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Original Research

Organic chromium modifies the expression of orexin and glucose transporters of ovarian in heat-stressed laying hens

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Abstract: In this study, the effect of the supplemental organic chromium (Cr) forms on the expression of ovarian orexin(hypocretin), glucose transporters (GLUTs), heat shock proteins (HSPs) andnuclear factor-kappaB (NF-κB)were investigated in laying hens (HS). Laying hens (n=1800; 16-wk-old; Lohmann LSL-Lite) were allocated to 6 random groups according to a 2×3 factorial trial scheme with two different environmental temperatures [Thermoneutral (TN groups; at either 22±2 °C 24 h/d) and heat stress (HS groups; at 34±2 °C for 8 h/d, 08:00 to 17:00 h, followed by 22°C for 16 h for a period of 12 wks)], andhens reared under both environmental conditions were fed either a basal diet or the basal diet supplemented with 1.600 mg of chromium-picolinate (CrPic, 12.43% Cr) and 0.788 mg of chromium-histidinate (CrHis, 25.22% Cr) per kg of diet, delivering 200 µg elemental Cr per kg diet. HS groups showed decreased levels of orexin and GLUTs(GLUT1, GLUT4), and increased NFκB, HSP60, HSP70 and HSP90 levels compared to the TN groups in ovarian tissue of hens ($P < 0.0001$ for all). However, dietary chromium supplementation (CrPic–CrHis) increasedorexin and GLUTs levels and significantly reduced the NF-κB and HSPs levels making them closer to those of thermoneutral group ($P < 0.0001$).In conclusion, CrPic and CrHis showed supported the relief and treatment of stress complications.

Key words: Heat stress; Chromium; Ovary; Orexin; GLUTs; NFκB.

Introduction

Heat stress is one of the most significant obstacles in the livestock industry, especially in poultry farms. Heat stress not only compromises health and well-being but it also negatively affects survival, performance and product quality (1,2). It has been reported that heat stress in poultry affects yield properties thatinclude growth rate, feed conversion ratio, and body weight, and it, therefore, has negative effects on animal performance and product quality (3,4). Heat stress also causes oxidative stress, which in turn is caused by the production of reactive oxygen types and a reduction in the concentration of vitamins and mineralsthat play a role in antioxidant defense systems (4,5).Several molecular mechanisms have been developed by organisms to inhibit any damage caused by sudden temperature changes and to maintain the structure of the cell and its enzymatic integrity (6,7).

Orexin was first discovered in 1998 by two independent research groups (8). Orexins, which regulate many physiological processes, are produced in numerous tissues including the kidneys, the pituitary gland, the thyroid gland, the testes, the ovaries, the jejunum and the lungs (9,10). In fact, orexins have been shown to regulate energy homeostasis (energy intake and consumption) (11), and both glucose and lipid metabolism (12).One of the most important features of orexin is that it is a stress modulator. However, the effect of the orexin system on the stress mechanism has not been clearly explained in many species (8).

It is known that glucose uptake is mediated by glucose transport proteins (GLUTs) (13,14). Recently, the profiling of changes in GLUT 1,3 and 4 expressions in the ovarian tissuesof poultry, sheep, cattle, rat, and mouse species have been reported in a number of studies (15,16). These reports also show that intra-ovarian factors are regulated by GLUTs expression during follicular development. These results show that ovarian GLUTs proteins are a regulatory mechanism that regulates glucose uptake (15,16).

HSPs, known as molecular chaperones, are stress proteins involved in subsequent cellular repair and cellular protection in case of a cellular damage (17,18). These chaperones facilitate protein folding and protect the protein from dangers such as misfolding, mixed assembly and protein breakdown (19).In addition, it plays an important role in development, modification, translocation, cell proliferation and differentiation, oncogenesis and protein folding (19,20). It is induced by environmental and physiological stresses especially due to factors such as heat stress, increase in reactive oxygen types, toxic chemicals, infections, acidosis, and energy depletion (21,22). HSPs are involved in many basic cellular mechanisms. They can interact with proteins involved in programmed cell death,such as cytochrome c, caspase or Apaf-1 (apoptotic protease activating factor 1) (23).

NFκB is a transcription factor found in every cell involved in inflammatory and innate immune responses such as cell survival, stress, proliferation, apoptosis and cell migration (24,25).Over the last few years, research

has shown that NF-κBcan be induced in genes affecting cell survival, cell adhesion, inflammation, innate immunity, differentiation, and growth. It has also been shown that it is a transcription factor that can be expressed anywhere (26-28).

