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Disturbance of antioxidant protection and natural resistance factors in rats with different availabilities of trivalent chromium

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Abstract: The aim of this study was to examine the influence of trivalent chromium ions (Cr^{3+}) supplementation on antioxidant status and immune defense of rats. During the 30 days of the experiment, the male rats (n = 10 per group) were supplemented with $CrCl_3$, dissolved in distilled drinking water in doses of 0, 70, or 140 μg Cr^{3+}/dm^3. Next, the contents of hydroperoxides of lipids (HPL) and thiobarbituric acid reactive substances (TBARS) in plasma were determined, as well as the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and reduced glutathione (GSH) in erythrocytes. Lysozymic and bactericidal activities of blood serum were also determined. Results of the experiment indicated that 140 μg Cr^{3+}/dm^3 increased HPL concentration by 22.1%, while the dose of 70 μg Cr^{3+}/dm^3 decreased concentration of TBARS by 20.3% ($P \leq 0.05$) in comparison with the control. Activity of SOD in rats supplied with both doses of Cr^{3+} significantly decreased, whereas activity of CAT, GPx, and GR significantly increased, similar to lysozymic and bactericidal activity. In general, the obtained results showed that a diet with chromium supplementation can strengthen the main mechanisms of lipid peroxidation defense by stimulation of enzyme activity of the antioxidant system and natural resistance factors.

Key words: Antioxidant enzymes, bactericide, lipid peroxidation defense, lysozymic, rats

1. Introduction

Chromium (Cr) is a metallic element that exists in many chemical valence states, but the most stable forms occurring in the environment are the 0 valence state (metal and alloys), trivalent chromium [Cr(III)], and hexavalent chromium [Cr(VI)]. Among them, the trivalent form of Cr is most abundant in nature, forming stable complexes with both organic and inorganic ligands. Chromium(III) can be found in drinking water as well as in most fresh foods, including bread, meat, and vegetables (1–3); however, the bioeffectiveness of it is still being discussed. On one hand, Cr(III) is widely used in tanning industrial processes and therefore many epidemiological studies point to frequent health problems such as cancer, dermatitis, asthma, chronic bronchitis, hypertension, chromosomal abrasion, back pains, metabolic syndrome, hemoglobin changes, and DNA detriment in lymphocytes in populations chronically exposed to Cr(III) (2,4,5). On the other hand, Cr(III) is considered to be relatively nontoxic and, moreover, it is widely accepted as an essential microelement in animal and human nutrition (1,3,5–7). Simultaneously, it seems that its supplementation in basal diets affects the other elements, with a synergistic effect on zinc levels but an antagonist effect on copper levels (8). The World Health Organization (7) and the European Food Safety Authority (1) estimated that the human daily intake of Cr(III) amounts to about 4.1 μg kg body $weight^{-1}$ day^{-1}; however, there is a large margin of safety of 4–5 orders of magnitude between the daily intake and "the No-Observed-Adverse-Effect Level". Furthermore, Cr(III) is very poorly absorbed via the gastrointestinal tract (0.4% to 2.8%) in both rats and humans (1). Therefore, many authors pay attention to the potential danger of chromium deficiency, which can cause intolerance and inability to utilize glucose for energy (diabetic and diabetic-like states), cardiovascular disease (increased aortic plaque formation and disturbance of hematological parameters), elevated blood lipids and high free fatty acid levels, low respiratory quotient, abnormalities of nitrogen metabolism, impaired growth, and decreased fertility and longevity in both humans and animals (5,6). These effects can stem from the fact that Cr^{3+} was proven to have dual properties as an antioxidant and prooxidant in the organism (4,9,10). However, the prooxidant effect of Cr(III), which involves the reduction of Cr(III) into Cr(II) (5,11,12) and causes lipid peroxidation (10,13,14), is known, as well. In response to this process, antioxidant

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defense mechanisms using superoxide dismutase (SOD) and reduced glutathione (GSH) (15,16) are activated.

Trivalent chromium ions (Cr^{3+}) are also regulators of the immune system and play an important role both in immune-stimulatory and immunosuppressive processes. They influence T and B lymphocytes, macrophages, production of cytokines, and immune response in humans and animals (17,18).

