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THE EFFECTS OF CHROMIUM MICROADDITIVE IN DIFFERENT DIETS FOR LAYING HENS (*Gallus gallus* L.) ON THE INTESTINAL DIGESTION AND CERTAIN BIOCHEMICAL BLOOD PARAMETERS

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Abstract

Cromium (Cr) is a biogenic element necessary for normal growth and development in animals and poultry. Cr regulates the synthesis of fats, carbohydrate exchange, and circulatory glucose concentration. Cr additives in the diets were reported to affect growth efficiency positively in broiler chicks (I.Z. Gubaydulina et al., 2018); supplementation of diets with Cr nanoparticles (100-200 ppb) stimulated mineral exchange in broilers. With the lack of the research related to the effects of Cr microadditives on the digestion in animals and the absence of the studies on animals with chronic intestinal fistulae, the aim of our study was to investigate the effects of Cr microadditive (100 ppb) as nanoparticles in different wheat-based diets for laying hens (with soybean cake or sunflower cake as main protein sources) on the intestinal digestion and certain biochemical blood parameters. The trial was performed on Hisex White chicken (*Gallus gallus* L.) (10-12 months of age, 5 birds per treatment, diet shifts in 7-10 day periods) with chronic duodenal fistulae. Cr (III) oxide (99.8 %) nanoparticles (d = 91 nm, specific surface area 9 m^2/g, Z-potential 93±0.52 mV) (Platina LLC, Moscow, Russia) were produced by plasmochemical synthesis. The activities of amylase, lipase and total proteases were determined in the duodenal digesta sampled in 1 hour after the feeding. The blood was sampled from the axillary vein in the morning from starved birds and centrifuged with sodium citrate to obtain serum. The activities of trypsin and alkaline phosphatase, concentrations of glucose, total protein, triglycerides, uric acid, alanine and aspartic acid transaminases in serum were determined using semiautomatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd, China). The activities of amylase and lipase in serum were determined using analyzer Chem Well 2900 (T) (Awareness Technology, USA) and reagent kits (Human GmbH, Germany). Cr microadditive was found to produce different effects within different diets. The digestibility of protein and fiber from the diets with sunflower cake tended to be higher in compare to soybean cake. When the birds were fed Cr-supplemented diets the serum amylase activity increased by 37.8-50.2 % irrespective of the diet, with simultaneous reduction in serum glucose concentration by 26.6-17.5 % evidencing the improvement in glucose assimilation. The latter is in agreement with the negative correlation between the activity of amylase in the duodenal digesta and serum glucose concentration (r = -0.72 and -0.45 for different diets, $p < 0.05$). The supplementation of soybean-based diet with Cr decreased the duodenal activity of lipase with simultaneous reduction in certain biochemical blood parameters by 22-40 %; however, these parameters remained within the physiologically normal ranges. The supplementation of sunflower-containing diet with Cr increased duodenal activities of amylase, lipase, and total proteases; it is probably related to the presence of antinutritive factors in sunflower cake. These results lead to the conclusion that the effects of Cr oxide nanoparticles depend on the composition of basic diet; this fact one should take into account in practice of poultry nutrition.

Keywords: *Gallus gallus* L., laying hens, duodenal digesta, activities of digestive enzymes, digestive enzymes in blood serum, chromium (III) oxide

Chromium refers to the biogenic elements that are found in the tissues of plants and animals and are necessary for the healthy growth and functioning of the body. Its most important biological role is to regulate fats synthesis, carbohydrate metabolism, and blood glucose. Chromium is part of the low molecular weight organic complex — glucose tolerance factor, which ensures its normal content in the blood. It acts as a regulator of the amount of sugar in the blood [1, 2], providing the normal activity of insulin. Chromium is involved in the metabolic control of cholesterol (part of trypsin) and serves as an activator of some enzymes, by participating in maintaining the normal functioning of the cardiac muscle and the functioning of blood vessels. Chromium also contributes to the excretion of toxins, salts of heavy metals, radionuclides [3].

Chromium biological activity is mainly due to the ability of Cr^{3+} ions to form complex compounds. Cr^{3+} ions are involved in stabilizing the structure of nucleic acids. Chromium affects the hemopoiesis and has the ability to activate trypsin since it is part of the crystalline trypsin in the form of a labile compound capable of splitting off chromium ions [4]. Chromium penetrates through the intestinal wall, and the rate of its absorption increases depending on the concentration, with a decrease in particle size and the presence of digestive agents (vitamins, phytates, amino acids) [5, 6].