Chromium (Cr) is related to the metabolism of carbohydrates, lipids, proteins and nucleic acids in different animal species. It has been stated that Cr supplementations added to diets have a positive effect on growth performance, carcass characteristics and meat quality in broilers under heat stress (29-31). Cris also a potent and effective antioxidant that reduces the efficiency and metabolism drop in heat-stressed poultry. These effects may be partly related to the replenishment of the body's Cr reserves (19,32). The use of organic forms of Cr may be helpful to explain the role of Cr in poultry exposed to heat stress (19,33). In this study, the changes in ovarianorexin, GLUTs, NF-κBand HSP expressions of different organic sources of chromium were investigated in laying hens.

Materials and Methods

Animals, diets and experimental design

A total of 1800 laying hens (16-wk-old, Lohmann LSL-Lite) were used in accordance with animal welfare regulations approved by the Veterinary Control and Research Institute (2014/5 No:5-01; Elazig, Turkey). The birds were reared in temperature-controlled rooms at either 22°C for 24 hours per day thermoneutral (TN), or 34°C for 8 hours (09.00 to 17.00 h) followed by 22°C for 16 hours per day heat stress (HS), during the 12-week experimental period. The laying hens in both TN and HS conditions were either not supplemented with Cr (C) or supplemented with 1.600 mg of CrPic (12.43% elemental Cr, Nutrition 21, NY) or 0.788 mg of CrHis (25.22% elemental Cr, Nutrition 21, NY) per kilogram. In order to avoid confounding effects of an organic portion of the Cr-chelates, the diets control, CrPic, and CrHis were supplemented with 1.401 mg picolinic acid+0.589 mg histidine, 0.589 mg histidine, and 1.401 mg picolinic acid per kilogram, respectively.In order to determine which of the two different chromium sources is more effective and at what dose, the dosage was chosen based on previously reported dosage in poultry (33).Each treatment was replicated in 75 cages. Feed and fresh water were offered ad libitum throughout the experimental period. Birds were exposed to an illumination program providing a light:dark cycle of 16 h:8 h per day.

Table 1. Ingredients and nutrient composition of the basal diet.

Ingredient	Amount (g/kg)
Maize	548.5
Soybean meal	282.6
Corn oil	46.6
Salt	3.3
DL-methionine	2.2
Limestone	95.3
Dicalcium phosphate	18.0
Vitamin-Mineral premix[1]	3.5
Nutrient composition (g/kg, dry matter basis)	
Metabolisable energy, kcal/kg[2]	2800
Crude protein	177.6
Calcium	40.4
Phosphorus	6.3
Methionine[2]	4.0
Lysine[2]	11.1

[2]Per kilogram contained: retinyl acetate, 1.8 mg; cholecalciferol, 0.025 md; dl-tocopheryl acetate, 1.25 mg; menadione sodium bisulfite, 2,5 mg; thiamine-hydrochloride, 1.5 mg; riboflavin, 3 mg; niacin, 12.5 mg; d-pantothenic acid, 5 mg; pyridoxine hydrochloride, 2.5 mg; vitamin B_{12}, 0,0075 mg; folic acid, 0.25 mg; choline chloride, 125 mg; Mn ($MnSO_4$-H_2O), 50 mg; Fe ($FeSO_4$-$7H_2O$), 30 mg; Zn (ZnO), 30 mg; Cu ($CuSO_4$-$5H_2O$), 5 mg; Co ($CoCl_2$-$6H_2O$), 0,1 mg; I (KI), 0,4 mg; Se (Na_2SeO_3), 0,15 mg. Moreover, the premix was reconstituted at the expense of $CaCO_3$ to contain histidinate (0.236 g) plus picolinate (0.560 g), CrPic (0.640 g) plus histidinate (0.236 g), and CrHis (0.315 g) plus picolinate (0.560 g) in the diets C, CrPic, and CrHis, respectively. [2]The Metabolizable energy, lysine, methionine and cysteine contents were calculated based on their tabular values listed for the feed ingredients.

Data and sample collection

At the end of the study, 15 birds from each group were killed by cervical dislocation. Samples of the ovaries were removed and stored (at−80°C) on ice for the subsequent determination of levels of orexin, GLUTs (GLUT1 and GLUT4), NF-κB and HSPs (Hsp60, Hsp70, and Hsp90).