Because trivalent chromium bioactivity is still fairly unknown and little discussed, this study was designed to examine the influence of trivalent chromium ion supplementation (Cr^{3+}) on antioxidant status and immune defense of the blood plasma of rats.

2. Materials and methods

2.1. Experimental design

Thirty male, 6-week-old, white laboratory Wistar rats were used in the experiment, conducted in compliance with agreement No. 21 of the Bioethical Commission of the Institute of Animal Biology NAAS (06.01.2010). During a 2-week acclimation period the animals were housed in individual cages at an animal facility in rooms maintained at 21 ± 1.5 °C and 65% humidity with a 12-h photoperiod, and they were fed a diet (Harlan Teklad LM-485 rodent diet) that consisted of 64% saccharose, 20% casein, 5% maize oil, and recommended doses of vitamins and microelements (Table 1). The rats were allowed to eat and drink distilled water ad libitum. During this phase of study, daily food and water intakes were established at 15 g diet/day and 20 mL/day, respectively. The Cr content in fodder was measured by means of atomic absorption spectrophotometer and established as 128 ± 26.5 μg/kg (i.e. 14.8 ± 3.06 μg/kg body weight).

After the acclimation period, experimental animals were weighed, and because there were no significant differences in average body weights (mean: 130 ± 1.9 g), the animals were divided into 3 groups (n = 10). During 30 days of experiment, rats in individual groups were supplemented with Cr(III) in the form of chromium chloride ($CrCl_3$, Sigma Aldrich) dissolved in distilled drinking water in doses of 0, 70, or 140 μg Cr^{3+}/dm^3, i.e. 0, 11, and 22 μg Cr^{3+}/day at daily water consumption of 20 ± 4.0 mL per rat.

After a 30-day experimental period, animals were ether anesthetized, blood samples were taken and centrifuged for 15 min at 3000 rpm, and plasma and erythrocytes samples were stored frozen at –20 °C following analyses. The contents of hydroperoxides of lipids (HPLs) and thiobarbituric acid reactive substances (TBARS) in plasma; SOD, catalase (CAT), glutathione peroxidase (GPx), and GSH contents in erythrocytes; and lysozymic and bactericidal activities of blood serum were determined.

Table 1. The doses of vitamins and microelements in diets of rats during the experiment.

Vitamin	Dose (mg/kg diet)	Mineral	Dose (mg/kg diet)
Alpha-tocopherol	110	$CaHPO_4$	30,000
Choline chloride	1650	NaCl	4000
D-Calcium pantothenate	66	$MgCl_2$	5000
Inositol	110	$ZnCO_3$	45
Menadione	49.5	$MnCO_3$	90
Niacin	99	KIO_3	1.75
PABA	110	$CuCO_3Cu(OH)_2.H_2O$	12.5
Pyridoxine HCl	22	Na_2SeO_3	0.22
Riboflavin	22	$FeCl_2.4H_2O$	71.175
Thiamin HCl	22		
Vitamin A acetate	19,800 units		
Calciferol (D_2)	2200 units		
Biotin	0.44		
Folic acid	1.98		
Vitamin B_{12}	0.33		

2.2. Processing

2.2.1. Concentration of HPLs

Determination of HPLs in biological material (plasma samples) was achieved by precipitating proteins with a solution of trichloroacetic acid and lipid extraction by ethanol followed by interaction of the studied extracts with ammonium thiocyanate (19). HPLs were determined in the following way: 1.5 mL of ethanol extract was completed by ethanol to 2.7 mL and shaken, and 0.02 mL of concentrated HCl and 0.03 mL of 1% salt Mora solution in 3% HCl solution were added to it. The mixture was shaken, and after 30 s, 0.2 mL of 20% solution of ammonium thiocyanate was added; afterwards, a raspberry color appeared. Optical density measurements were carried out within 10 min after the addition of ammonium thiocyanate by means of a spectrophotometer at the wavelength of 480 nm. The control sample was prepared like a research sample, but instead of blood plasma, 0.2 mL of bidistilled water was taken. The content of HPLs in biological material was expressed in terms of optical density at 480 nm in 1 mL of plasma (plasma ΔD_{480}/mL).

