Cadmium-induced oxidative damage and protective action of fractioned red beet (*Beta vulgaris*) root juice in chickens

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Abstract. Cadmium (Cd) is one of the most dangerous environmental bioaccumulative pollutants that affects many organs in humans and animals. Present investigation was conducted to evaluate the protective effect of fractioned red beetroot juice on Cd-induced oxidative stress in chickens. The named red beetroot juice fraction (BJF) was received by juice ultrafiltration. Per oral administration of BJF for 10 days followed by dietary Cd exposure (50 mg kg^{-1} of diet) was evaluated in the *in vivo* experiments in chickens. The prominent increase of Cd concentration in blood plasma, liver and kidney provoked the rise of oxidative processes activity in organs. BJF treatment attenuated the Cd-induced oxidative stress. The changes of oxidative stress markers - the reduction of hepatic and kidney malondialdehyde amount, the increase of glutathionperoxidase level in liver and blood catalase activity indicated the possible antioxidative influence of BJF. Chickens exposed to Cd showed no evidence of clinical toxicity, but exhibited some features of adverse action of this heavy metal. The increase of uric acid concentration in blood serum is associated with protein catabolic processes intensified by Cd affect. Suppressive effect of Cd on the immune response in chickens manifested in alteration of cell and humoral immunity parameters. The data of the most investigated oxidative stress markers, biochemical and immunological indices in Cd-exposed chickens were almost back to the values, when received BJF *per os* during 10 days. Administration of fractioned red beetroot juice to Cd-treated chickens prevented the oxidative impact of this heavy metal and provided immunomodulating effect.

Key words: oxidative stress, cadmium, red beetroot juice, antioxidative effect, chickens.

INTRODUCTION

Heavy metal cadmium (Cd) is a naturally occurring element that is present everywhere in the environment – in almost all soils, surface waters, plants and wildlife. In comparison with other heavy metals, Cd possesses high mobility in soil and is taken up by plants in various degrees (Kah et al., 2012; Brzóska et al., 2016). Cd content in the environment is increasing, because this element is continuously released from its natural sources (the Earth's crust), and introduced through various human activities, including industrial processes, it does not undergo biodegradation (Moulis & Thévenod, 2010).

Cd intake by humans mainly occurs via the food chain and this heavy metal is considered one of the most dangerous occupational and environmental poisons. Hazards

from Cd are associated with its high bioaccumulative capacity. Adverse human, animal and plant physiological effects of Cd are numerous (Bernhoft, 2013). At the cellular level, the pro-oxidative Cd action results in oxidative damage to the cellular macromolecules and cellular structures (Curcic et al., 2014; Rahman et al., 2017). Disbalanced antioxidant system and developed oxidative stress produce injury to healthy tissues and immune cells by free radicals, which results in compromised immune functions in chickens (Vasiljeva, Berzina & Remeza, 2011). Cd is among the immunosuppressive agents, it excessive and even low doses in daily food can cause diverse physiological and immunological disturbances in human, animals and poultry (Bokori & Fekete, 1995; El-Boshy et al., 2015).

Since Cd-induced damage in an organism is believed to be irreversible, the question of primary prevention is of great importance. Efficient beneficial results may be achieved by addition of nutrients with antioxidative properties to the diet (Nair et al., 2013). Antioxidants are known to play a vital role in the health due to cell protection from damage induced by oxidative stress (Surai, 2003; Brzóska & Rogalska, 2013).

Cultivated forms of red beet (*Beta vulgaris L.*) have been used for medical purposes since ancient times. In recent years, interest in the biological activity of red beets and its potential utility as a functional nutrition and disease prevention has been growing. Red beetroot juice is also considered to promote therapeutic treatment in a number of clinical pathologies associated with oxidative stress and inflammation (Clifford et al., 2015; Cho et al., 2017).

