added to the feed of the clinoptilolite group ($n = 14$) once a day for 30 days, and the cows were kept under observation until the clinoptilolite mixed in the feed was completely consumed. The animals were kept in a separate barn complex during the experiment. Clinoptilolite was not added to the feed of seven cows in the control group, and previous care and feeding practices were continued. The natural clinoptilolite material was purchased from Gordes Zeolite Mining (Nat-min 9000; Bayraklı, Izmir, Turkey). The NH_4 ion exchange capacity of the clinoptilolite material was 1.7–2.1 mEq/g, and its chemical composition was SiO_2 67.11% (w/v), Al_2O_3 11.84% (w/v), Fe_2O_3 1.47% (w/v), MgO 1.15% (w/v), CaO 2.18% (w/v), Na_2O 0.38% (w/v), and K_2O 3.44% (w/v).

Before the trial (day 0) and at the end of the 30-day trial period, in the early morning, before feeding and watering, milk (manual milking), blood (from the tail vein), and urine samples (manual massage to the perineum) were collected from the 21 cows used in the study. In addition, samples were taken from the feed, water, and grass given to the cows.

All animals were fed twice daily a mixed ration containing grass, corn silage, and commercial concentrate pellets *ad libitum*. The ration for dry and lactating cows contained 16% and 19% crude protein, respectively. The source of grass given to the cows consisted of purchased grass grown and harvested in different sources by several different farmers. Milk, feed, grass, and water samples collected during the study were kept in a freezer at −80 °C until analysis. Samples removed from the freezer before analysis were allowed to thaw at room temperature, and the concentrations of Pb, Cd, Zn, Cu and Fe were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES) device (SpectroBlue, Germany). The quantitative measurement of the analysed elements (Cd, Cu, Fe, Pb and Zn) was evaluated by drawing calibration curves in analytical standard units of 0–0.01–0.02–0.04–0.06–0.08–0.01–0.2–0.4–0.6–0.8–1 and 2 ppm (by creating 13 calibration points, including blank) (Merck, Germany) using the external calibration method. The analytical standard measurements are summarised in Table 1. Approximately 4 mL of the milk samples was then mixed with 10 mL of 65% HNO_3 for element analysis of the samples. Microwave extraction using a CEM MARS 6 microwave oven (CEM Corporation, Matthews, NC, USA) was performed with the following parameters: power: 290–1800 W, ramp time: 20 min, hold time: 15 min, temperature: 200 °C (Aluc and Ekici, 2019). The instrumental conditions used during ICP-OES measurement are summarised in Table 1. Heavy metal levels and recoveries in milk measurements are shown in Table 2. Repeated analysis of the samples showed good accuracy (relative standard deviation, RSD, ≤1%), and the recovery rates ranged from 83% to 98%.

A total of 7 mL of blood was taken from the tail vein of each of the 21 cows used in the study: 5 mL into tubes without anticoagulants and 2 mL into tubes with ethylenediaminetetraacetic acid (EDTA).

Haematological analysis values of blood samples with EDTA were determined using a Mindray BC5000 haematology analyser (Shenzhen, China) within 2 h following blood collection.

Serum was separated from blood samples and immediately transferred to the laboratory and stored in Dappen dishes at −80 °C.

Serum albumin, glucose, urea, uric acid, creatinine, total protein, aspartate aminotransferase (AST) and total bilirubin were measured using a Mindray BS120 automatic biochemistry analyser.

Serum IL-1β and IL-6 levels were measured using commercial ELISA test kits (Sun Red Biotechnology Company, China, Cat. No. 201-04-0157 and Cat. No. 201-04-0008).

The SOD level was measured with a Cayman 706002 commercial test kit (Cayman Chemical, Ann Arbor, MI, USA), and serum MDA level was measured using an ELISA commercial test kit (Sun Red Biotechnology Company, China, Cat. No. 2.01-04-0255).

Physical, chemical, and microscopic analyses of the 4 mL of urine collected in sterile urine collection containers were performed within 2 h. The remaining urine was placed in 5 × 1.5-mL plastic tubes reserved for urinary creatinine/protein ratio measurement and stored at −80 °C until analysis.

