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CONCENTRATION-DEPENDENT ANTIOXIDANT/PRO-OXIDANT ACTIVITY OF ASCORBIC ACID IN CHICKENS

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The effects of ascorbic acid supplementation on biomarkers of oxidative stress, cadmium accumulation in organs, immune system activity and kidney function in chickens were investigated. The treatment groups of chickens were fed either plain diet or diet supplemented with ascorbic acid at 100, 500, 1000 and 2000 mg/kg for four weeks. Liver and kidney tissues were assayed for cadmium concentration, and the hepatic levels of ascorbic acid and dehydroascorbic acid (DHAA; the oxidised form), malondialdehyde, glutathione, activity of glutathione peroxidase, blood serum uric acid, creatinine, lysozyme and circulating immune complexes were measured. Supplementation with a high dose of ascorbic acid (1000 and 2000 mg/kg in the diet) caused an imbalance between pro-oxidative and antioxidative activities, and induced a suppressive effect on innate immunity. The results suggest that oxidative stress compromises renal function. We observed that ascorbic acid increased cadmium accumulation in a dose-dependent manner.

Key words: *ascorbic acid, oxidative stress, cadmium, chicken.*

INTRODUCTION

Ascorbic acid (AA) has antioxidant effects and can protect tissue against damage caused by strong oxidants that contain reactive oxygen species. AA forms a redox system and is also connected with the glutathione (GSH) system (Winkler *et al*., 1994; Kohen and Nyska, 2002). Oxidative stress decreases AA concentration in tissues, and therefore, supplementation of AA is required in diet. However, AA may alter the pro-oxidant-antioxidant balance in poultry, depending on its dose and the concentrations of redox-active transition metal ions (iron, cadmium, copper, lead, mercury and chromium) in tissue (Poljsak *et al*., 2005). Increased oxidative stress has been implicated in heavy metal overload conditions. For example, cadmium induces a significant increase in reactive oxygen species (Gobe and Crane, 2010).

Cadmium is a toxic metal and targets the liver and kidneys following acute and chronic intoxication, which may induce cell death via apoptosis. Dietary supplementation with antioxidants is an important consideration for limiting renal oxidative stress and progression of chronic kidney disease (Brown, 2008). After necrotic cell death, a burst of uric acid is released from the cells, which induces acute inflammatory responses (Kono *et al*., 2010).

Inflammation is triggered by circulating immune complexes (CIC) that enter the tissue (Clynes *et al*., 1999). Uric acid is normally excreted from the body via the kidneys (80%) and intestines (20%). Heavy metal poisoning increases the concentration of uric acid in human serum, whereas therapeutic dosages of AA facilitate the excretion of uric acid (Mitch *et al*., 1981). The concentration of serum creatinine can also be used to estimate renal dysfunction (Eisner *et al*., 2010).

Only scanty and contradictory information is available concerning the oxidative stress response of metabolic organs to a range of doses of ascorbic acid (Duarte and Lunec, 2005; Poljsak *et al*., 2005).

The aim of the present study was to determine the effect of dietary supplementation of ascorbic acid, using a wide range of doses, on the content of a heavy metal, such as cadmium, in the liver and kidney, and on the oxidative status, innate immunity and kidney function markers in chicks.

MATERIALS AND METHODS

Animals. New-hatched Lohmann brown cockerels were obtained from the Latvian poultry company BALTICOVO.

Experimental design. All of the experimental procedures were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia, authorisation reference number 13, from 22 December 2008). The chickens were housed in cage units with free access to food and water. The experiment was conducted using one- to thirty-day-old chickens.

The animals were divided into five groups of 25 animals each. Control group chickens (Control) received a basal wheat-barley diet containing all of the necessary nutrients without any ascorbic acid supplementation. The chickens of the other four groups were provided with the same basal diet that was supplemented with one of the following doses of ascorbic acid, AA (Sigma-Aldrich, St. Louis, Missouri, USA): 100 mg/kg (100 AA, second group), 500 mg/kg (500 AA, third group), 1000 mg/kg (1000 AA, fourth group), and 2000 mg/kg (2000 AA, fifth group).

