INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 6, p. 370-375, 2019

RESEARCH PAPER OPEN ACCESS

Effects of raw rock phosphate rich in heavy metals on oxidative stress markers in layers

Anwar Ul Haq, Zahed Mahmood*, Muhammad Ali, Abdul Ghaffar, Muhammad Yameen

Department of Biochemistry, Government College University, Faisalabad, Pakistan

Key words: Raw rock phosphate, Dicalcium phosphate, Oxidative stress parameters, Laying hens

http://dx.doi.org/10.12692/ijb/14.6.370-375 Article published on June 30, 2019

Abstract

The present study was planned to evaluate the effects of various concentrations of raw rock phosphate (RRP) rich in heavy metals on oxidative stress markers in blood samples obtained from laying hens. A total of 192 commercial laying hens of the same weight were allocated under the Completely Randomized Design to 6 treatments with 4 replicate cage boxes of 10 chicks each. The birds were maintained in cages for a period of 21 to 45 weeks of age. The RRP [A representative sample from Tarnawai-Lagarban, Danna and Kakul at Khyber Pakhtoon Khawa, Pakistan] was incorporated into a standard diet based on corn, rice broken, rice polishing, soya bean meal, sunflower meal and corn gluten meal (60%) to replace the amount of P provided by the DCP at 0, 25, 50, 75, and 100% level. A control diet was formulated without phosphate. Oxidative stress parameters (TAS, catalase, SOD and GSH-Px) showed decreasing ($P<0.05$) trend in birds fed raw rock phosphate-based diets as compared to those birds fed control (bone meal) and 100% DCP diets, while TOS and MDA values in birds fed with raw rock phosphate-based diets increased than those of control and 100% DCP diets. It may be concluded that raw rock phosphate-based diets at 75 and 100% replacing DCP may cause buildup of free radicals in the serum, enrichment of lipid peroxidation and hampering the activities of antioxidant enzymes which accordingly stimulates oxidative stress and damages the antioxidant function in hens.

* **Corresponding Author:** Dr. Zahed Mahmood ✉ drzahiduaf2003@yahoo.com

Introduction

Commercial poultry production is related with different stresses accountable for decreasing productive and reproductive performance of growing chicks, breeders and commercial layers. A growing body of testimony indicates that most of stresses in poultry production at the cellular level are associated with oxidative stress (Surai, 2016). The imbalance of pro-oxidants and the endogenous antioxidant mechanisms in living tissues leads to uncontrolled oxidative damage to cellular components, known as oxidative stress (Kohen and Nyska, 2002).

Present study is related to raw rock phosphate (RRP) based layer diet containing heavy metals caused stress in chickens. An excess of heavy metals can cause oxidative damage to membrane lipids, DNA and proteins, and their oxidation may ultimately lead to cellular dysfunction and tissue injury (Valavanidis *et al*., 2006). The catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are three important enzymes of intracellular antioxidants in cells. The SOD converts superoxide anion radical into hydrogen peroxide, whilst GSH-px and CAT change hydrogen peroxide into water (Weydert and Cullen, 2010).

No such studies have been reported in the literature so far and hence, it is first study of its kind to evaluate the effects of RRP on oxidative stress biomarkers in laying hens exposed to heavy metals under feeding trial. Thus, it is necessary to understand the mechanisms of metal toxicity in laying birds, and the concentrations that cause effects on oxidative stress biomarkers. The aim of this pre-clinical study trial was to evaluate the effects of various concentrations of RRP rich in heavy metals on oxidative stress markers in blood samples obtained from laying hens.

Materials and methods

Birds and diets

A total of 192 commercial laying hens (Hy-Line W-98) of approximately the same weight were allocated under the Completely Randomized Design to 6 treatments with 4 replicate cage boxes of 10 chicks each. The birds were maintained in cages for a period of 21 to 45 weeks of age. The raw rock phosphate [A representative sample from Tarnawai-Lagarban, Danna and Kakul at Khyber PakhtunKhwah, Pakistan] was incorporated into a standard diet based on corn, rice broken, rice polishings, soyabean meal, sunflower meal and corn gluten meal (60%) to replace the amount of P provided by the DCP at 0, 25, 50, 75, and 100% level (Table 1). A control diet was formulated without phosphate. Six experimental diets were formulated to include adequate levels of all nutrients needed to satisfy the chicks' minimum requirements (NRC, 1994).