Red beet contains betalain pigments belonging to the group of cationic antioxidants. Betalains are divided into two groups: red betacyanins (predominantly betanin) and yellow betaxanthins (vulgaxanthine I and vulgaxanthine II). Red betanin is primarily responsible for the antioxidant ability of red beets (Czapski et al., 2009). Betanin is both a free radical scavenger and an inducer of an antioxidant defense mechanism in cells. Its effect is dose dependent (Esatbeyoglu, 2014). Attempts to concentrate the fraction of juice betalains by gel filtration on Sephadex using juice extracts in water or ethanol were unsuccessful (Lee et al., 2005). Despite the failure of mentioned experiment the idea of red beetroot fractionation seems prospective. Recently, using the modern method of membrane separation (diafiltration) (Mereddy et al., 2017), as well as ultrafiltration (Krumina et al., 2016), allowed to obtain more concentrated pigment fractions of beet juice. In these cases, freshly squeezed beet juice was used. Ultrafiltration is more suitable for industrial use in comparison with chromatography. Ultrafiltration as industrial technology has several advantages over chromatography. Ultrafiltration use is cheaper, has higher productivity and provides possibility to prepare natural compounds product with more accurate parameters (i.e. cut off point).

Present investigation was conducted to evaluate the protective effect of red beetroot juice fraction (BJF) in chickens exposed to Cd.

MATERIALS AND METHODS

Ethics statement

All experimental procedures were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia, authorisation reference number 13, December 22, 2008).

Animals and experimental design

35-day-old male Lohmann brown chickens were obtained from SIA BALTICOVO (Iecava, Latvia) and used for the laboratory investigations. Chickens were divided into four groups of 7 heads each. Food and water were provided *ad libitum* for 10 days. Group 1 (Control) received the fool-fed basal diet without any supplements. Group 2 (+Cd) was given the same diet with addition of Cd 50 mg kg^{-1} (as $CdCl_2$, Sigma, EU). Group 3 (+BJF) received the basal diet without any supplements and each chicken was administered by 1 mL of BJF *per os* daily. Group 4 (+Cd+BJF) was fed the same diet supplemented with Cd as group 2 and administered by 1 mL of BJF *per os* daily. The chosen high experimental level of dietary Cd exposure was based on our previous unpublished experimental data and had hazard effect for 35–45-days old chickens. It allows investigate a risk of health damage by this heavy metal. In accord with our previous study the best administered dose of BJF for chicken was 1 mL per one head (Smirnova et al., 2017). Red beet (*Beta vulgaris*) root juice fraction (BJF) was produced using the laboratory equipment**.** The red beetroots were washed, skinned, shredded, and the juice was extracted mechanically by juice press. The juice was deproteinized using heating at 85ºC for 10 min followed by centrifugation. The supernatant was fractionated with ultrafiltration using ultrafilter membrane with cut-off-point 150 KDa. 100 mL of the obtained fraction was contained: 65.80 mg of betanin, 40.10 mg of vulgaxanthine-I, 7.80 g of sucrose, 11.00 mg of ascorbic acid, 1.20 mg of lysozyme and 0.20 mg of iron. Obtained ultrafiltrate BJF was used in the experiment.

At the end of experiment, chickens were weighed and sacrificed by decapitation in accordance with the Recommendation for Experimental Animals of the European Convention (Close et al., 1997). Whole blood, blood plasma, liver, kidneys of chickens were collected and used for analyses.

Biochemical assays

Cd determination of the tissue samples was performed after dry ashing in atomic absorption spectrophotometer Perkin-Elmer (model AAnalyst 700), according to the procedures of the AOAC (1999). The antioxidant status was evaluated by measuring the level of lipid peroxidation product malondialdehyde (MDA) in liver and kidney homogenates by the thiobarbituric acid reaction (Surai et al., 1996). Activity of glutathionperoxidase (GSH-Px) in liver homogenate was measured based on the method described by Pinto and Bartley (Pinto & Bartley, 1969) with some modifications. Catalase (CAT) activity in blood was estimated by the method of Aebi (Aebi, 1984). The determination of uric acid in blood serum was performed enzymatically (Trivedi et al., 1978). Hemoglobin (Hb) level was estimated by cyanohemoglobin method with commercial kit (SIA 'Divi Dent', Latvia). Total protein concentration in blood plasma was determined spectrophotometrically by the biuret method, using a commercial kit (Boehringer, Germany).

Immunological analyses

Immune cell T- and B- populations were estimated as responses to T- and B-markers: sheep erythrocytes for T-cells and zymozan (C3 complexes) for B-cells. Serum lysozyme content was evaluated by nefelometric assay (Shugar, 1952) with some modification, by absorptiometric determination of the decrease in turbidity of a suspension of *Micrococcus lysodeicticus.* Nonspecific circulating immune complexes

(CIC) in serum were estimated spectrophotometrically using precipitation with polyethylenglycol (Riha et al., 1979).