2.2.2. Concentration of TBARS in plasma

The concentration of TBARS, an indicator of lipid peroxidation in plasma and tissues, was measured by a reaction with thiobarbituric acid (20). The quantifying of reacted compounds with thiobarbituric acid was determined by comparing the absorption of light rays with a standard curve of TBARS equivalents formed by hydrolysis of acid 1,1,3,3-tetramethoxypropane.

2.2.3. Activity of SOD in erythrocytes

Determination of SOD activity was carried out by means of the method described by Beauchamp et al. (21), which is based on binding of radicals present in the sample of superoxide, generated by enzymatic reactions of xanthine and xanthine oxidase via SOD, which is present in the sample, by nitroblue tetrazolium. Finally, 600 µL of SOD reaction mixture containing 0.1 mM xanthine, 0.1 mM EDTA, 50 mg of bovine serum albumin, and 25 mmol/L nitroblue tetrazolium was added to 125 µL of supernatant or 125 µ: of standard solution of SOD, and subsequently 25 µL of 9.9 nM xanthine oxidase was added to each tube at 30-s intervals. Each sample was incubated for 20 min at 25 °C, and then the reaction was stopped by adding 0.5 mL of 0.8 mM solution of $CuCl_2$ in 30-s intervals. The amount of created formazan was determined spectrophotometrically at the wavelength of 560 nm.

2.2.4. Protein concentration

Protein concentration was determined by the Lowry method.

2.2.5. Activity of CAT in erythrocytes

The activity of CAT was determined by the Aebi method (22), where the principle of analysis was based on the determination of constants of the decomposition rate of hydrogen peroxide by CAT. The methodology of determining the activity of CAT is as follows: 1 mL of 40 mM H_2O_2 was added to 2 mL of the sample in a phosphate buffer (50 mM, pH 7.0, prepared by mixing 0.681 g of KH_2PO_4 in 100 mL of distilled water and 1.335 g of $Na_2HPO_4 \times 2H_2O$ in 150 mL of distilled water). Concentration reduction of H_2O_2 was determined spectrophotometrically at the wavelength of 240 nm for 3 min.

2.2.6. Activity of GPx in erythrocytes

The activity of GPx was determined at the wavelength of 340 nm by recording of NADPH concentration decrease. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM NADPH, 1 unit/mL GPx, 1 mM GSH, and 0.25 mM H_2O_2. The source of the enzyme (0.1 mL of hemolyzed erythrocytes) was added to 0.8 mL of this mixture, and the mixture was incubated at 25 °C 5 min before the reaction start, which was caused by the addition of 0.1 mL of hydrogen peroxide. Absorption of light rays at the wavelength of 340 nm was recorded for 5 min. Activity was calculated in µM GSH for 1 min per 1 g of protein.

2.2.7. Activity of glutathione reductase in erythrocytes

Glutathione reductase (GR) activity was determined spectrophotometrically by the recovery speed of glutathione in the presence of NADPH. The activity of GR was determined in a reaction environment that contained 2 mL of phosphate buffer, 0.2 mL of EDTA, 0.5 mL of oxidized glutathione, 0.2 mL of hemolysate, and 0.1 mL of NADPH. Activity of the enzyme was determined by the reduction of NADPH at 37 °C during 10 min with a spectrophotometer at the wavelength of 340 nm. GR activity was expressed in µM NADPH for 1 min per 1 g of protein (23).

2.2.8. Concentration of GSH in erythrocytes

The concentration of GSH was measured by using dithionitrobenzoate acid (DTNBA) and Ellman reagent. The method was based on the formation of colored products as a result of the 5.5-dithiobis-2-nitrobenzoate acid reagent reaction and free sulfhydryl groups of erythrocytes that absorb light rays at 412 nm.

One milliliter of sample was deprived of protein by adding a solution containing 1.67 g of metaphosphoric acid, 0.2 g of Na_2EDTA, and 30 g of NaCl in distilled water. Next, 2.4 mL of Na_2HPO_4 and 0.3 mL of DTNBA were added to each sample, and the supernatant was purified by centrifugation (10 min, 3000 rpm). The formed 5-thio-2-nitrobenzoate acid was proportional to the concentration of GSH, determined spectrophotometrically at the wavelength of 412 nm and at 25 °C, in comparison with the control samples.