Table 1. Conditions of the inductively coupled plasma optical emission spectrometer (ICP-OES)

Display name	BEC	DL	Standard error	Correlation coefficient	Range
Cd 226.502	0.00722 ppm	0.00021 ppm	0.00211 ppm	0.9985	0.00021–0.096 ppm
Cu 324.754	0.04790 ppm	0.00089 ppm	0.00089 ppm	0.9997	0.00089–0.096 ppm
Fe 238.204	0.02800 ppm	0.00101 ppm	0.00268 ppm	0.9998	0.00101–0.480 ppm
Pb 220.353	0.08330 ppm	0.00099 ppm	0.00089 ppm	0.9997	0.00099–0.096 ppm
Zn 202.613	0.00605 ppm	0.00016 ppm	0.02380 ppm	0.9994	0.00016–2.400 ppm

Parameters	Value
Plasma power	1435 W
Pump speed	30 rpm
Coolant flow	13 L min^{-1}
Auxiliary flow	0.80 L min^{-1}
Nebuliser flow	0.75 L min^{-1}
Number of replicates	3
Integration time	3 s
Sample uptake rate (μL min^{-1}) (speed)	0.3 rps

BEC: Background Equivalent Concentration; DL: detection limits.

Table 2. Heavy metal levels (ppm) and recoveries in the milk measurements

	Cu	Pb	Zn	Cd	Fe
Standard addition	0.100	0.100	0.100	0.100	0.100
Measured value	0.95 ± 0.324	0.96 ± 1.026	0.98 ± 0.491	0.83 ± 0.430	0.91 ± 0.265
Recovery (%)	95	96	98	83	91

Samples from the freezer were allowed to thaw at room temperature before analysis, and the urine creatinine/protein ratio was determined using an Mindray BS300 autoanalyser.

Before performing statistical analysis, data were examined as parametric test assumptions. Descriptive statistics for each variable were calculated and presented as 'mean ± standard error of mean'. To test the differences in each parameter between sampling times in groups, a two-way mixed analysis of variance (ANOVA) was used. The effects of group, time of sampling and their interaction on heavy metal levels, proinflammatory cytokine, oxidative stress, liver and kidney functions, and blood parameters were analysed using the following model with repeated measures:

$$Y_{ijk} = \mu + G_i + T_j + (G \times T)_{ij} + e_{ijk}$$

where, Y_{ijk}, dependent variable; μ, overall mean; G_i, effect of group (i = control and treatment); T_j, effect of time of sampling (j = day 0 and day 30); $(G \times T)_{ij}$, interaction between group i and time of sampling j; and e_{ijk}, residual error.

When a significant difference was revealed, any significant terms were compared by simple effect analysis with Bonferroni adjustment. $P < 0.05$ was considered as significant in all analyses. All statistical analyses were performed using IBM SPSS Statistics software, Version 23.0.

RESULTS

The Cu, Pb, Zn, Cd and Fe values measured in the water sample taken from the dairy cattle farm were as follows: <0.001, 0.068, <0.001, <0.001, 1.375 ppm, respectively; 0.6325, 1.6775, 3.7125, 0.04125 and >97.132 ppm in the feed sample, respectively; and 0.3025, 0.1237, 0.9212, <0.000 and 15.07 ppm in the grass sample, respectively.

The Cu, Pb, Zn, Cd and Fe values measured in the milk samples taken from the control and experimental groups on days 0 and 30 are shown in Table 3.

Serum total cholesterol, uric acid, total protein, albumin, AST, urea, creatinine, urine creatinine (uCr)/urine total protein (uTP) ratio, glucose, triglyceride, HDL, IL-1β, IL-6, monocyte/HDL cholesterol ratio, SOD, and MDA values measured in the control and treatment groups of cows on days 0 and 30 are shown in Table 4.

Results are presented as the arithmetic mean ± standard deviation. The different letters (a, b) in the same line indicate a statistically significant difference ($P < 0.05$) and different letters (A, B) in the same column indicate a statistically significant difference ($P < 0.05$).

White blood cell (WBC), neutrophil, lymphocyte, monocyte, eosinophil, basophil, haemoglobin (Hb), and red blood cell (RBC) values in the control and treatment groups on days 0 and 30 are shown in Table 4.

DISCUSSION

Pb and Cd, which have been studied and evaluated for many years and which are important elements in environmental pollution, enter the body via plants, water and air, and can cause acute or chronic toxicity (Aslani et al., 2012; Bilgücü et al., 2016; Briffa et al., 2020). The recommended levels of the standards for milk are not given as numbers because they differ from region to region and country to country. In many countries, milk standards for elements vary. These are as follows: for Pb values, this could vary between 0.02 and 0.1 ppm (Ministry of Labour, Health and Social Affairs of Georgia, 2001; Pavlovic et al., 2004; European Commission, 2006) and between 0.05 and 0.3 ppm for Cu (Işık et al., 1996; Yetişmeyen, 2000; Tekinşen, 2000; European Commission, 2001); values are between 2 and 6 ppm for Zn (Pechová et al., 2008) and the standard for Fe is 0.7 ppm (Storelli et al., 2007; Safonov, 2020).