Statistical analysis

All statistics were performed using the software *Statistica* 7. Results of Cd content and biochemical parameters are presented as mean ± SE. Multiple group comparision was done using *one-way* ANOVA and *Post-hoc* Tukey HSD test.

RESULTS AND DISCUSSION

In general, the variance analysis revealed that there was a statistically significant difference between the treatment groups of the experiment for most of analyzed parameters, except MDA level in chicken liver (Table 1).

Table 1. Results of multiple group comparison with one-way ANOVA, analysing Cd content and biochemical parameters in chickens under Cd exposure and administration with red beetroot juice fraction (BJF) (in bold – significant P values, $P < 0.05$, n = 28, df = 3)

Parameter	F*, stated	P-value	F, critical
Cd, blood plasma	250.097	**3.12346E-18**	3.009
Cd, liver	1899.165	**1.19352E-28**	3.009
Cd, kidney	703.131	**1.64691E-23**	3.009
MDA, liver	2.174	0.11734499	3.009
MDA, kidney	51.925	**1.20918E-10**	3.009
GSH-px	27.599	**5.95067E-08**	3.009
Catalase	5.994	**0.003371862**	3.009
Hemoglobin	34.762	**6.73753E-09**	3.009
Total protein	45.681	**4.49054E-10**	3.009
Uric acid	29.528	**3.18001E-08**	3.009
T- lymphocytes	64.860	**1.17286E-11**	3.009
B(C3)- lymphocytes	10.276	**0.000153855**	3.009
Lysozyme	22.609	**3.56601E-07**	3.009
CIC	28.842	**3.95871E-08**	3.009

* Fisher's value.

The Cd content in blood plasma, liver and kidney of chickens did not differ between the control group and the 3rd group, which received BJF (Table 2). Additional Cd administration provided a significant increase of this heavy metal concentration in chickens: by 2.3 times in blood plasma, 80 times and 93 times in liver and in kidney, respectively, compared to the control. After additional per oral intake of BJF reduced Cd accumulation in chickens of the 4th group was found: in blood and liver by 1.3 times and by 1.2 times, respectively. On contrary, there was a slight increase in Cd content by 1.1 times in kidney of the group 4 compared to the single Cd treatment (group 2). The observed increase of Cd concentration in kidneys can be associated with the growing intensity of removing of this heavy metal from organism (Zabulyte et al., 2007).

A marked increase of Cd concentration in blood plasma, liver and kidneys of chickens is accompanied by enhance of oxidative processes in organs (Erdogan et al., 2005; Berzina et al., 2007). MDA is one of the final products polyunsaturated fatty acids peroxidation in cells. This is the final product of lipid peroxidation. An increase in free

radicals causes overproduction of MDA followed by cell oxidative injury (Babu et al., 2006; Moitra et al., 2014). Cd triggers adverse effects in organs *via* oxidative stress induction. With Cd dietary overload, peroxidation of membrane lipids causes liver and kidney injury by free radical generation, as evidenced by an increase in MDA production (Nemmiche, 2017). It is known that one of the most sensitive organs to the action of Cd is kidney (Surai, 2003). Addition of Cd in chicken diet resulted in a significant increase of MDA level in kidney by 25.4% (Table 3). Administration of BJF to Cd-treated chickens slightly reduced this effect in kidneys. MDA level decreased by 7.0% in comparison with the group 2. The level of hepatic MDA in chickens of (+ BJF) and (+ Cd + BJF) groups did not differ from the control data.

Table 2. The effect of red beetroot juice fraction (BJF) administration on cadmium (Cd) concentration in chicken's organs after Cd dietary intake

Group	Cd, $\mu g \cdot g^{-1}$		
	Blood plasma	Liver	Kidney
1. Control	$0.044 \pm 0.002^{a*}$	0.12 ± 0.01^a	0.20 ± 0.03^a
2. + Cd	0.103 ± 0.005^c	9.61 ± 0.48^c	18.6 ± 1.56^b
3. + BJF	0.046 ± 0.007^a	0.10 ± 0.05^a	0.19 ± 0.08^a
4. + Cd + BJF	0.080 ± 0.003^b	8.05 ± 0.35^b	20.6 ± 1.65^b

* Statistically different within column according to Post-hoc Tukey's test ($P < 0.05$).