2.2.9. Determination of lysozymic activity of serum by nephelometric method

Lysozymic activity was determined by the change in optical density of microbial frozen *Micrococcus lysodeikticus* under the influence of lysozyme test solution. First, 0.03 mL of tested serum was added to 1.47 mL of prepared *Mc. lysodeikticus* microbial culture flushing and the mixture was incubated in a thermostat at 37 °C for 1 h. Nephelometry was conducted at the wavelength of 540 nm. Percentage of lysozyme activity was determined by numerical parameters. To this end, percentage of light penetration of initial microbial frozen (20%) was calculated from the rate of studied frozen light penetration (24).

2.2.10. Bactericidal activity of serum

Bacterial activity of serum is an integral indicator of the natural humoral resistance, which indicates the ability of blood to refine itself. Bactericidal activity of blood in relation to microorganisms is associated with the presence in serum of nonspecific protective components (normal antibodies, lysozymes, complements, properdin, interferon, bacterial lysines) and other factors. Flushes from daily cultures of *Escherichia coli* were prepared with saline, the density of which was determined by photoelectrocolorimeter. One milliliter of investigated serum was added to 4.5 mL of sterile meat peptone broth, and in the control sample 1 mL of saline was used. One drop of *E. coli* culture was then added to both samples. To determine optical density, 2 mL from each sample was taken. The remaining mixtures were incubated at 37 °C for 3 h. Calculation of the results was done by finding the difference between the final and initial optical densities (25).

2.3. Statistical analysis

Statistical analysis was performed on statistically significant differences between experimental groups by dispersive analysis (one-way ANOVA) and Duncan test using SPSS 10.1. The value of the results was ascertained at $P \leq 0.05$. Figures were prepared using Grapher 7.0 (Golden Software Inc., USA).

3. Results

Results of the experiment indicated the influence of Cr(III) availability on lipid peroxidation. Findings revealed that a dose of 140 μg Cr^{3+}/dm^3 increased HPL concentration by 22.1%, while a dose of 70 μg Cr^{3+}/dm^3 decreased by 20.3% ($P \leq 0.05$) the concentration of TBARS in the blood of rats in comparison with animals with no Cr(III) supply (Table 2).

Moreover, larger availability of Cr(III) caused changes in the activity of antioxidant enzymes in the blood of rats (Figure 1). In comparison with the control group, activity of SOD in the blood of rats supplied with 70 and 140 μg Cr^{3+}/dm^3 decreased by 1.6 and 2.6 times ($P \leq 0.01$, Figure 1a), whereas activity of CAT increased by 1.27 ($P \leq 0.05$, Figure 1b) and 1.46 ($P \leq 0.01$, Figure 1b) times, GPx by 1.12 and 1.21 times ($P \leq 0.01$, Figure 1c), and GR by 1.28 ($P \leq 0.01$) and 1.14 times ($P \leq 0.05$, Figure 1d), respectively. However, supplementation of Cr(III) did not increase GSH concentration in the blood of rats significantly ($P > 0.05$, Figure 1d).

Administration of chromium to rats was also accompanied by an increase of lysozymic and bactericidal activity of blood serum (Table 3). Lysozymic activity was definitely higher in the serum of rats treated with chromium at doses of 70 and 140 μg Cr/dm^3, by 17.0% ($P \leq 0.05$) and 26.2% ($P \leq 0.01$), respectively, and bactericidal activity was respectively higher by 16.3% ($P \leq 0.05$) and 26.2% ($P \leq 0.01$) compared with animals not receiving chromium.

4. Discussion

Although the essentiality of chromium in animal and human nutrition is now well accepted, the recommended intake is still under discussion because this element is absorbed in the gastrointestinal tract at less than 3% (1). For this reason, Yoshida et al. (26) indicated the lowest adverse effect level of water Cr(III) supplementation to be 100 μg/g Cr^{3+}. However, in the discussed experiment, changes in the antioxidant system of rats were observed

Table 2. Influence of accessibility of chromium on the content of lipid peroxidation (LPO) products in blood of rats (mean ± SE, n = 10).

LPO products	Dose of chromium supplied in drink water		
	0 μg Cr/dm^3	70 μg Cr/dm^3	140 μg Cr/dm^3
HPLs (ΔD^{480}/mL)	0.73 ± 0.027^a	0.70 ± 0.002^a	0.57 ± 0.009^b
TBARS-active products (nmol/mL)	7.29 ± 0.07^a	6.13 ± 0.11^b	7.59 ± 0.11^a

Values in rows with different superscripted letters differ significantly at $P \leq 0.01$.