Physiological and biochemical study. At the end of the experiment, the cockerels were sacrificed by decapitation in accordance with the recommendation for the euthanasia of experimental animals of the European Convention (Close *et al*., 1997). Peripheral blood samples, liver and kidney were collected for analysis.

Lipid peroxidation in chicken tissues was determined by thiobarbituric reaction. The results were expressed as μmol malondialdehyde (MDA) per gram of fresh tissue (Surai *et al*., 1996). The total vitamin C, and dehydroascorbic acid (DHAA) concentrations in chick liver were estimated by the 2.4-dinitrophenylhydrazine colorimetric method; the reduced ascorbic acid (AA) content was calculated by subtraction (Surai *et al*., 1999). The glutathione level in the liver was measured spectrophotometrically using 5.5-ditiobis-para-nitro-benzoic acid (Beutler *et al*., 1963). The activity of glutathione peroxidase (GSH-Px, EC 1.11.1.9) in liver was assayed by the coupled enzyme procedure using hydrogen peroxide as the substrate (Pinto and Bartley, 1969). The concentration of cadmium in chicken liver and kidney were determined using atomic absorption spectrophotometry (Anonymous, 2000). Uric acid concentration in blood serum was estimated by the carbonate method using colorimetric procedure (Eichhorn *et al*., 1961). The creatinine content in blood serum was measured using the colorimetric method with alkaline picrate, based on the Jaff reaction (commercial biochemical kit, Divi-Dent). The indices of non-specific humoral immune responses were examined in chickens immunized intraperitoneally with 0.1 ml 10% sheep red blood cells in PBS seven days before the end of experiment. Serum lysozyme level was estimated using a mo dified bacteriolytic method (Грант и др., 1973) based on absorptiometric determination of the decrease in turbidity of a suspension of *Micrococcus lysodeikticus*, as described by Shugar (1952). Nonspecific circulating immune complexes in serum were estimated spectrophotometrically after precipitation with polyethyleneglycol 6000 (Riha, 1979).

Statistical analysis. The data were expressed as the means ± SD. The differences between the control and four experimental groups were analysed using analysis of variance (ANOVA) followed by the Dunnett's test and Tukey's post hoc test. A P value of 0.05 was considered statistically significant.

RESULTS

The level of reduced AA and DHAA in chick liver (Fig. 1) depends on the AA dose in diet. The reduced AA content relative to the DHAA content decreased more than two times in chickens receiving high doses of AA (1000 and 2000 mg/kg of diet). Tables 1 and 2 show that the ascorbic acid concentration in the diet is correlated with the accumulation of cadmium in liver and kidney. The concentration of cadmium in the liver increased when ascorbic acid was supplemented in the diet at doses of 500 ($P < 0.0002$), 1000 ($P < 0.0001$) and 2000 mg/kg ($P < 0.0001$). The content of this heavy metal in kidney significantly increased when ascorbic acid was added to the diet at a dose of 2000 mg/kg ($P < 0.02$). Moreover, the ascorbic acid at doses of 500 mg/kg, and particularly at 1000 and 2000 mg/kg, significantly decreased the level of GSH. However, ascorbic acid decreased the activity of GSH-Px at doses of 1000 and 2000