It is known about the positive effect of chromium supplements in feeding young chickens [7, 8]. It is shown [9, 10] that the introduction of chromium nanoparticles into the diet of broiler chickens in the doses of 100-200 ppb stimulates the exchange of chemical elements. The contrary opinion [11-14] that chromium can have a negative effect on the body exists as well. Since the response to a certain amount of heavy metal is different, when chromium is introduced into the diet in the doses of 100 ppb, both stimulation and inhibition of some processes occur, which directly depend on its amount in the feed.

In the present paper on laying hens with duodenal fistula, it was shown for the first time that when a chromium microadditive is introduced into the diet, the activity of enzymes in the digestive tract of chickens which react differently to the presence of heavy metal in different feed ingredients, changes. The blood biochemical parameters (the activity of amylase, trypsin, glucose, triglycerides, total protein, uric acid, alkaline phosphatase) change as well.

The work objective was to study the effect of Cr_2O_3 additive on digestion and blood biochemistry of laying hens when the additive is introduced in microdoses (100 ppb of feed) into feed of different composition.

Techniques. The experiments were performed with Hisex White laying hens (*Gallus gallus* L.) aged 10-12 months (Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, 2018). All manipulations were carried out in accordance with the requirements of the European Convention for the protection of vertebrates used for experimental and other scientific purposes (ETS No. 123, Strasbourg, 1986; https://www.msu.ru/bioetika/doc/konv.doc). To collect the duodenum digesta, the poultry was operated by implanting a cannula in the duodenum, opposite to the confluence of the pancreatic and bile ducts. Surgical operations were performed with the use of sedatives and painkillers. The chicken was fixed in the left lateral position in a special machine.

The incision was made on the right side of the last rib on the edge of the lateral process of the keel bone at 4-5 cm. The duodenum was extracted, the place of ducts confluence in the duodenum was found and a purse suture of 0.5-0.6 cm was placed opposite to it. The incision was made inside the purse suture, a cannula was inserted and a purse suture was tightened. The area around the implanted cannula was carefully treated, additional purse suture was put, if necessary. The bowel was immersed deep into the thoracoabdominal cavity and the

surgical wound was sutured with knotted sutures, capturing all the layers. After the operation, the poultry had access to water for 16-18 hours but did not receive the feed. After 5-7 days after surgery, when the poultry health was fully restored, physiological experiments have been started.

The physiological experiment was performed by the method of group-periods (5 animal units in each group), formed based on the principle of analogues. The experiment included two periods (7-10 days each): during the control period, the poultry did not receive an additive, during the experimental period, Cr_2O_3 ultrafine preparation (LLC Platinum, Russia; produced by plasma chemical synthesis, d = 91 nm, specific surface area 9 m^2/g, Z-potential 93±0.52 mV, Cr_2O_3 content 99.8%) at a dose of 100 ppb was added to the main diet. In the first series of experiments, soy cake was included in the main diet, in the second series this was sunflower cake. To obtain significant results, at least three digestion experiments were performed on each chicken in each test period.

The feed was prepared in accordance with zootechnical norms. The poultry received 30 g per bird in the morning on an empty stomach, the rest was fed during the day. After 1 h after feeding, duodenal chyme (5 ml) was sampled, the samples were immediately centrifuged (5 min at 5000 rpm) and diluted with a cooled Ringer solution (1:10).

Amylase activity in chyme was determined by starch hydrolysis [15] using a photometer KFK-3 (Zagorsk Optical and Mechanical Plant, Russia) at λ = 670 nm and expressed in milligrams of cleaved starch per 1 ml of chyme for 1 min. Lipolytic activity was measured on a semi-automatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd., China) using a kit of reagents for lipase (OOO DIACON-VET, Russia). Protease activity was determined with casein as a substrate by Hammerstein (EMD Millipore Corp., Billerica, USA) with colorimetry (KFK-3 at λ = 450 nm) [16].