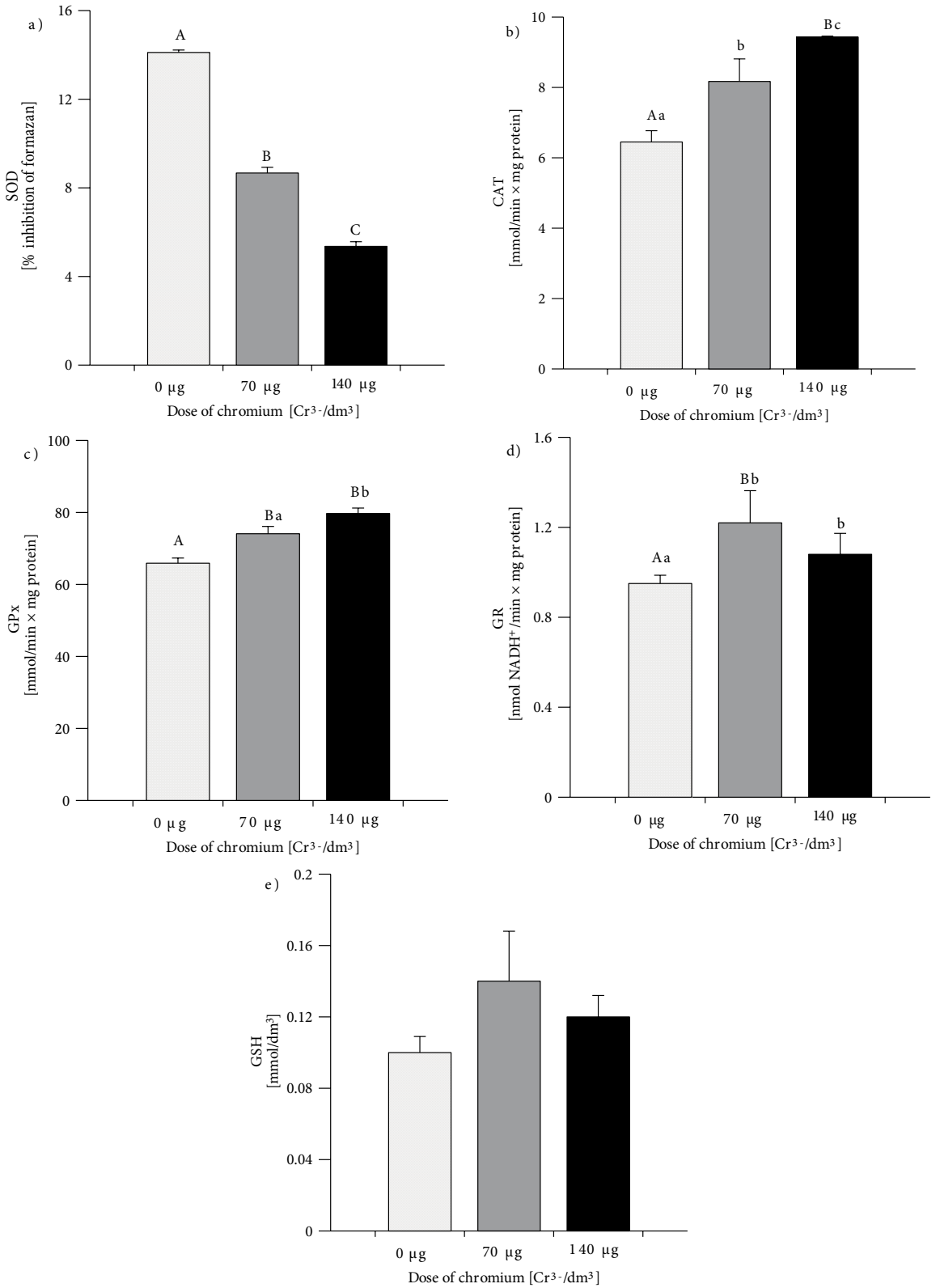

Figure 1. Activity of antioxidant enzymes: a) superoxide dismutase (SOD), b) catalase (CAT), c) glutathione peroxidase (GPx), d) glutathione reductase (GR), and e) GSH concentrations in blood of rats water supplied with Cr(III) at doses of 0, 70, and 140 µg Cr^{3+}/dm^3. A, B, C: bars with different uppercase letters differ significantly at $P \leq 0.01$. a, b, c: bars with different lowercase letters differ significantly at $P \leq 0.05$.

