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Impact of Zinc-Induced Moulting on Certain Health Biomarkers in White Leghorn Layers (*Gallus domesticus*)

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ABSTRACT

The study was aimed to evaluate the effect of Zn-induced moulting (3 g of ZnO/kg diet) for three consecutive weeks on certain indicators of oxidative stress including total oxidant status and total antioxidant capacity in serum and tissues (Liver, Heart, kidney and muscles); while serum concentrations of homocysteine, ceruloplasmin, paraoxonase and arylesterase in commercial White Leghorn layers. The extent of DNA damage in lymphocytes was also assessed in experimental birds. After an acclimatization period of three weeks, the birds were divided into moulted (ML; n= 12) and non-moulted layers (NML; n= 12). The ML group revealed higher levels of TOS in Liver ($P<0.05$), Heart ($P<0.01$) and kidney ($P<0.05$). The TAC values in ML group were only higher ($P<0.01$) for serum while significantly lower for heart ($P<0.05$), kidney ($P<0.01$) and muscle ($P<0.01$) compared to NML group. Serum homocysteine and DNA damage were significantly increased ($P<0.01$), whereas, paraoxonase and arylesterase were decreased ($P<0.01$) in ML group in comparison to the NML group. Although zinc-induced (3 g/kg per day) moulting is a stressful procedure which can effectively increase the DNA damage and total oxidant status in some organs, an increased total antioxidant values in serum can be counted as the beneficial aspect of ZnO induced moulting.

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INTRODUCTION

Layers farming is the most organized and valuable sector of the poultry industry in Pakistan providing as a source of cheaper dietary protein as eggs and meat (Masoud et al. 2024). Moulting helps to improve egg quality and production in spent layers towards the second production cycle. Moulting is a natural process along with a management tool for rejuvenation of reproductive system involving reproductive quiescence, periodic shedding and replacement of feathers in all species of domesticated and non-domesticated birds (Idris et al., 2023; Olayiwola & Adedokun, 2025). This natural process has been reproduced on commercial level by adopting various artificial moulting techniques for inducing moult to get better and prolonged egg production in egg-laying hens during their subsequent production cycles (Anwar et al., 2012a, 2012b; Anwar et al., 2018; Onbaşılar et al., 2025). For the purpose, various techniques have been implied including water and food deprivation alone/or both namely ‘fast induced moulting’; dietary manipulation of certain nutrients such as copper sulphate, zinc sulphate, zinc oxide (ZnO) alone or with low dietary calcium, aluminium oxide; and hormone-induced moulting by using the progesterone and thyroxine (Yousaf et al., 2007; Ga et al., 2022). Fast induced moulting has long been used for inducing moult in spent layers but this method of moulting has been criticized by advocates of animal welfare due to possible outcome of stress (Khan et al., 2013; Sabzian-Melei et al., 2022). Among various dietary manipulation techniques used for induction of moulting, zinc-induced moulting has been reported to exhibit beneficial effects on laying hens in terms of better post-moult performance owing to the possible role of zinc in reproductive traits (Anwar et al., 2013; Dong et al., 2025). This protocol has been less criticized, reported to be less stressful and superior to forced moulting by many researchers and the poultry welfare advocates (Khan et al., 2011; Khan et al., 2012; Silva-Mendonça et al., 2015; Adli et al., 2025). Effects of various supplementations for alleviating stress after zinc-induced moulting on overall production parameters, eggshell quality, immunity, mineral deposition, hormonal profile and immunocytochemistry of pituitary at various stages of production during second and third egg-laying cycle of spent layers have already been reported by many researchers (Anwar et al., 2012a, 2012b, 2013). However, the possible role of zinc-induced moulting on serum and tissue oxidative and anti-oxidative status at the time of moulting is still lacking.

Harmful oxidative free radicals are constantly produced from every cell of the body during normal physiological and metabolic processes (Sohail et al., 2011; Hashemitabar & Hosseinian, 2024). The increased oxidative stress in poultry birds could be due to many underlying stress factors and moulting phenomenon is also one of them (Sahin et al., 2009; Olayiwola & Adedokun, 2025). Augmented production of oxidant species due to these stressful conditions leads towards lowered production of various enzymes, minerals and vitamins which are a vital part of antioxidant defence system of the body (Sohail et al., 2013; Dong et al., 2025). Consequently, keeping in view the significant role of Zn-induced moulting in oxidative stress, the current study was conducted to evaluate the effect of Zn-induced moulting on certain indicators of oxidative stress at tissues and serum level (total oxidant status and total antioxidant capacity in serum and tissues; serum concentrations of homocysteine, ceruloplasmin, paraoxonase and arylesterase) in commercial White Leghorn layers.