The blood was sampled from the axillary vein in the morning from starved birds and centrifuged with sodium citrate for 3 min at 5000 rpm. The activities of amylase and lipase were determined using an analyzer Chem Well 2900 (T) (Awareness Technology, USA) and reagent kits (Human GmbH, Germany). The activity of trypsin [17] was determined using a semiautomatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd., China).

Biochemical blood tests were performed on a semi-automatic flow biochemical analyzer BS-3000P (SINNOWA Medical Science & Technology Co., Ltd., China) with a reagent kit (OOO DIACON-VET, Russia).

The conditions of keeping and feeding poultry during the period of the experiments conform to the standards of ARRTPI (Guide for Optimizing Mash Recipes for Poultry. Sergiev Posad, 2014). Feeds were prepared on the basis of wheat and barley. Feed No. 1 contained 19.4% of soybean cake, feed No. 2 21.4% of sunflower cake. The amount of available energy in the feeds was the same (265 kcal/100 g). The sunflower cake feed contained 2.11% more crude fiber and 1.07% more crude fat.

JMP Trial 14.1.0 (SAS, USA) software was used for statistical processing of (https://www.jmp.com/en_nl/of-fers/free-trial.html), by which the mean value (*M*), the standard error of the mean (±SEM) and the correlation (*r*) were calculated, the significance of the differences was assessed by the Student *t*-test. The differences were considered statistically significant at $p < 0.05$.

Results. It is known that pancreatic enzymes adapt to the quality of food entering the body [18]. In our experiments, replacing soy cake with sunflower cake in the diet of laying hens, led to a change in the activity of digestive enzymes in the duodenal chyme (Table 1).

1. Activity of digestive enzymes in the duodenal digesta of Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition (M±SEM, n = 5)

Indicator	Feed No. 1		Feed No. 2	
	control	test	control	test
Amylase, mg · ml^{-1} · min^{-1}	1426±144.8	1086±70.0	666±147.6	1747±187.3*
Lipase, U/l	3231±484.0	1762±187.6*	2669±144.5	4573±644.5*
Proteases, mg · ml^{-1} · min^{-1}	66±6.0	65±3.6	65±4.3	80±8.0*

N o t e. For a description of groups and feeds, see the Techniques section.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

Amylase activity decreased by 53.3% ($p < 0.05$) when replacing soybean cake with sunflower cake in the diet. The activity of lipase and total proteases remained unchanged. Chromium microadditive, introduced into the diet on the background of soybean cake, reduced the activity of amylase by 24.0%, lipase by 45.5% ($p < 0.05$) compared to the control (feed No. 1). The addition of chromium to the feed with the sunflower cake (feed No. 2) led to an increase in the activity of amylase by 162.3% ($p < 0.05$), lipase by 71.4% ($p < 0.05$), protease by 23.1% ($p < 0.05$). Consequently, the chromium microadditive acted as a stimulant of the secretory function of the pancreas at low enzymatic activity in the gut, and inhibited lipase activity at high enzymatic activity.

Digestibility of nutrients reflected the adaptation of digestive enzymes to feed ingredients and microelements (Table 2).

2. Digestibility of nutrients in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition (M±SEM, n = 5)

Digestibility, %	Feed No. 1		Feed No. 2	
	control	test	control	test
Protein	91.5±0.25	92.1±0.46	89.9±0.33*	90.7±0.25
Fiber	33.3±2.40	31.6±2.04	20.9±2.10*	23.1±1.78
Fat	91.2±0.38	90.4±0.34	93.4±3.10	91.3±0.47

N o t e. For a description of groups and feeds, see the Techniques section.
* Differences between controls are statistically significant at $p < 0.05$.

Protein digestibility when replacing soybean cake with sunflower cake (control periods) decreased by 1.5% ($p < 0.05$). The amount of lysine excreted with poultry manure in the control period (feed No. 2) exceeded the same indicator for feed No. 1 by 25.0% ($p < 0.05$), the content of methionine in the control periods did not change significantly and amounted to 0.001±0.0002 g. The crude fiber in the feed containing soybean cake was digested by 12.4% better ($p < 0.05$) compared to sunflower cake. Consequently, against the background of higher activity of digestive enzymes, the digestibility of protein and fiber for soybean cake was higher than for sunflower cake. The introduction of chromium nanoparticles into the diet did not significantly affect the digestibility of nutrients when using different protein ingredients in the feed.