Table 3. Influence of accessibility of chromium on lysozymic and bactericidal activity of serum (mean ± SE, n = 10).

Activity of blood serum,%	Dose of chromium supplied in drinking water		
	0 μg Cr/dm^3	70 μg Cr/dm^3	140 μg Cr/dm^3
Lysozymic	30.21 ± 0.86^{Aa}	35.34 ± 1.80^{b}	38.12 ± 1.34^{B}
Bactericidal	41.53 ± 1.86^{Aa}	48.29 ± 2.03^{b}	52.40 ± 2.17^{B}

A, B: values in rows with different uppercase letters differ significantly at $P \leq 0.01$.
a, b: values in rows with different lowercase letters differ significantly at $P \leq 0.05$.

in the case of both 70 and 140 μg Cr^{3+} addition. First, a decrease of lipid peroxidation was observed. This is consistent with the opinion that Cr(III) may be attributed to participation in redox reactions (9). Redox reactions are crucial for normal functioning and integrity of cells and tissues in the organism (27). It is known that the reaction to stress generates reactive oxygen forms, including superoxide anion radicals (O^{2-}), hydroxyl radicals (HO^{-}), and hydrogen peroxide (H_2O_2), which cause lipid peroxidation, especially in membranes, and can cause tissue damage (13). Therefore, chromium deficiency can induce severe atherosclerosis and disrupt blood lipid levels while Cr(III) supplementation can improve lipid metabolism, decrease cholesterol content, and increase the amount of triacylglycerols, which are less exposed to lipid peroxidation due to their chemical structure (11). On the other hand, Ścibior et al. (14) did not observe any changes of lipid peroxidation caused by Cr supplementation.

This hypothesis can be supported by a decrease of SOD activity, while an increase in activities of CAT, GPx, and GR in erythrocytes of the blood of rats supplied with Cr^{3+} was observed. It is well known that SOD is one of the enzymes that interrupt the chain of oxygen-dependent free-radical reactions in cells of aerobic organisms. Therefore, enzyme activity is associated with intensity of lipid peroxidation and depends on the amount of intermediates accumulated in the tissue (28). It not only prevents the development of free radical reactions and accumulation of superoxide anions and peroxides but also supports a high activity of redox processes (15,16). By action of Cr^{3+}, SOD activity decreases, as it is related to the intensity of lipid peroxidation and depends on the amount of accumulated intermediates in the tissue (16). Simultaneously, there was an increase in the activity of CAT, GPx, and GR, which participated in the reduction of H_2O_2, a product of the reaction of SOD with active forms of oxygen (free radicals) (16). Their increasing activity may also result from regulatory effects of these enzymes on gene expression (9). At the same time, stimulation of defense is supported by increasing the GSH concentration in the blood of rats supplied with chromium (29). This is consistent with the observation of other authors who established an increase of its content in the lungs of rats with hyperlipidemia (30) or under the influence of vitamin C synthesis with L-gulonic lactone in rats' liver (8).

One of the important factors that testify to nutrition value is the state of the immune system and its components. Such indicators as bactericidal activity and lysozymic serum activity, as well as humoral factors of nonspecific resistance, show protective effect. It was found during the experiment that Cr(III) supplementation can induce higher resistance to the antigenic load, which is evidenced by an increase of lysozymic and bactericidal activity. This corresponds with the results of Şahin et al. (8) such that total serum immunoglobulin levels in rabbits treated with a higher dose of Cr(III) were growing. Moreover, a number of other reports indicated a weakening of natural resistance factors, which takes place in animals fed a chromium-deficient diet (9). It is caused by deterioration of membrane cells receptors' activation to insulin, which becomes less supplied with energy and functionally weaker.

In general, the obtained results show that a diet supplemented with chromium can strengthen the main mechanisms of lipid peroxidation defense by the stimulation of enzyme activity of the antioxidant system and natural resistance factors. This proves the indispensability of chromium supplied in animal nutrition and emphasizes its particular biological role.

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