MATERIALS AND METHODS

Experiment

Twenty-four commercial caged housed White Leghorn layers (67 weeks old) were procured from the commercial layer farm (Rana Poultry Farm, Faisalabad) and brought to the research station, Institute of Physiology and Pharmacology, University of Agriculture Faisalabad, Pakistan. The project proposal was approved by the members of the "Institutional Review Board and Ethical Committee for the Use of Animals in Research" at the Faculty of Veterinary Science, University of Agriculture Faisalabad, Pakistan (No. 14388-91). The evaluation committee believes that the likelihood and degree of discomfort or harm to the animal subjects expected during this study are not greater than those usually faced in daily life or during the performance of routine physical examinations or tests. The birds were provided with basal feed ration 100 g/bird per day (Table 1), ad-libitum water, light duration of 16 hours and caged housing having one bird per cage during acclimatization period. After an acclimatization period of three weeks, the birds were divided into moulted (ML; n= 12) and non-moulted layers (NML; n= 12) as described by Idris et al. (2023). The moulted birds (ML) were provided with light duration of 12 hours and layer ration 35 g/bird per day during the period of moulting. At the end of the twenty-one days zinc-induced moulting trial, remaining birds (n=12) were slaughtered for the collection of blood and tissue samples. Blood samples were collected in sterilized test tubes without anticoagulant for serum separation and with anticoagulant heparin (50 U/mL) to isolate lymphocytes for comet assay. The test tubes were kept in refrigerator, then centrifuged at 2000 × g for 20 minutes. Serum was harvested and stored at -20ºC till further analysis.

Preparation of tissue homogenate

The organs including spleen, liver, heart, kidney and muscle samples were separated from the birds at the time of sampling. Tissue homogenate (10% w/v) was prepared by triturating 0.1 g of tissue in 1000 µL of tissue homogenate buffer in a tissue homogenizer. Tissue Homogenate Buffer was prepared by using 1mM EDTA, 0.25 M sucrose, 15 mM Tris-HCL (pH 7.4) and 1mM DL-dithiothreitol by adopting the methodology as prescribed by Ruiz-Gutiérrez et al. (2001).

Table 1: Ingredients and composition of basal feed ration (g/100g of diet)

Ingredients	Inclusion level (%)
Corn	40
Rice Tips	10
Maize Gluten 30 %	6
Soyabean Meal	8
Fish Meal	6
Rice Polishing	11
Limestone Powder	7
Canola Meal	10
D.C.P	1.5
Vitamin mineral Premix[a]+ Amino acid[b]	0.5
Total Nutritive Value	100
Crude Protein	16
Energy	2795 Kcal

[a]Premix / kg feed: Cholecalciferol 2200 U; Riboflavin 5.5 mg; Vitamin A 8300 U; D-calcium pantothenic acid 15 mg; Niacin 36 mg; Choline 500 mg; Folic acid 0.5 mg; Vitamin B1 1 mg; Pyridoxine 2.2 mg; Biotin 0.05 mg; Vitamin K 2 mg; Vitamin E 8 U; Vitamin B12 0.02 mg; Manganese 80 mg; Zinc 60 mg; Iron 60 mg; Copper 5 mg; Cobalt 0.2 mg; Iodine 1 mg; Selenium 0.15 mg; [b]Amino Acids/kg feed: Lysine 0.72 g; Methionine, 0.143 g; Threonine, 0.35 g.

Laboratory analyses

The serum and tissue samples were analyzed in the laboratory to determine total oxidant status (TOS), total antioxidant capacity (TAC), and serum homocysteine (Hcy), ceruloplasmin, paraoxonase and arylesterase levels by adopting prescribed methodologies. Biosystem (Biosystems BTS-330, S.A. Costa Brava, Barcelona, Spain) spectrophotometer was used for analyses of TAC, TOS and other serum metabolites. However, UV/VIS spectrophotometer (Model U-2001, Hitachi instruments, Schaumburg, IL) was used for the spectrophotometric analyses of ceruloplasmin activity.