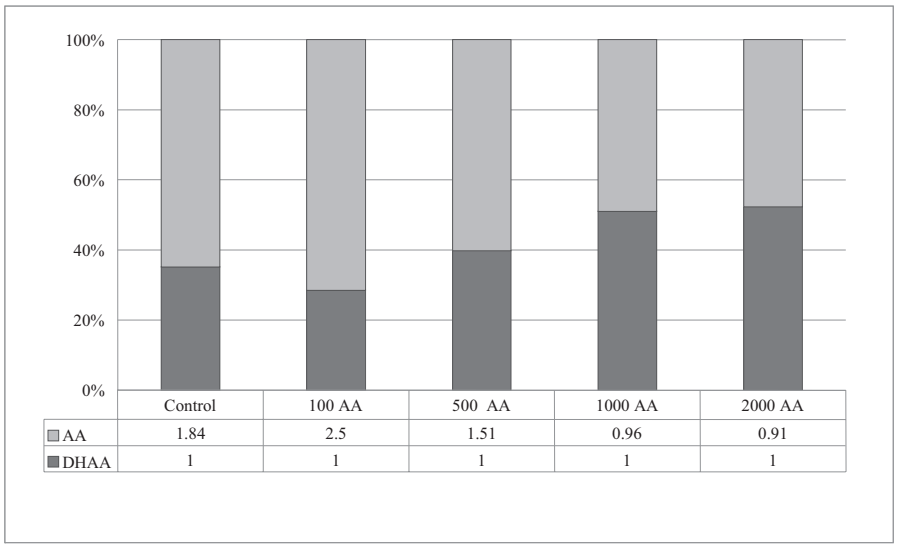

Fig. 1. Proportion of dehydroascorbic acid and reduced ascorbic acid to total vitamin C in liver of chickens receiving diet with different ascorbic acid levels from hatching to 30 days of age

Table 1

HEPATIC CADMIUM CONCENTRATION, GLUTATHIONE LEVEL, GLUTATHIONE PEROXIDASE ACTIVITY AND MALONDIALDEHYDE FORMATION IN CHICKENS THAT RECEIVED DIETS SUPPLEMENTED WITH ASCORBIC ACID (mean ± SD)

Group	Cd, μg/g	GSH, μmol/g	GSH-Px, μmol·GSH/min/g	MDA, μmol/g
1. Control	0.22 ± 0.02	4.83 ± 0.27	4.46 ± 0.46	29.56 ± 1.68
2. 100 AA	0.26 ± 0.04	4.92 ± 0.36	4.49 ± 0.54	32.05 ± 1.42
3. 500 AA	0.38 ± 0.06*	4.17 ± 0.18***	4.37 ± 0.27	31.51 ± 3.45
4. 1000 AA	0.40 ± 0.05**	3.91 ± 0.23**	3.30 ± 0.15**	34.68 ± 1.47*
5. 2000 AA	0.46 ± 0.03**	3.75 ± 0.31 **	3.20 ± 0.11**	42.17 ± 3.73**

Cd, cadmium; AA, ascorbic acid; GSH, glutathione; GSH-Px, glutathione peroxidase and MDA, malondialdehyde. Statistically significant differences from the control group: *P = 0.0002; **P < 0.0001 and ***P = 0.0006.

Table 2

CONCENTRATION OF CADMIUM AND PARAMETERS OF RENAL FUNCTION IN CHICKENS THAT RECEIVED DIET SUPPLEMENTED WITH ASCORBIC ACID (MEAN ± SD)

Group	Cd, μg/g wet wt of kidney	Uric acid, μmol/l of blood serum	Creatinine, μmol/l of blood serum
1. Control 1	0.20 ± 0.00	131.59 ± 18.33	67.40 ± 2.31
2. 100 AA	0.20 ± 0.00	116.59 ± 5.82	57.12 ± 0.29***
3. 500 AA	0.26 ± 0.02	117.42 ± 12.49	60.73 ± 0.25***
4. 1000 AA	0.30 ± 0.04	167.38 ± 27.48*	119.51 ± 6.54***
5. 2000 AA	0.32* ± 0.03	175.71 ± 14.15**	126.59 ± 3.31***

Statistically significant differences from the control group: *P = 0.02; **P = 0.0008 and ***P < 0.0001.