In the analysis of milk and dairy products for heavy metals and mineral substances, while significant changes in the mineral levels could not be determined, it has been reported that Pb and Cd were above the acceptable level. It is difficult to determine the source of heavy metal exposure due to its multifactorial nature (Aslani et al., 2012; Pilarczyk et al., 2013; Bilgücü et al., 2016). Similarly, in the milk

Table 3. Cu, Pb, Zn, Cd and Fe values measured in the milk samples

Parameters	Groups Control (n = 7) Treatment (n = 14)	Sampling day		P value		
		0	30	Group	Time	G × T
Cu (ppm)	Control	0.034 ± 0.031	0.038 ± 0.043			
	Treatment	0.021 ± 0.020	0.071 ± 0.018			
Pb (ppm)	Control	0.098 ± 0.01	0.105 ± 0.01^{A}	0.051	0.037	0.008
	Treatment	0.104 ± 0.01^{a}	0.076 ± 0.01^{b,B}			
Zn (ppm)	Control	2.96 ± 0.49	2.95 ± 0.45			
	Treatment	3.42 ± 0.32	2.84 ± 0.38			
Cd (ppm)	Control	<0.000	<0.000			
	Treatment	<0.000	<0.000			
Fe (ppm)	Control	0.59 ± 0.36	0.50 ± 0.36			
	Treatment	0.56 ± 0.34	0.68 ± 0.34			

Results are presented as the arithmetic mean ± standard deviation.
a, b: Different letters in the same line indicate a statistically significant difference ($P < 0.05$).
A, B: Different letters in the same column indicate a statistically significant difference ($P < 0.05$).

Table 4. Serum total cholesterol, uric acid, total protein, albumin, AST, urea, creatinine, uCr/uTP ratio, glucose, triglyceride, HDL, IL-1β, IL-6, monocyte/HDL cholesterol ratio, SOD and MDA, WBC, neutrophil, lymphocyte, monocyte, eosinophil, basophil, Hb and RBC values