Total oxidant status (TOS; µmol/l of H_2O_2 equivalent)

The total oxidant status (TOS) in the serum and tissues of spent layers was estimated by adopting the described by Erel (2005). The total oxidant concentration in the serum sample was determined from the calibration curve prepared from different concentrations of hydrogen peroxide (H_2O_2) and TOS was expressed as µmol of hydrogen peroxide (H_2O_2) equivalent/*l*. The precision level of the TOS assay was up to< 3% and Intra assay coefficient of variance (CV) were kept under 10 %. The linearity of the assay was up to 200 µmol H_2O_2 equivalent /*l*.

Total antioxidant capacity (TAC; mmol/l of Trolox equivalent)

The method described by the Erel (2004) was used for the estimation of TAC in serum and tissue samples. The reaction rate was calibrated with trolox (Vitamin E synthetic analogue) which was used to form the standard curve for measuring TAC and was expressed as mmol/*l* equivalent of trolox. The precision level and intra assay CV for assay was below 3%. The linearity of the assay was 6 mmol trolox equivalent /*l*.

Homocysteine (Hcy; µmol/l)

The enzymatic activity of Hcy was determined spectrophotometrically through commercially available kit of Diazyme Laboratories (Ref. DZ568A; Gregg Court, Poway, CA USA) as per manufacturer's instructions. The intra assay CV was 2.2 to 5.5 percent and the detection limit of the assay was 1.5 µmol/*l*.

Ceruloplasmin activity (Unit/l)

Serum ceruloplasmin activity (U/*l*) was estimated and calculated by adopting the method elaborated by Anwar et al. (2012a) and using the formula as described by the Schosinsky et al. (1974). The minimum level of detection of assay was between 7 to 10U/*l*. The linearity of assay was 440 U/*l* in measured sample and CV was ≤ 4.2 %.

Paraoxonase activity (PON; Unit/min/l)

The method described by Juretić et al. (2006) was used for the estimation of paraoxonase activity in the serum samples. The final enzymatic activity of paraoxonase was expressed in Unit/min/*l* and the activity of paraoxonase was measured by using formula mentioned in reference methodology. Intra assay CV was below 10 % and the reaction rate/hydrolysis rate of paraoxon was stable up to 5 min. The minimum level of activity for this assay ranged from 80 to 100 U/min/*l*.

Arylesterase activity (KUnit/l)

The enzymatic activity of arylesterase was determined by adopting the methodology as described by Juretić et al. (2006). The enzymatic activity of the arylesterase was calculated by using the prescribed formula given in reference method. Intra assay CV was below 7% and initial rate of hydrolysis of paraoxon was stable up to 5 min.

Comet assay

The extent of DNA damage in blood lymphocytes of moulted and non-moulted hens was examined by performing single cell microgel electrophoresis or comet assay (Singh et al., 1988).

Statistical analysis

Statistical analysis was performed using Statistical Package for Life Sciences, Version 17 (SPSS Inc., Chicago, IL, USA). The results are reported as mean ± SE. Independent t-test was implied to monitor the difference between moulted and non-moulted group of layers.

RESULTS

Overall results for the status of the serum and tissue health biomarkers in spent moulted layers (ML) and non-moulted layers (NML) are given in Table 2.

The DNA damage level in terms of comet tail lengths (CTL's) did increase significantly in the lymphocytes of spent layers supplemented with high dietary zinc oxide (ZnO; 3 g/kg diet) in ML group as compared to the NML group.

The ML group revealed higher TOS levels in serum and all organs under study. Statistical significance was, however, noticed for liver (P<0.05), heart (P<0.01) and kidney (P<0.05). Regarding TAC, values in heart (P<0.05), kidney (P<0.01) and muscle (P<0.01) were significantly lower in ML than that in NML group. However, TAC level in serum of ML group was found significantly higher (P<0.01) as compared to NML group.

Serum homocysteine (Hcy) was significantly higher (P<0.01) whereas paraoxonase and arylesterase were lower (P<0.01) in ML group as compared to that in NML group. The concentration of ceruloplasmin was not significantly different among experimental groups.

Table 2: Overall status of health biomarkers (mean±S.E) in serum and tissues of the moulted and non-moulted White Leghorn layers.