Western blot analysis

Ovary protein expressions (orexin, GLUT1, GLUT4, NF-κB, HSP60, HSP70, and HSP90) were determined by a western blot analysis as described by Sahin *et al*(34).For transcription factor analyses, accurately weighed ovarian samples were homogenized in 1:10 (w/v) of 10 mM of Tris-HCl buffer (pH of 7.4) containing 0.1 mM ofNaCl, 0.1 mM of phenylmethylsulfonyl fluoride and 5 µM of soluble soybean powder (Sigma, St. Louis, MO) as a trypsin inhibitor. After centrifuging at 15,000 × g at 4°C for 30 minutes, the supernatant was transferred into fresh tubes to be immediately assayed. Supernatants were mixed with Laemmli's sample buffer and boiled for 5 minutes. Aliquots containing 20 µg of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subsequently transferred to a nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH). The nitrocellulose blots were washed twice for 5 minutes in phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin in PBS for 1 hour prior to the application of the primary antibody. Antibodies against GLUT1, GLUT4 (Santa Cruz Biotechnology Inc, CA, USA), orexin, NF-κB, HSP60, HSP70 and HSP90 (Abcam, Cambridge, UK) were diluted (1:1000) in the same buffer solution containing 0.05% Tween-20. The nitrocellulose membrane was incubated at 4°C overnight with protein antibody. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam). Specific binding was detected using diaminobenzidine and hydrogen peroxide as substrates. Protein loading was controlled using β-actin antibody (Sigma, St. Louis, MO). Samples were analyzed in quadruplicate for each experimental condition, and

protein levels were determined densitometrically using an ImageJ analysis system (National Institute of Health, Bethesda, MD).

Statistical analysis

Data were analyzed by two-way ANOVA using the PROC GLM procedure (SPSS v.15). The linear model to explain group effects was: yijk = μ + Ei + Sj + (E*S)ij + eijkwhere y = response variable; μ = population mean; ET = environmental temperature, i = 1,2; S = CrHis or CrPic supplementation, j = 1,2,3; and e = residual error [N (σ, μ; 0, 1)]. Statistical significance was considered to be $P \leq 0.05$.

Results

The effect of heat stress and Cr supplementation on protein levels isshown in Figure 1.Ovary orexin (57.17% vs. 109.55%), GLUT1 (72.09% vs. 113.623%) and GLUT4 (66.15% vs. 94.28%) levels were lower, whereas of ovary NF-κB (160.94% vs. 89.14%), HSP60 (201.49% vs. 79.17%), HSP70 (295.32% vs. 107.76%) and HSP90 (210.62% vs. 93.46%) was higher in the heat-stressed laying hens than the control laying hens ($P< 0.0001$ for all). Evaluating the stress groups with each other, orexine (30.74%), GLUT1 (39.05%) and GLUT4 (49.62) levels significantly increased in the CrPic group compared to the control group ($P<0.0001$), whereas NF-κB (24.53%), HSP60 (25.76%) ($P<0.0001$ for both), HSP70 (4.22%)and HSP90 (13.89%) significantly decreased ($P < 0.05$ for both)**.** In addition, in the stress groups, orexin (46.07%), GLUT1 (50.80%) and GLUT4 (81.29%) levels significantly increased in the CrHis group compared to the control group ($P <0.0001$). In contrast, levels of NF-κB (39.06%), HSP60 (40.17%) ($P<0.0001$ for both), HSP70 (27.01%) and HSP90 (33.56%) were significantly reduced by CrPic and CrHis supplementation, being CrHis superior to CrPic ($P< 0.05$ for both).