Table 3

PARAMETERS OF INNATE HUMORAL IMMUNITY IN CHICKENS THAT RECEIVED DIET SUPPLEMENTED WITH ASCORBIC ACID (mean ± SD)

Group	Lysozyme activity in blood serum, μg/ml	Circulating immune complexes (CIC) in blood serum, extinction units ± 100
1. Control	10.75 ± 0.20	4.13 ± 0.44
2. 100 AA	6.36 ± 0.54*	4.20 ± 0.89
3. 500 AA	3.12 ± 0.19*	2.43 ± 0.35*
4. 1000 AA	2.88 ± 0.10*	3.07 ± 0.71**
5. 2000 AA	1.85 ± 0.10*	1.47 ± 0.19*

Statistically significant differences from the control 1 group: *P < 0.0001 and **P = 0.01.

mg/kg. Increased MDA formation in the liver was detected with ascorbic acid doses of 1000 and 2000 mg/kg.

Animals that were fed a diet that was supplemented with ascorbic acid at doses of 1000 and 2000 mg/kg had a higher concentration of uric acid in blood serum (Table 2). In addition, chickens that received ascorbic acid at doses 1000 and 2000 mg/kg displayed increased serum levels of creatinine compared to the control group (Table 2).

Parameters of innate humoral immunity in the treated chickens are shown in Table 2. Lysozyme activity in blood serum of chickens that were fed diets enriched with ascorbic acid at all doses was significantly decreased compared to that in the control group. The serum CIC level was decreased in chickens that were fed diets containing ascorbic acid at doses of 500, 1000 and 2000 mg/kg compared to that in the control group. Dose-dependent effects on lysozyme activity and CIC level were observed.

DISCUSSION

The focus of the present study was to provide a better understanding of the effects of dietary ascorbic acid supplementation to chicken. Chicken as an experimental model is especially useful in biological studies due to its high intensity of some metabolic processes (Klandorf *et al*., 1999). Birds differ from primates and guinea pigs by their capacity to synthesise ascorbic acid endogenously. However, a dietary supplementation of ascorbic acid can be beneficial for poultry to improve performance and egg production.

Ascorbic acid can act as an antioxidant, contributing to the total antioxidant capacity of an animal's antioxidant defence system. Intake of ascorbic acid in a low dose (100 mg/kg diet) in our experiment indicated some increase of antioxidant activity in chicken organs. Our study demonstrated that hepatic GSH levels and GSH-Px activity were decreased in chickens that received 500, 1000 and 2000 mg/kg of ascorbic acid compared to those in the control group. The concurrent significant increase of MDA levels in chickens in the 1000 and 2000 mg/kg AA groups indicated an imbalance between anti-oxidative and pro-oxidative responses in liver. Elevated levels of MDA, which is an end product of polyunsaturated fatty acid oxidation, suggested that ascorbic acid at a dose of 1000 and 2000 mg/kg in the diet exhibited pro-oxidative effects.

The fraction of the dehydroascorbic acid of total vitamin C might provide useful information about the level of oxidative stress. Consequently, the ratio of DHAA to total ascorbate could be a marker of oxidative stress (Lykkesfeldt *et al*., 1997). The reduction of DHAA to AA occurs intracellularly and depends on both ascorbic acid and the dominant intracellular reductant glutathione. In the present study, we found AA overload to be a predictor of high liver concentrations of the oxidised form of ascorbic acid, DHAA.

We demonstrated that ascorbic acid stimulated cadmium accumulation in organs in a dose-dependent manner. This may be due to stimulation of the intestinal absorption of cadmium by ascorbic acid. The uptake of cadmium into enterocytes is mediated by the divalent metal transporter 1 (DMT1), which is a proton-coupled transporter (Park *et al*., 2002). The capacity of DMT1 transport is optimal at pH 5.5 (Gunshin *et al*., 1997). Therefore, the absorption of cadmium was possibly increased by ascorbic acid via a decreased luminal pH.