Cd is known to cause oxidative stress by increasing lipid peroxidation. This heavy metal may damage enzymatic antioxidative defense system and disturb oxidative/antioxidative status of the cells (Nair et al., 2013). GSH-Px is selenium-dependent enzyme that catalyses the reduction of hydroxyperoxides by glutathione. Its main function is to protect against the damaging effect of endogenously formed hydroxyperoxides (Galazyn-Sidorczuk et al., 2012). It was established, that Cd exposure to rats caused the inhibition of GSH-Px and CAT activities in kidneys and liver (Jihen et al., 2009). The results of our study demonstrated that the level of hepatic GSH-Px decreased in Cd-treated groups (2 and 4) by 14.6% and 11.5%, respectively, compared to the control.

Table 3. The effect of red beetroot juice fraction (BJF) administration on oxidative stress indices in organs of Cd-treated chickens

Group	MDA, $mM \, g^{-1}$		GSH-Px, $mM \, GSH \, min^{-1} \, g^{-1}$	Catalase, $k \, g^{-1} \, Hb$
	Liver	Kidney	Liver	Blood
1. Control	$17.7 \pm 0.76^{a*}$	13.8 ± 0.59^a	9.60 ± 0.23^a	56.4 ± 2.52^a
2. + Cd	18.3 ± 0.68^a	17.3 ± 0.56^b	8.20 ± 0.28^b	50.6 ± 2.25^b
3. + BJF	17.6 ± 0.83^a	14.1 ± 0.27^a	9.51 ± 0.19^a	57.0 ± 1.98^a
4. + Cd+BJF	17.4 ± 0.69^a	16.1 ± 0.60^b	8.50 ± 0.36^b	56.9 ± 2.00^a

* Statistically different or similar within column according to Post-hoc Tukey's test ($P < 0.05$).

CAT is a common antioxidant enzyme, which is produced naturally in almost all living organisms. It is very important enzyme in protecting the cell from oxidative damage by reactive oxygen species. The effects of the exposure to Cd on CAT activities have been studied rather extensively and are depending on the experimental conditions (Wang et al., 2015). Cd stimulates the formation of reactive oxygen species, thus causing

oxidative damage to erythrocytes and various tissues resulting in loss in membrane functions (Sarkar et al., 1998). The activity of CAT in blood of Cd-exposed chickens significantly decreased by 10.3 % compared to the enzyme activity of the control group (Table 3). It characterizes the disturbing action of Cd on enzyme state. BJF administration to chickens of the group 4 showed the increase of CAT activity to the same level as in control data. The restored changes of GSH-Px and CAT activities in chicken tissues of group 4 indicated a protective effect of BJF against toxic impact of Cd. It is notable that MDA level and investigated enzymes activities did not change in chickens administered by BJF alone (group 3) compared to the control.

There was no difference between Cd-treated chickens and control group in blood hemoglobin level and concentration of total protein in blood plasma (Table 4). After intake of BJF an increase of hemoglobin index and total protein level in blood plasma of chickens was found compared to the control. Administration of BJF alone also increased hemoglobin level in chicken blood.

Table 4. The influence of red beetroot juice fraction (BJF) intake on hemoglobin and protein metabolic indices in Cd-treated chickens

Group	Blood	Blood plasma	
	Hemoglobin, g dl^{-1}	Total protein, mg L^{-1}	Uric acid, g dl^{-1}
1. Control	$8.63 \pm 0.12^{a*}$	28.95 ± 0.33^a	2.51 ± 0.28^a
2. + Cd	8.63 ± 0.06^a	28.93 ± 0.80^a	3.31 ± 0.12^b
3. + BJF	9.15 ± 0.18^b	29.01 ± 0.46^a	2.60 ± 0.17^a
4. + Cd+ BJF	9.21 ± 0.14^b	32.74 ± 1.07^b	2.82 ± 0.07^a

* Statistically different within column according to Post-hoc Tukey's test ($P < 0.05$).