Blood Sample collection and biomarker analyses

At the end of trial period, blood sample (3mL) was collected in blood collection vacutainer from each hen under the study in different treatment groups and control group. Collected blood samples were allowed to clot and then centrifuged at 3500 rpm for 10 minutes to separate serum for oxidative biomarkers determination. Oxidative stress parameters including total antioxidant status (TAS), total oxidant status (TOS), Malondialdehyde Assay (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in blood serum were analyzed after homogenization (1:10 w/v) in a stock buffer (1.15% KCl in 0.01M PBS (pH 7.4) with 0.02M EDTA).

Serum TAS and TOS were measured using novel automated method developed by Erel (2004 & 2005) with a spectrophotometer (UV-1603, Shimadzu). The results are expressed in terms of mmol/l and µmol/l, respectively. Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (TBARS), was assessed following the methodology described by Placer *et al*. (1966) with a spectrophotometer (UV-1603, Shimadzu). As the MDA is one of the most abundant carbonyl products of lipid peroxidation, the amount of MDA produced was used as an index of lipid peroxidation. The CAT (EC1.11.1.6) activity was assayed following the methodology described by Clairbone (1985), based on the decomposition of hydrogen per oxide (H_2O_2) in molecular oxygen and water by this enzyme. The rate of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240nm with a spectrophotometer (UV-1603, Shimadzu).

The assay mixture consisted of 950µL of potassium phosphate buffer (0.05M, pH7.0), 500µL of H_2O_2 (0.03M) and 50µL of sample. The results were expressed as IU/L. The activities of SOD (EC1.15.1.1) and GPx (EC1.11.1.9) were determined with the Ransel and Ransod kits (Randox Laboratories), respectively using spectrophotometer (A25-Autoanalyzer, BioSystems), following descriptions of Reglero *et al.* (2009) with some modifications. To determine the SOD and GPx activities, homogenized samples were diluted at 1:20 and 1:25(v:v) with Ransel diluting agent and Ransod sample diluents (Randox Laboratories), respectively. The SOD and GPx activities were expressed as IU/mL and U/L, respectively.

Statistical Analysis

The results were expressed as mean ± standard error (SE) and one way analysis of variance (ANOVA) for statistical significance was carried out using statistical package for social sciences (SPSS) version 12.0 analysis system. Significant differences in the concentrations of the elements were determined by applying ANOVA. Tukey multiple-comparison test was used to compare the differences in the mean values of the elements. Differences were considered to be significant at $p < 0.05$.

Results and discussion

Oxidative stress parameters (TAS, CAT, SOD and GSH-Px) showed decreasing ($p<0.05$) trend in birds fed RRP-based diets as compared to those birds fed on control (bone meal) and 100% DCP diets, while TOS and MDA values in birds fed RRP-based diets increased than those of control and 100% DCP diets (Table 2). Such changes of oxidative stress parameters in this study might be on account of heavy metals and F in the diets of RRP (Table 1). Inadequate literature is available on the influences of higher dietary F contents on oxidative damage parameters in the poultry. Findings of the present trial are in line with results of few other studies (Liu *et al.*, 2003; Chen *et al.*, 2011; Deng *et al.*, 2014), which indicated that high dietary F decreased the actions of catalase, GSH-Px & SOD and increased content of MDA in broiler chicken's serum, advocating of F introverted the actions of blood serum antioxidants as well as raised the reactive oxygen species (ROS) levels in the chickens' blood circulatory system.

Table 1. Composition of experimental diets containing 0 to 100% raw rock phosphate (RRP) in place of acidulated phosphate rock (DCP) during production phase (21 to 45 wks).