The data we obtained earlier indicate a change in the activity of digestive enzymes in blood of meat chickens when various biopreparations are used in the diet [19]. In these experiments, biochemical parameters of blood in poultry did not have significant differences when using different ingredient composition of feed. The exception was triglycerides, the amount of which was 2 times higher with feed No. 2 compared to feed No. 1 (Table 3), indicating better fat absorption and the same lipase activity in the duodenal digesta. When chromium was added together with soy protein, an increase in amylase activity in blood comprised 37.8% ($p < 0.05$), the remaining indicators tended to decrease compared to the control: for trypsin — by 23.8% ($p < 0.05$), for glucose — by 26.3% ($p < 0.05$), for alkaline phosphatase — by 26.1% ($p < 0.05$), for total protein — by 21.4%

($p < 0.05$), for triglycerides — by 22.2% ($p < 0.05$), for uric acid — by 25.7% ($p < 0.05$), for alanine aminotransferase (AlAT) — by 29.9% ($p < 0.05$), for aspartate aminotransferase (AsAT) — by 40.1% ($p < 0.05$). When using chromium microadditives with sunflower cake, the activity of amylase increased by 50.1% while reducing glucose by 17.2% ($p < 0.05$).

3. Blood biochemical parameters in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition ($M \pm SEM$, $n = 5$)

Parameter	Feed No. 1		Feed No. 2	
	control	test	control	test
Amylase, U/l	288±18.8	397±32.7*	271±25.0	407±35.2*
Lipase, U/l	58±2.8	62±7.5	61±3.0	63±0.2
Trypsin, U/l	202±43.7	154±16.8	95±37.9	135±53.6
Glucose, mmol/l	11.3±0.40	8.3±0.51*	12.0±0.33	9.9±0.42*
Alkaline phosphatase, u/l	1155±161.3	853±97.3	1293±68.8	1635±185.6
Total protein, g/l	41.9±1.91	33.0±1.12*	42.4±1.71	38.7±2.30
Triglycerides, mmol/l	1.8±0.30	1.4±0.21	3.6±0.40	4.3±0.40
Uric acid, mmol/l	245±34.7	182±19.1	233±21.1	180±16.2
Alanine aminotransferase, U/l	7.7±0.91	5.4±0.53*	7.9±0.54	7.5±0.91
Aspartate aminotransferase, U/l	197.2±8.81	118.3±16.63*	211.2±6.65	190.2±11.21

N o t e. For a description of groups and feeds, see the Techniques section.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

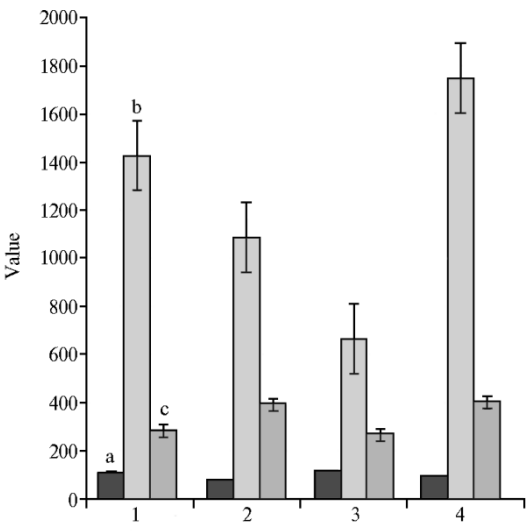

Fig. 1. Blood glucose concentration (mmol/l) (a) **and amylase activity** (U/l) (b)**, and amylase activity in duodenal digesta** ($mg \cdot ml^{-1} \cdot min^{-1}$) (b) **in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition:** 1 — control (feed No. 1), 2 — test (feed No. 1), 3 — control (feed No. 2), 4 — test (feed No. 2). For a description of groups and feeds, see the Techniques section. Indicators for glucose are increased by 10 times.

A relationship has been established between amylase activity in the duodenal chyme and the glucose content: the higher the activity of the enzyme, the less glucose contained in the blood (Fig. 1). If the correlation in the control was not significant, then in the experiment the correlation coefficient between the activity of amylase in the duodenal chyme and glucose in blood remained steadily negative: for feed No. 1 $r = -0.72$ ($p < 0.05$), for feed No. 2 — $r = -0.45$. This fact allows us to suggest that chromium significantly affects carbohydrate metabolism due to the better use of glucose by the body. Chromium is known to regulate glucose homeostasis by activating insulin receptors, thereby enhancing signal transduction and increasing insulin sensitivity [20-22].