Parameters	Non-Moulted	Moulted
Comet Tail length's	23.74±0.619	25.91±0.618*
Total Oxidant Status (TOS; µmol/L)		
Serum	1.26±0.15	1.32±0.06^{NS}
Spleen	0.63±0.15	0.66±0.10^{NS}
Liver	0.36±0.033	0.55±0.084*
Heart	0.061±0.003	0.86±0.07**
Kidney	0.19±0.04	0.45±0.10*
Muscle	0.11±0.03	0.14±0.03 ^{NS}
Total Antioxidant Capacity (TAC; mmol/L)		
Serum	0.45±0.05	1.64±0.01**
Spleen	1.06±0.11	0.92±0.04 ^{NS}
Liver	0.40±0.03	0.33±0.06^{NS}
Heart	0.90±0.018	0.84±0.013*
Kidney	0.51±0.05	0.32±0.02 **
Muscle	0.86±0.10	0.49±0.08**
Serum Enzymatic Activity		
Paraoxonase (Unit/L)	443.77±7.34	343.57±10.6**
Arylesterase (Unit/L)	182.1±9.64	147.26±5.29**
Ceruloplasmin (Unit/L)	26.22±6.13	23.71±1.19^{NS}
Homocysteine (µmol/L)	21.01±2.17	76.06±3.95**

*Significant at (P≤0.05) **Significant at (P≤0.01) NS = non-significant.

DISCUSSION

Zinc-induced moulting has widely been adopted as a management tool for better results regarding health, production and reproduction of laying hens. Various studies have proved this technique as a less harmful/stressful method than other conventional methods for the induction of moult (Khan et al., 2011; Mishra et al., 2022). In the current study, the increased TOS at some tissues level, and decreased TAC at tissue level in moulted group showed that Zn-induced moulting apparently didn't show any significant impact to maintain the balance between oxidative and anti-oxidative capacity at tissue level of moulted group. However, increased serum TAC in ML group reveals the replenishing efficacy of Zn inclusion in the moulting diet (Sahin & Kucuk, 2003). Moulting stress and lack of proper nutrient availability because of lower feed intake and suppression of appetite center after zinc supplementation in Zn-induced moulting could be suggested as the underlying factor for increased oxidative stress in serum and tissues of ML group (Park et al., 2004).

Previously, the dietary $ZnSO_4$ (60 mg/kg) have been demonstrated for increased TAC and decreased oxidative stress in heat stressed Japanese quails (Sahin & Kucuk, 2003) by increasing bodily Zn, vitamin C and E along with decline in malondialdehyde (MDA; oxidative stress biomarker). Zinc enhances antioxidant capacity and prevents lipid per-oxidation by decreasing the augmented production of free radicals (Park et al., 2004).

Zinc enhances TAC at serum level that might be attributed to an increased concentration of Zn in serum of the ML group (Sahin et al., 2006; Yang et al., 2024).

Increased TOS at tissue level in current study is similar as reported by Lü & Combs (1988) who revealed that dietary Zn in chicken decreased antioxidant status at tissue level by interfering in vitamin-minerals based antioxidant defense system. Similarly, Hughes & Samman (2006) demonstrated that increasing the supplemented dose of Zn, lowered the activity of antioxidant enzyme-superoxide dismutase (SOD), resulting in an augmented generation of reactive oxygen species (ROS). The production of these ROS or pro-oxidant species because of failure of antioxidant defense system disturbs overall balance of oxidative-antioxidant capacity at cellular level, leading towards the oxidative damage (Lü & Combs, 1988; Yeni et al., 2005). So, Zn given to induce molt failed to establish high TAC at tissue level and resulted in an increased TOS in spent layers which could be because of interference of ZnO with other antioxidant defence systems like SOD in addition to the stress levied upon the birds due to moulting phenomenon.

Homocysteine is an abnormal amino acid which is an oxidative stress biomarker synthesized during methionine metabolism (da Costa et al., 2005). It was found to be significantly higher in ML groups of our study which is in line with earlier published reports (Hong et al., 2000). It has been established that homocysteine has a direct relationship with Zn concentration in body as any increase or decrease in Zn concentration effects methionine synthase activity which in turn is responsible for corresponding change in plasma homocysteine concentration. The deficiency of Zn has been reported to increase methionine synthase activity in rats which in turn is responsible for lowering plasma homocysteine level (Hong et al., 2000). We have already reported that feeding ZnO for inducing moult in layers exhibits an increasing effect on serum homocysteine levels, which was sustained until 5 % production level after moulting (Anwar et al., 2012a). The increased enzymatic activity of homocysteine in serum has been correlated with stressful conditions like heat stress, moulting stress etc. in birds (Sahin et al., 2006). In the present study, an increased oxidative stress in terms of enhanced TOS and decreased methionine synthase activity after high dietary ZnO (3 g/kg) can be attributed to an increased serum homocysteine levels in moulted spent layers (Hong et al., 2000; Vázquez-Lorente et al., 2022).