Items (% unless noted)	Control	DCP:RPR				
		100:0	75:25	50:50	25:75	0:100
Corn	52.00	52.00	52.00	52.00	51.70	51.40
Rice broken	10.00	10.00	10.00	10.00	10.00	10.00
Rice polishings	6.34	6.34	6.24	6.28	6.30	6.31
Soyabean meal	17.00	17.00	17.00	17.00	17.00	17.00
Sunflower meal	4.00	4.00	4.03	3.83	3.96	4.00
Corn gluten meal (60%)	5.00	5.00	5.00	5.00	5.00	5.00
Lime stone	3.00	3.00	2.92	2.84	2.76	2.68
DCP	-	2.00	1.50	1.00	0.50	-
Raw phosphate rock	-	-	0.65	1.31	1.96	2.61
Bone meal	2.00	-	-	-	-	-
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Premix*	0.30	0.30	0.30	0.30	0.30	0.30
DL-Methionine	0.06	0.06	0.06	0.06	0.06	0.06
Total	100	100	100	100	100	100
Calculated						
ME Kcal/kg	2800	2800	2800	2800	2800	2800
Lysine	0.85	0.85	0.85	0.85	0.85	0.85
Methionine	0.40	0.40	0.40	0.40	0.40	0.40
Analyzed						
Crude protein	16.50	16.50	16.50	16.50	16.50	16.50
Crude fiber	4.90	4.90	4.90	4.90	4.90	4.90
Ether extract	3.50	3.50	3.50	3.50	3.50	3.50
Total P	0.61	0.61	0.61	0.61	0.61	0.61
Available P	0.40	0.32	0.32	0.32	0.32	0.32
Calcium	3.58	3.56	3.52	3.56	3.57	3.56
F mg/kg	32.0	0.25	148	292	439	645
Heavy metals (mg/kg)						
Cd	0.42	0.44	0.46	0.61	0.94	1.15
Cr	3.47	3.45	3.60	4.21	5.44	5.85
Cu	8.20	8.40	9.48	10.31	10.74	12.99
Mn	58.20	55.50	68.20	82.24	85.34	89.43
Ni	1.40	1.56	2.04	2.31	2.54	2.94
Zn	61.30	58.60	20.95	34.99	38.09	42.18
Pb	2.84	3.13	4.91	5.19	6.10	6.79
Fe	98.11	104.44	118.24	122.47	126.14	132.06

*The vitamin-mineral premix provided per kilogram of diet: vitamin A, 5,000IU; cholecalciferol, 750IU; vitamin E, 7.5mg; menadione, 0.63mg; thiamine, 0.25mg; riboflavin, 1.6mg; pyridoxine, 0.5mg; vitamin B_{12}, 4.0µg; niacin, 12.5mg; calcium pantothenate, 1.8mg; butylated hydroxytoluene, 63mg; Cu, 3mg; Fe, 40mg; Zn, 30mg; Mn, 30mg; I, 1.2mg; Co, 0.36mg; Se, 0.24mg

Table 2. Effects of replacing dicalcium phosphate (DCP; on a P basis) with raw rock phosphate (RRP) on different oxidative stress parameters.

Items	Control	RRP replacing DCP (%)					P-value
		0	25	50	75	100	
TAS (mmol/l)	1.45± 0.01^{a}	1.42± 0.02^{a}	1.29± 0.02^{ab}	1.21± 0.03^{ab}	1.13± 0.02^{b}	1.12± 0.05^{b}	0.04
TOS (µmol/l)	1.43± 0.03^{b}	1.40± 0.02^{b}	1.53± 0.02^{b}	1.62± 0.01^{ab}	1.84± 0.04^{a}	1.87± 0.08^{a}	0.03
MDA (nmol/l)	9.57± 0.02^{c}	9.23± 0.06^{c}	9.72± 0.02^{c}	12.54± 0.01^{b}	13.87± 0.05^{a}	13.95± 0.04^{a}	0.04
CAT (IU/l)	72.5± 7.00^{a}	71.6± 5.60^{a}	70.4± 5.80^{a}	56.6± 3.30^{ab}	33.1± 2.90^{b}	32.2± 3.00^{b}	0.03
SOD (IU/ml)	7.6± 0.09^{a}	7.0± 0.04^{a}	6.8± 0.08^{a}	6.0± 0.07^{ab}	5.5± 0.05^{b}	5.3± 0.10^{b}	0.04
GSH-Px (U/L)	102± 7.00^{a}	101± 6.90^{a}	98± 5.00^{a}	87± 1.00^{ab}	64± 2.00^{b}	60± 1.89^{b}	0.03

$^{a-c}$Means within a row without common superscripts are significantly different (P < 0.05).