Triglycerides are esters that serve as the main constituents of fat in all living organisms. Getting into the gut together with food, triglycerides are turned to glycerin and fatty acids by lipase of gastric juice and pancreas [23], but the issues of fat metabolism in the poultry body are not fully studied [24]. According to our data, the increase in lipase activity in the chyme increased the number of triglycerides in blood (Fig. 2).

The value of the lipase-fat ratio in blood, when used feed with soy additive in the diet of laying hens, is almost 2 times higher than the control indicators with the addition of sunflower cake. A direct relationship was observed between lipase

activity in the duodenal chyme and triglycerides, i.e., the better the hydrolysis of fats in the bowel was, the more lipids entered the blood (correlation coefficient r ranged from 0.42 to 0.63; $p < 0.05$). Microadditive chromium enhanced these processes, improving the absorption of fats.

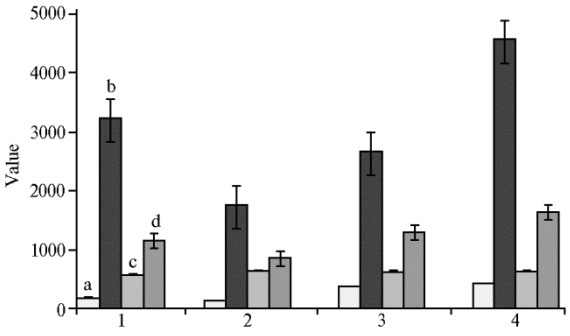

Fig. 2. Blood concentration of triglycerides (mmol/l) (a)**, activity of lipase** (U/l) (b) **and alkaline phosphatase** (U/l) (c)**, lipase activity in duodenal digest** (U/l) (d) **in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition:** 1 — control (feed No. 1), 2 — test (feed No. 1), 3 — control (feed No. 2), 4 — test (feed No. 2). For a description of groups and feeds, see the Techniques section. Indicators for triglycerides are increased 100-fold, for blood lipase 10-fold.

The activity of alkaline phosphatase, which is secreted in the liver, depends on the lipase content in the duodenal chyme [25]. A relationship between lipase activity in duodenal digesta and alkaline phosphatase activity in blood was found (see Fig. 2). The correlation between these indicators was strong in some cases ($r = –0.90$, $p < 0.05$).

Total blood protein is a relatively stable indicator in healthy animals; its fluctuations can be associated with low protein content in the feed or with a disease. Blood proteins are mainly represented by albumins, which provide maintenance of oncotic blood pressure. In our experiment, the decrease in the total protein content within the physiological norm was associated with the introduction of chromium microadditives into the feed (Fig. 3). At the same time, the amount of uric acid in the blood and AlAT activity changed, indicating a normalization of the liver and pancreatic function [26].

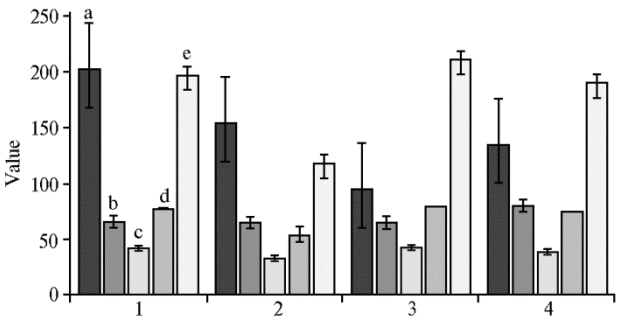

Fig. 3. Blood trypsin activity (U/l) (a)**, total proteins** (g/l) (b)**, alanine aminotransferase activity** (U/l) (c)**, aspartate aminotransferase activity** (U/l) (d)**, and duodenal protease activity** ($mg \cdot ml^{-1} \cdot min^{-1}$) (e) **in Hisex White cross laying hens (*Gallus gallus* L.) fed with dietary chromium oxide Cr_2O_3 nanoparticles as influenced by the feed ingredient composition:** 1 — control (feed No. 1), 2 — test (feed No. 1), 3 — control (feed No. 2), 4 — test (feed No. 2). For a description of groups and feeds, see the Techniques section. Indicators for alanine aminotransferase are increased 10-fold.