Paraoxonase and arylesterase are antioxidant enzymes and usually work as a single enzyme (Elkiran et al., 2007). In current study, concentration of both enzymes decreased significantly in the ML group as compared to the NML group. Paraoxonase and arylesterase play a pivotal role in protection of body against lipid per-oxidation and have been reported to have an inverse relation with the oxidative stress of the body (Rozenberg et al., 2003; Juretić et al., 2006). The lowered concentration can be attributed to an increased oxidative stress after moulting (Anwar et al., 2012a). However, possible role of dietary zinc for inhibiting the activity of these antioxidant enzymes cannot be neglected as dietary Zn under its normal physiological concentration has been reported to inhibit the enzymatic activity of these enzymes (Debord et al., 2003).

Zinc-induced moulting by dietary ZnO @ 3g/kg for 3 weeks increased TOS in tissues of spent layers. Though the balance between oxidative levels enhanced in spent layers at a tissue level, Zn enhanced serum antioxidant capacity. Furthermore, Zn-induced moulting resulted in a significant decrease in paraoxonase and arylesterase activity in moulted spent layers which is an index of stress in these birds. Supplementation of ZnO is a beneficial mineral supplement to be included in the moult diet being an antioxidant, which will be helpful in alleviation and early recovery from moulting stress. This early recovery from moulting stress will also be fruitful for early production recovery in second production cycle with a better performance.

Dietary zinc provides protection against DNA damage by up regulating the zinc associated proteins that are involved in DNA repair mechanisms. The DNA damage level in terms of comet tail lengths (CTL's) increased significantly in the lymphocytes of moulted layers as compared to the non-moulted layers. Zinc regulates the transcription and replication of DNA through the zinc-associated proteins like tumor suppressor protein p^{53}. The p^{53} protein is involved in regulating cell proliferation, DNA repair and apoptosis and can arrest the cell cycle at G1 phase allowing the sufficient DNA repair before the start of cell division (Lane, 1992; Levine, 1997). Zinc inclusion above the normal physiological requirement may result more harm than benefits. In-vitro high zinc and vitamin C supplementation results increased oxidative DNA damage in human lymphocytes as compared to the lymphocytes supplemented with low level of zinc and vitamin C (Harréus et al., 2005). The increase in CTL's in the lymphocytes of zinc supplemented layers might be due to the increased oxidative DNA damage to lymphocytes as a result of 3 g/kg dietary zinc supplementation. Therefore, some lower doses of ZnO should be evaluated to achieve possible production goals after induced moulting.

Limitation and future direction

The study was the part of a research project that was funded by the Higher Education Commission of Pakistan to evaluate the impact of different zinc supplementation strategies to ameliorate the impact of zinc-induced moulting in White Leghorn layers. Although the results related to oxidative biomarkers presented in this paper gives a snapshot of the impact of moulting on oxidative status of spent layers, the results consisted of very important information regarding physiology of moulting in spent layers. This was a longitudinal study where authors have a limited control over the study to collect samples during trial. The collection of data more frequently during zinc-induced moulting would give a better understanding related to changes on the health and physiology of spent layers during zinc-induced moulting.

Conclusion

The supplementation of dietary zinc oxide (3 g/kg diet) for 3 weeks resulted in increased oxidative stress and increased DNA damage in spent layers. Supplementation of zinc to spent layers with different lower level of ZnO should also be further experimented to maximize the beneficial effect of zinc oxide (ZnO) supplementation and to minimize the stress on spent layers.

DECLARATIONS

Competing Interest: The authors declare that they have no competing interest with anyone.

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Conflict of Interest: The authors declare no conflifct of intrest in this study.

Data Availability: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics Statement: The institutional review board and ethical committee to use animals in research at the faculty of Veterinary science of the University of Agriculture, Faisalabad, Pakistan approved all the experimental processes that involved animals and were performed in respect of the national and international standards on the handling and use of laboratory animals.

Author's Contribution: Conceptualization, MI and HA; Methodology, MI and HA; Statistical analysis, MI and AI; Investigation and Data Curation, MI, HA; Writing-original draft preparation, MI; writing-review and editing, MI, HA, FM, UF, AI and MAA All authors have read and agreed to the published version of the manuscript.

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