TAS: Total antioxidant status; TOS: Total oxidant status; Catalase: CAT; MDA: Malondialdehyde Assay;

SOD: Superoxide Dismutase, GSH-Px: Glutathione Peroxidase

Heavy metals possess lethal prospective and capacity to create ROS particularly whilst at high contents that may perhaps cause metal-linked oxidative strain which may direct to oxidative damage to proteins, lipid & DNA (Valko *et al.*, 2005). The present results corroborate those obtained by Mashkoor *et al.* (2016), who reported that levels of CAT and TAS were declined (p<0.05) in chicks treated with Cr than those for control group. Likewise, such reduction in CAT in the Cr-treated rats and fish was also reported by Molina-Jijon *et al.* (2011) and Shaheen and Akhtar (2012). Similarly, Wu *et al.* (2013) noted the reduction in GSH-Px, CAT and SOD activities in the Ni-induced broiler groups as compared to without Ni group.

Conversely, contents of MDA were higher in the Ni supplemented broiler groups than that of control group. Moreover, some studies indicated higher lipid peroxidation occurred after exposure of Pb in broilers (Hoffman *et al.*, 2000; Mateo and Hoffman, 2001; Mateo *et al.*, 2003) and after Hg exposure in rats and birds (Huang *et al.*, 1996; Hoffman *et al.*, 2005).

The SOD, GSH-Px and CAT were recognized as the first line of cellular protection against oxidative damage which work together in the disintegration of H_2O_2 and O_2^- to less detrimental forms. In this sense, SOD catalyses the conversion of O_2^- into H_2O_2 & O_2 after that GSH-Px & CAT catalyze the disintegration of H_2O_2 (Halliwell and Gutteridge, 1999).

In the present study, serum antioxidant enzymes activities in the birds fed RRP-based diets particularly at 75 and 100% RP groups were decreased as compared with other groups. Such reduction in enzymes activities may direct to extreme accessibility of H_2O_2 and $\bullet O_2$ in biological systems which ultimately will create OH^- implicated in the beginning as well as proliferation of lipid peroxidation (Halliwell and Chirico, 1993). Consequently, the MDA may cause cross-linking in nucleic acids, lipids & proteins (Halliwell and Chirico, 1993). This MDA production provokes increase of membrane fragility along with modification of membrane fluidity (Chen and Yu, 1994). Furthermore, Marnett (1999) reported that MDA hampers different enzyme affects & applies mutagenicity as well as carcinogenicity via constituting DNA adducts (segments of DNA bound to a cancer-causing chemical). Thus current results clearly revealed that increasing concentrations of MDA in the birds' serum caused through heavy metals found in RP-based diets. Since a delayed biomarker of oxidative stress, the enhanced yield of MDA involves the augmentation of lipid peroxidation with deposition of lipid peroxides in the body which thus decreases antioxidative role in hens.

Conclusion

It may be concluded that RRP-based diets at 75 and 100% replacement with DCP may cause buildup of free radicals in the serum, enrichment of lipid peroxidation and hampering the activities of antioxidant enzymes which accordingly stimulates oxidative stress and damages the antioxidant function in hens.

Acknowledgments

Authors are highly thankful to Higher Education Commission of Pakistan and the Department of Biochemistry, Government College University Faisalabad for their valuable support in this research completion.

References

Chen JJ, Yu BP. 1994. Alteration in mitochondrial membrane fluidity by lipid peroxidation products. Free Radical Biology and Medicine **17,** 411-418.
DOI: 10.1016/0891-5849(94)90167-8

Chen T, Cui HM, Cui Y, Bai CM, Gong T. 2011. Decreased antioxidase activities and oxidative stress in the spleen of chickens fed on high-fluorine diets. Human and Experimental Toxicology **30,** 1282-1286.

Clairbone A. 1985. In: Greenwald RA. (Ed.), Catalase Activity. CRC Handbook of Methods in Oxygen Radical Research. CRC Press, boca Raton, FL.

Deng Y, Hengmin C, Xi P, Jing F, Zhicai Z, Junliang D, Qin LK. 2014. Effects of high dietary fluoride on serum biochemistry and oxidative stress parameters in broiler chickens. Health **6,** 1840-1848.