The analysis of the enzyme content in the blood is widely used in medicine to assess the state of the heart and liver. Fernando De Ritis found in 1957 that special significance has not only the AlAT and AsAT activity in blood but also their ratio (de Ritis ratio). Fluctuations in this indicator in humans are in the range from 1 to 2. In the test chickens, its value was extremely high, 21.8-26.7. Probably, the calculation of the de Ritis ratio for chickens should be refused, but it is important to note that the correlation analysis we performed showed a direct and fairly stable relationship between AsAT and AlAT, for which the value of r reached 0.98 ($p < 0.05$). With the addition of chromium oxide nanoparticles, the de Ritis ratio decreased slightly in the test group poultry compared to the control. Unlike tissue enzymes, pancreatic enzymes hydrolyze proteins, fats, and carbohydrates in the

gastrointestinal tract to monomers, and then enter the blood, where they participate in the regulation of metabolism. As known [27], there is a direct relationship between the activity of trypsin in the blood and the amount of glucose: the more trypsin in the blood, the higher the glucose content. This correlation was confirmed in studies on poultry in the postprandial period [28].

Our experimental data are also consistent with the results of studies [1, 2], which show that chromium regulates blood glucose homeostasis by activating insulin receptors and thus, as already noted, modulates signal transduction and increases insulin sensitivity [29, 30]. As for lipid metabolism, unlike Oganyan et al. [3], no significant effect of chromium microadditive on blood triglyceride concentration was found in this investigation. However, it was found that chromium microadditive acts as a stimulator of duodenal enzymes activity at low enzymatic activity in the gut, and at high enzyme activity it inhibits lipase activity. Consequently, it is reasonable to suppose that at low enzymatic activity, chromium supplementation has a positive effect on lipid metabolism. In protein metabolism when Cr_2O_3 was added to the feed, there were some changes: total protein decreased within the physiological norm (by 24.1%) when the chickens received the diet with the soybean cake, and protease activity increased by 23.1% when sunflower cake was used, but the digestibility of crude protein did not change significantly.

In experiments on rats [31], a comparative analysis of different chromium forms in the diet showed that the activity of amylase in the pancreas increases at a dose of chromium oxide nanoparticles 300 ppb. The use of chromium picolinate (CrPic) in a similar dose in feed for rats stimulates the activity of lipase and protease. The increased dose (500 ppb) of $CrCl_3$ and CrPic reduces lipase activity in the duodenum and reduces the activity of amylase and lipase in blood, which indicates the depressing effect of high doses of chromium on enteropancreatic circulation of digestive enzymes and metabolic imbalance of Mg and Fe in the blood. The amount of triglycerides decreases at the maximum doses of chromium in the form of chloride and picolinate, which confirms their participation in lipid metabolism. In addition, rats showed a significant increase in the frequency of DNA damages in peripheral blood and liver leucocytes after exposure to chromium oxide at doses of 300 and 1000 mg/kg [32].

The results we obtained on chickens are consistent with the action of chromium oxide nanoparticles on rats. The advantage of the our experiments is that this is the first to study the effect of chromium oxide in vivo on cannulated chickens under the influence of different protein sources.

Thus, dietary chromium microadditive to different protein components of the ration has an ambiguous effect in laying hens of the Hisex White cross. In the experimental period, regardless of the diet, with the introduction of chromium oxide supplements, the activity of blood amylase increases by 37.8-50.2% ($p < 0.05$) while the glucose content in the basal period reduces by 26.6-17.5% ($p < 0.05$), which indicates an improvement in glucose absorption. The activity of amylase in the duodenal digesta and the amount of glucose in the blood correlate, the correlation coefficients in the test period for different diets comprised –0.72 ($p < 0.05$) and –0.45. For a wheat diet with the addition of soy cake, the chromium oxide reduce the activity of lipase in the bowel digesta while the blood biochemical parameters of laying hens reduced by 22-40% ($p < 0.05$) within the physiological norm. The use of chromium oxide together with sunflower cake increases the activity of amylase, lipase and protease in the duodenum, which is associated, apparently, with the anti-nutrients in sunflower cake. This indicates the role of the basic diet in the action of chromium oxide on the bird body.

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