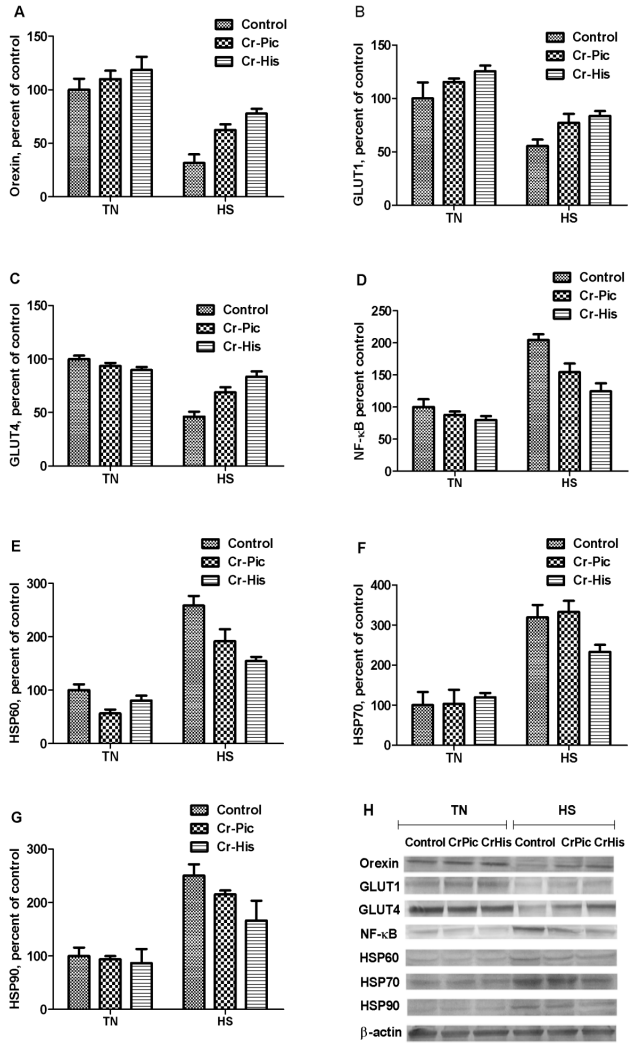

Figure 1. The effect of chromium on orexin (Panel A), GLUT1 (Panel B), GLUT4 (Panel C), NFκB (Panel D), HSP60 (Panel E), HSP70 (Panel F) and HSP90 (Panel G) protein expression levels of ovarian tissue in heat stressed laying hens. The intensity of the bands was quantified by densitometric analysis (Panel H). Data are expressed as a ratio of the normal control value (set to 100%). The bar represents the standard mean error. Blots were repeated at least 3 times (n=3) and a representative blot is shown. β-actin was included to ensure equal protein loading. Means on the bars with no common superscript differ significantly at the level $p< 0.01$ using Fisher's multiple comparison test.

Discussion

There are many studies on the harmful effects of stress on poultry (35-37). From previous studies,the problems caused by heat stress in the livestock sector are known to cause significant economic losses, especially in poultry farming. Heat stress in females negatively affects the oogenesis, oocyte maturation, fertilization, and embryo development and implantation rates (38,39). Numerous biochemicaland structuralchanges occur in the heart, liver, kidneys and other organs as a result of stress in animals (40,41). In line with the study reports by Sahin *et al* (34)and Zeferino *et al* (39), analyses in this study have shown that laying hens exposed to heat stress are affected in a negative way. The harmful effects of heat stress and reactive oxygen types can be prevented by antioxidant vitamins and minerals acting through various mechanisms (42,43). Chromium, which has important functions in proteins, carbohydrates and lipid metabolism, is one of the basic minerals consumed in the diet of mammals in particular. Chromium has been reported to be effective in insulin and cell membranes sensitive to insulin. It has also been reported that the lack of chromium may lead to impairment in glucose tolerance (44,45). In addition, chromium chelates relieve oxidative stress (43,45). In a quail heat stress model, the CrPic supplementation-administered on its own or as a combination-improved body weight gain, feed consumption, carcass characteristics and feed conversion rates when compared to the control diet, the CrPic-only administered diet or the biotin-fortified group (33). In a study conducted by Torki*et al* (46) in laying hens, the dietary CrPic and C vitamin supplementation led to significant interactions between the yellow color in egg, the eggshell mass, and eggshell thickness. Hens with dietary CrPic supplementation were found to have lower serum glucose, total cholesterol, and triglyceride concentrations, but they had higher serum albumin and total protein concentrations than those of the other groups. On the other hand, in human estimated safe and adequate daily dietary intake is suggested by the National Research Council to be between 50μg to 200μg/day (47). There are also many studies

on chromium toxicity (48,49,50). In humans exposed to dissolved hexavalent chromium aerosols and mists (as chromium trioxide mist) for intermediate durations, nasal irritation, ulceration, and mucosal atrophy and rhinorrhea have been reported, with the lowest observed adverse effect level (LOAEL) values ranging from 0.09 to 0.1 mg chromium(VI)/m3. In addition, studies in rodents have shown that the upper respiratory tract is a primary target of exposure to inhaled chromium trioxide mist, with LOAEL values ranging from 0.49 to 3.63 mg chromium(VI)/m3 (48).