Erel O. 2004. A novel automated method to measure total antioxidant response against potent free radical reactions. Clinical Biochemistry **37,** 112-119.

Erel O. 2005. A new automated colorimetric method for measuring total oxidant status. Clinical Biochemistry **38,** 1103-1111.

Halliwell B, Chirico S. 1993. Lipid peroxidation: Its mechanism, measurement, and significance. American Journal Clinical Nutrition **57,** 715-724.

Halliwell B, Gutteridge J. 1999. Free radicals in biology and medicine, 3rd ed. Oxford University Press, USA.

Hoffman DJ, Heinz GH, Sileo L, Audet DJ, Campbell JK, LeCaptain LJ. 2000. Developmental toxicity of lead-contaminated sediment to mallard ducklings. Archive of Environmental Contamination and Toxicology **39,** 221-232.

Hoffman DJ, Spalding MG, Frederick PC. 2005. Subchronic effects of methyl- mercury on plasma and organ biochemistries in great egret nestlings. Environmental Toxicology and Chemistry **24,** 3078-3084.

Huang YL, Cheng SL, Lin TH. 1996. Lipid peroxidation in rats administrated with mercuric chloride. Biological Trace Element Research **52,** 193-206.

Kohen R, Nyska A. 2002. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicologic Pathology **30,** 620-650.

Liu GY, Chai CY, Cui L. 2003. Fluoride Causing Abnormally Elevated Serum Nitric Oxide Levels in Chicks. Environmental Toxicology and Pharmacology **13,** 199-204. http://dx.doi.org/10.1016/S1382-

Marnett LJ. 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutation Research **424,** 83-95. doi:10.1016/S0027-5107(99)00010-X

Mashkoor J, Khan A, Khan MZ, Hussain I. 2016. Chromium toxicity and oxidative stress in broiler chicks and its amelioration with vitamin E and bentonite. International Journal of Agriculture and Biology **18,** 1103-1108.

Mateo R, Beyer WN, Spann JW, Hoffman DJ, Ramis A. 2003. Relationship between oxidative stress, pathology, and behavioral signs of lead poisoning in mallards. Journal of Toxicology and Environmental Health Part A **66,** 1371-1389.

Mateo R, Hoffman DJ. 2001. Differences in oxidative stress between young Canada geese and mallards exposed to lead-contaminated sediment. Journal Toxicology and Environmental Health Part A **64,** 531-545.

Molina-Jijon E, Tapia E, Zazueta C, El Hafidi M, Zatarain-Barrón ZL, Hernández-Pando R, Medina-Campos ON, Zarco-Márquez G, Torres I, Chaverri JP. 2011. Curcumin prevents Cr (VI)-induced renal oxidant damage by a mitochondrial pathway. Free Radical Biology and Medicine **51,** 1543-1557.

National Research Council (NRC). 1994. *Nutrient requirements of poultry*. 9th Revised Edition, National Academy Press, Washington, DC.

Placer AZ, Linda LC, Johnson B. 1966. Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. Analytical Biochemistry **16,** 359-364.

Reglero MM, Taggart MA, Monsalve-González L, Mateo R. 2009. Heavy metal exposure in large game from a lead mining area: effects on oxidative stress and fatty acid composition in liver. Environmental Pollution **157,** 1388-1395.

Shaheen T, Akhtar T. 2012. Assessment of chromium toxicity in *Cyprinus carpio* through hematological and biochemical blood markers. Turkish Journal of Zoology **36,** 682-690.

Surai PF. 2016. Antioxidant systems in poultry biology: superoxide dismutase. Journal of Animal Research and Nutrition **1,** 8.

Valavanidis A,Vlahogianni T, Dassenakis M, Scoullos M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety **64,** 178-189.

Valko M, Morris H, Cronin MT. 2005. Metals toxicity and oxidation stress. Current Medicinal Chemistry **12,** 1161-1208.

Weydert CJ, Cullen JJ. 2010. Measurement of superoxide dismutase, catalase, and glutathione peroxidase in cultured cells and tissue. Nature Protocols **5,** 51-66.

Wu B, Cui H, Peng X, Fang J, Zuo Z, Deng J, Huang J. 2013. Investigation of the serum oxidative stress in broilers fed on diets supplemented with nickel chloride. Health **5,** 454-459.