Parameters	Groups Control (n = 7) Treatment (n = 14)	Sampling day		P value		
		0	30	Group	Time	G × T
Cholesterol (mg dL^{-1})	Control	137.14 ± 19.73	141.71 ± 19.73			
	Treatment	169.00 ± 13.95	153.00 ± 13.95			
Uric acid (mg dL^{-1})	Control	0.68 ± 0.08	0.68 ± 0.08			
	Treatment	0.69 ± 0.06	0.75 ± 0.06			
Total protein (g dL^{-1})	Control	7.20 ± 0.61	7.47 ± 0.61			
	Treatment	7.71 ± 0.43	7.83 ± 0.43			
Albumin (g dL^{-1})	Control	3.45 ± 0.28	3.34 ± 0.28^{B}	0.002	0.172	0.048
	Treatment	3.67 ± 0.24^{b}	$4.23 \pm 0.24^{a,A}$			
AST (U/L)	Control	91.86 ± 24.83	111.57 ± 24.83			
	Treatment	104.86 ± 23.37	103.79 ± 24.83			
Urea (mg dL^{-1})	Control	43.00 ± 5.11	42.00 ± 5.11	0.003	0.196	0.131
	Treatment	50.43 ± 3.61	63.07 ± 3.61			
Creatinine (mg L^{-1})	Control	1.22 ± 0.38	1.35 ± 0.38			
	Treatment	1.33 ± 0.37	1.32 ± 0.37			
uCr/uTP	Control	2.29 ± 0.15	2.07 ± 0.15	<0.001	0.025	0.570
	Treatment	1.80 ± 0.11	1.43 ± 0.11			
Glucose (mg dL^{-1})	Control	71.57 ± 6.43	77.29 ± 6.43	0.002	0.071	0.418
	Treatment	84.93 ± 4.49	99.64 ± 4.49			
Triglyceride (mg dL^{-1})	Control	14.29 ± 2.55	15.00 ± 2.55	0.005	0.034	0.072
	Treatment	16.64 ± 1.92	24.93 ± 1.92			
HDL cholesterol (mg dL^{-1})	Control	172.86 ± 18.19	157.29 ± 18.19			
	Treatment	174.07 ± 15.83	192.71 ± 15.83			
IL-1β (ng mL^{-1})	Control	226.91 ± 36.16	220.15 ± 36.16			
	Treatment	218.62 ± 34.51	189.08 ± 34.51			
IL-6 (ng mL^{-1})	Control	243.17 ± 18.13	247.89 ± 18.13			
	Treatment	243.60 ± 12.82	223.35 ± 12.82			
Monocyte/HDL cholesterol ratio	Control	0.007 ± 0.004	0.007 ± 0.004			
	Treatment	0.010 ± 0.003	0.005 ± 0.003			
MDA (nmol g^{-1} protein)	Control	4.40 ± 0.28	4.55 ± 0.28	0.555	0.042	0.138
	Treatment	4.18 ± 0.20	5.06 ± 0.20			
SOD (U/g protein)	Control	55.34 ± 4.22	56.80 ± 4.22^{A}	0.002	0.119	0.048
	Treatment	50.38 ± 2.99^{a}	$37.25 \pm 2.99^{b,B}$			
WBC (10^9/L)	Control	9.05 ± 0.94	10.09 ± 0.94			
	Treatment	8.99 ± 0.67	8.59 ± 0.67			
Neutrophil (10^9/L)	Control	4.31 ± 1.03	4.94 ± 1.03			
	Treatment	4.76 ± 0.73	4.06 ± 0.73			
Lymphocyte (10^9/L)	Control	3.85 ± 0.71	5.26 ± 0.71			
	Treatment	4.50 ± 0.53	4.63 ± 0.53			
Monocyte (10^9/L)	Control	1.16 ± 0.55	1.02 ± 0.55			
	Treatment	1.18 ± 0.39	0.92 ± 0.39			
Eosinophil (10^9/L)	Control	0.22 ± 0.18	0.29 ± 0.18			
	Treatment	0.27 ± 0.16	0.29 ± 0.16			
Basophil (10^9/L)	Control	0.034 ± 0.026	0.021 ± 0.026			
	Treatment	0.049 ± 0.019	0.084 ± 0.019			
Hb (g dL^{-1})	Control	9.99 ± 0.51	9.90 ± 0.51			
	Treatment	9.92 ± 0.45	9.56 ± 0.45			
RBC (10^{12}/L)	Control	5.89 ± 0.44	5.99 ± 0.44			
	Treatment	6.07 ± 0.41	5.58 ± 0.41			

AST: aspartate aminotransferase; uCr/uTP: urine creatinine/urine total protein; HDL: high-density lipoprotein; MDA: malondialdehyde; SOD: superoxide dismutase; WBC: white blood cells; Hb: haemoglobin; RBC: red blood cells.

samples collected in this study, the basic minerals 'copper, iron and zinc' were found to be at the normal levels specified in the Communiqués of the Ministry of Agriculture (2008, 2014). However, as reported also by other researchers (Aslani et al., 2012; Pilarczyk et al., 2013; Bilgücü et al., 2016), the Pb level in milk samples was found to be above the acceptable value in this study. In order to find the possible causes of Pb exposure in cows, we believe that it would be useful to perform repeated analyses of feed, grass, water and soil samples in terms of Pb concentration; samples would be

collected at regular intervals in areas where Pb pollution has been observed.

Many variants of proinflammatory cytokine responses, oxidative stress, liver and kidney functions, and haematological parameters in heavy metal exposure continue to be investigated today (Aslani et al., 2012; Abdou and Hassan, 2014; Dhaliwal and Sushma, 2016; Andjelkovic et al., 2019; Chibowska et al., 2020). In this study, changes in the parameters indicated by the above researchers were investigated in cows that were thought to have experienced Pb exposure, and the effect of clinoptilolite on these parameters was evaluated.

It is important to consider that Pb exposure causes a significant increase in all cytokines, including IL-1 beta and IL-6, due to inflammation (Dyatlov and Lawrence, 2002; Chibowska et al., 2020). The lack of significant changes in serum IL-1 beta and IL-6 values in the cows in this study was attributed to the fact that the intensity of Pb exposure was not at a level that would cause a severe inflammatory reaction in the body. However, the not so significant decrease in IL-1 beta and IL-6 values in the serum samples collected 30 days after adding clinoptilolite to the feed – compared to the pre-trial values – was attributed to the possible therapeutic effect of clinoptilolite on systemic inflammatory conditions. This result can show us how to limit excessive free radical production inducing the inflammatory response. In addition, free radicals induced by metals disrupt the transcription signalling pathways and can cause a proinflammatory cytokine response.