This study indicated a close relationship between serum lysozyme and CIC levels, which are inflammatory markers, in chickens that were fed diets that were supplemented with 500, 1000 and 2000 mg/kg AA. The bulk of CIC most likely contains antibodies against autoantigenic proteins, microbes and nuclear antigens, whereas lysozymes in serum predominately originate from macrophages (Torsteinsdottir *et al*., 1999; Wiik, 2003). Proteins that are modified by reactive oxygen species have been shown to elicit antibodies in a variety of diseases (Kurien and Scofield, 2008). The significant reduction of serum lysozyme and CIC levels in chickens that received 2000 mg/kg AA in the diet suggested that immune system function was suppressed, which may be associated with oxidative damage of macrophages and plasma cells.

Our study demonstrated that a high dose of ascorbic acid intake caused elevated levels of serum uric acid and creatinine. The antioxidant activity of ascorbic acid at low doses may improve kidney function by protecting renal tubular uric acid and creatinine secretory transporters from oxidative injury. Excessive ascorbic acid intake likely had an adverse effect on renal function, leading to reduced tubular excretion through the dysregulation of renal tubular uric acid and creatinine secretory transporters via ROS.

Uric acid is a powerful antioxidant (Waring, 2002). The observed high serum uric acid levels in chickens in the 1000 and 2000 mg/kg AA groups suggests that these chickens cannot manage ROS effectively and that transient impairment of renal functions may occur.

In summary, the results of our study demonstrated that high doses of ascorbic acid in the diet exhibited a pro-oxidative effect. The "threshold" concentration of ascorbic acid for this effect was 1000 mg/kg in the chicken diet. Furthermore, consistent exposure to high doses of dietary ascorbic acid (1000 and 2000 mg/kg) induces loss of normal antioxidant reserves and immunosuppression, rendering the animals more susceptible to renal disturbances. In addition, a stimulatory effect of ascorbic acid on cadmium accumulation in organs and pro/anti-inflammatory activities was demonstrated.

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ASKORBĪNSKĀBES ANTIOKSIDATĪVĀ/PROOKSIDATĪVĀ AKTIVITĀTE ATKARĪBĀ NO KONCENTRĀCIJAS CĀĻU ORGANISMĀ

C vitamīns piedalās daudzos vielmaiņas procesos. Tas stiprina organisma imūnsistēmu, piedalās bioloģiskās oksidēšanās un reducēšanās procesos. Askorbīnskābe un dehidroaskorbīnskābe veido redoks sistēmu un ir saistītas ar glutationa sistēmu. Dažādu stresu ietekmē askorbīnskābes koncentrācija audos samazinās. Tādēļ barību vajag bagātināt ar šo vitamīnu. Darba mērķis bija pētīt dažādus oksidatīvā stresa biomarķierus cāļu organismā, kadmija akumulāciju orgānos, imūnsistēmas aktivitāti un nieru funkcijas izmaiņas askorbīnskābes ietekmē atkarībā no tās koncentrācijas barībā. Eksperimentos izmantojām vienu dienu vecus Lohmann Brown gailīšus, kurus sadalījām piecās analogās grupās. Cāļi saņēma kombinēto barību bez vai ar askorbīnskābes piedevām (0, 100, 500, 1000 un 2000 mg/kg) četru nedēļu laikā. Konstatējām, ka askorbīnskābe nelielā koncentrācijā (100 mg/kg barības) cāļu organismā darbojas kā antioksidants. Pētījumā pierādīts, ka, palielinot šī vitamīna daudzumu barībā (1000 un 2000 mg/kg), dzīvnieku organismā mainās antioksidatīvo – prooksidativo procesu līdzsvars, un tā rezultātā attīstās oksidatīvais stress: aknās palielinās C vitamīna oksidētās formas — dehidroaskorbinskābes daudzums, samazinās glutationa līmenis un glutationperoksidāzes aktivitāte, uzkrājas nepiesātināto taukskābju oksidēšanas gala produkts malondialdehīds, inducējās nespecifiskās imunitātes supresija, traucētas nieru funkcijas. Novērojām, ka askorbīnskābe, atkarībā no koncentrācijas, veicina smago metālu, t.sk. kadmija akumulāciju cāļu aknās un nierēs.