Cd exposure caused the intensification of protein catabolic processes. It is mainly related to a risk of damage in kidneys (Ferraro et al., 2010; Akesson et al., 2014; Wallin et al., 2014). Uric acid in blood plasma, a product of protein catabolism, may be considered as a marker of kidney function in organism (Cohen et al., 2007; Braun & Sweazea, 2008). It is in accord with the data of our study. Significant increase of uric acid concentration in blood plasma was recorded for chickens of the group 2 by 31.9% (Table 4). The elevated accumulation of uric acid in chickens of group 2 suggests Cd-induced disturbance of kidney excretion function associated with a change in purine metabolism intensity. Although uric acid acts as an antioxidant and has a free-radical scavenging effect, when accumulates to a high level in blood it can cause health problems (Mielcarz et al., 2006). The BJF intake provided a decline in Cd adverse effect on protein metabolism in chickens. The BJF administered alone did not significantly influence the protein catabolic processes in chickens.

Suppressive Cd action on chicken immune response was manifested by a significant decrease of cell-mediated parameters in the group 2 (Table 5). Compared with the control values the count of T-lymphocytes decreased by 58.3%, and B (C3)-lymphocyte number – by 33.3%.

The influence of Cd on humoral immunity also was unfavourable. The data of serum lysozyme and CIC illustrated the response of nonspecific immunity on Cd exposure and BJF intake in chickens. Lysozyme concentration in Cd-treated chickens was significantly lower than in control group. The level of nonspecific antigen-antibody circulating complexes in blood serum of Cd-exposed chickens increased by 19.8%

compared to the control. The results of immunological studies showed that intake of BJF eliminated a Cd immunosuppressive impact in chickens, but it was not notably effective when administered alone.

Table 5. Cd-induced immunological changes and the effect of red beetroot juice fraction (BJF) administration in chickens

Group	Blood		Blood serum	
	T-lymphocytes, %	B(C3)-lymphocytes, %	Lysozyme, $\mu g\ mL^{-1}$	Circulating immune complexes (CIC), extinction x 100
1. Control	$36.0 \pm 3.0^{a*}$	15.0 ± 1.8^a	8.0 ± 1.23^a	3.1 ± 0.11^a
2. + Cd	15.0 ± 4.0^b	10.0 ± 2.1^b	4.7 ± 0.88^b	3.7 ± 0.14^c
3. + BJF	34.0 ± 2.0^a	16.0 ± 3.0^a	7.8 ± 1.08^a	3.2 ± 0.15^{ab}
4. + Cd+ BJF	31.0 ± 3.2^a	12.0 ± 2.0^b	5.0 ± 0.76^b	3.3 ± 0.12^b

* Statistically different within column according to Post-hoc Tukey's test ($P < 0.05$).

The results of biochemical and immunological studies are in accordance with chicken growth parameters. Chickens exposed to Cd showed no evidence of clinical toxicity. After 10 days of Cd treatment, chickens body weight did not differ from the control data. Chicken body weight of the control and Cd-treated groups was 475.4 ± 24.5 g and 474.4 ± 60.2 g, correspondingly. BJF administration had a positive influence on the chicken body weight both of the 3rd (485.3 ± 51.2 g) and the 4th Cd-exposed (484.1 ± 39.9 g) groups.

The observed suppressive action of Cd supplementation in chicken diet during 10 days was expressed by an increase of Cd accumulation in kidneys, liver and blood plasma; development of oxidative stress in organs; disturbance of protein metabolic processes; decrease of cell-mediated and humoral immune response.

Administration of 1 mL BJF *per os* daily alone had no significant effect on the investigated parameters, but prevented the Cd-induced damage in chickens. Experimental betalain rich fraction from red beetroot juice was obtained using ultrafiltration. Antioxidative capacities of BJF provide pigments of betalain group, including betanin. Betanin is both a free radical scavenger and an inducer of antioxidant defense mechanism in cells (Esatbeyoglu et al., 2014). The similar protective effect of plant extract (*Fragaria ananassa*) against Cd adverse impact also was observed in rats, because of its polyphenolic composition, described by Elkhadragy et al. (2018).

CONCLUSION

Administration of red beetroot (*Beta vulgaris*) juice fraction to Cd-treated chickens during 10 days protected against Cd-induced oxidative damage and provided immunomodulating effect.

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