As reported by Dhaliwal and Sushma (2016), low SOD levels and high MDA levels had been detected [control group SOD (U/mg Hb) 23.31 ± 3.97, MDA (nmol g^{-1} Hb) 414.4 ± 70.2, study group SOD (U/mg Hb) 16.33 ± 2.54, MDA (nmol g^{-1} Hb) 491.11 ± 113] before the experiments on cows in this study were conducted, and these levels were thought to have indicated the presence of oxidative stress. It was determined that the cause of oxidative stress might be due to Pb exposure in the cows of this study since there were no clinical signs of other diseases and no elements other than Pb were detected in the milk. It has been reported that there is a positive correlation between the severity of liver lesions and changes in serum ALT and AST levels in animals exposed to heavy metals (Aslani et al., 2012; Abdou and Hassan, 2014). In this study, changes in serum AST levels detected in the cows due to mild liver impairment were thought to have been caused by possible Pb exposure.

In cases in which heavy metal stress occurs, an increase in serum triglyceride and a decrease in HDL were reported to have resulted from the use of lipids for gluconeogenesis to meet an increased energy demand (Abdou and Hassan, 2014; Bekus et al., 2016). Similar findings were also found in the serum samples in this study, in which the significant increase in triglyceride values in serum samples 30 days after clinoptilolite application – compared to the pre-trial values – was considered evidence that the energy requirement was still provided by lipids.

In animals exposed to Pb, an increase or decrease in serum blood urea nitrogen and an increase or decrease in serum creatinine have previously been observed (Aslani et al., 2012; Abdou and Hassan, 2014; Dhaliwal and Sushma, 2016; Andjelkovic et al., 2019). In our study, it was determined that the serum creatinine value was high and that the uCr/uTP value was low in samples collected before the experiment. Thirty days after adding clinoptilolite to the feed, a significant decrease in uCr/uTP and no significant changes in the serum creatinine levels were attributed to an increase in urinary protein excretion.

In the samples collected on the 30th day of this study, serum IL-1 beta and the monocyte/HDL cholesterol ratio were found to be lower than the day 0 values. This result may be attributable to the fact that the amount of Pb entering the body may be blocked by clinoptilolite and due to the possibility of clinoptilolite exhibiting a systemic anti-inflammatory effect, as previous researchers have also suggested (Papaioannou et al., 2005; Enhos et al., 2018; Pavelic et al., 2018; Onat et al., 2020).

The reported decrease in serum albumin in the case of Pb exposure was also observed in the serum samples in this study (Aslani et al., 2012; Abdou and Hassan, 2014; Andjelkovic et al., 2019). A significant increase in serum albumin determined at the end of the experiment was attributed to the reported systemic anti-inflammatory effect of clinoptilolite (Pavelic et al., 2018).

In experimentally-induced acute Pb toxicity in rats, the declining trend in the RBC and Hb values and the increasing trend in the WBC and neutrophil values were also found to be similar to the increasing trend in the WBC and neutrophil values in the blood samples in this study (Aslani et al., 2012; Abdou and Hassan, 2014; Andjelkovic et al., 2019).

In accordance with previous reports, clinoptilolite significantly adsorbs Pb in the rumen and abomasum and has an absorptive and detoxifying effect in the intestine, while also exhibiting a local and systemic anti-inflammatory effect. In this study, a significant decrease in Pb concentration and positive changes in serum antioxidant and anti-inflammatory parameters were attributed to the positive effect of clinoptilolite in milk samples collected at the end of the 30-day trial period from cows that had clinoptilolite added to their feed (Papaioannou et al., 2005; Pavelic et al., 2018).

In conclusion, although we determined that the Pb level was high in the milk samples collected from the dairy cows in our study, we consider this to be the result of chronic Pb exposure in the animals since clinical findings specific to high levels of Pb were not seen in the cows and because we could not determine the source of Pb exposure. Both the decrease in serum SOD values and the increase in MDA values in these cows are indicative of oxidative stress development in the animals. The changes we observed in the serum urea, creatinine, uCr/uTP, albumin and total protein values in our study may be related to structural and/or functional disorders that may have developed in the kidneys, depending on Pb exposure. Therefore, we believe that adding clinoptilolite to cow feed at a dose of 200 mg kg^{-1} body weight for 30 days reduces the amount of Pb in milk; in addition, significant improvements in serum oxidative stress

parameters and parameters indicative of kidney function show that clinoptilolite can be successfully used in disorders caused by chronic Pb exposure.

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