Orexins regulates stress-related diseases, nutrient uptake, stimulation and behavioral responses (51). The studies show that orexin is activated in response to stressful conditions and plays a key role in physiological and behavioral responses to stress (52). In a study by Greene *et al* (8), it was found that heat stress resulted in a significant decrease in liver orexin mRNA levels in quail, in agreement with this study. Similarly, hypothalamus orexin mRNA levels were significantly reduced compared to the thermoneutral temperature group in broiler chickens (53). In the present study, we have found a decrease in ovary orexin levels of laying hens reared under heat stress. However, ovary orexin level was increased by chromium supplementation in the laying hens kept under heat stress condition. There are no previous studies related to investigating the effects of Cr supplementation on the ovary orexin levels in laying hens to compare with this study.

Localization, expression, and regulation of GLUTs proteins are specific to tissues and cells. In a study by Zhang *et al* (13) on the effects of gonadotropin, glucose transports and apoptosis in rat ovaries, GLUT1, GLUT3, and GLUT4 were detected. Consistent with the high expression of GLUT1-4, gonadotropin has been reported to have a potent stimulatory effect on serum glucose uptake. In the current study, ovarian tissue levels of GLUT1 and GLUT4 decreased in the control group exposed to heat stress. However, GLUT1 and GLUT4 were significantly increased by Cr supplementation in the heat-stressed laying hens (Figure 1). Due to lack of prior studies, investigating the effects of supplemental Cr on these protein levels in the ovarian of laying hens kept under HS condition; present data are not comparable with the literature. Thus, the beneficial effect of Cr supplementation on increased levels of GLUTs is significant and implies Cr has a potential role in preventing the development of ovarian damage. In addition, a previous study done in rats reported that Cr has a significant increase in retina GLUTs levels (54).

NF-κBis known as a family of eukaryotic transcription factors that control many biological processes such as inflammation, cell cycle, apoptosis, development, and regulation (55). Cellular responses mediated by other transcription factors such as NF-κB and AP1 play an important role in the cancer mechanism (56). In a study performed by Sahin *et al* (42), the activation of the liver NF-κBlevels in both the thermoneutral and the heat stress groups decreased in the treatment of tomato powder, compared to the control level. Similar to our results, in another study conducted by Akdemir *et al* (19) in quail, for those quail exposed to heat stress, the liver NF-κB, levels increased compared to those kept in the thermoneutral environment by about one-and-a-half times. In the same study, it has been reported that the inhibition of the transcription factors studied was increased due to the increase of the CrHis dose.

Orhan *et al* (57) found that hepatic expression of HSP60, HSP70, and HSP90 was increased in quail exposed to heat stress compared to thermoneutral temperature quails. Additionally, in another study-in line with our study-itwas reported that an increase in the CrHis dosage in the diet reduced HSP60 (28.4%),HSP70 (30.9%), and HSP90 (22.3%) levels in chicken liver tissues when compared to the groups in the thermoneutral condition (19). Moreover, in a study on the CrHis in experimental diabetes-induced rats through the application of STZ, kidney HSPs, MDA, and 8-isoprostane levels increased significantly. In the study treatment group, kidney tissue HSP60 and HSP70, and MDA and 8-isoprostanes – markers of lipid peroxidation – decreased significantly. The study, which is in parallel with our research, reported the ability to reduce nephropathy damage due to the antioxidative properties of CrHis (58).

In conclusion, in the experimental model for heat-stressed laying hens,by comparison to the thermoneutral control group, it was found that formsof chromium added to the diets led to an increase in the levels of orexin and GLUTs found in the ovary, and in contrast to the control group, a reduction in stress-induced tissue damage with the inhibition of NF-κBand HSP protein expressions.The positive effect of Cr supplementation was more pronounced when Cr was chelated with histidinate than when chelated with picolinate.However, it should be noted that we evaluated the effect of only two concentrations of Cr on the expressions of orexin, GLUTs, HSPs and NF-κB. Thus, further studies are needed to test the effect of awiderange of concentrations of Cr.

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Author's Contribution

KS participated in the study design and drafting the manuscript. OO, MT, ZT, and CO, participated in the data collection and assays and data analysis. NSparticipated in the data analysis and statistical analysis for the variables and drafting the manuscript. All authors read and approved the final manuscript.

Interest Conflict

No potential conflict of interest was reported by the authors.

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