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Impact of crystalline progesterone on serum biochemical parameters in Lohmann Brown layers

Ahmed, B.[1]* and Yahaya, A.[2]

[1]Department of Animal Science, Umaru Musa Yar'adu University P.M.B 2218, Katsina State, Nigeria.
[2]Department of Animal Science and Technology, Federal University of Technology Owerri, Imo State, Nigeria.

*Corresponding author. Email: bishirahmed944@gmail.com; Tel: +2347030286505.

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ABSTRACT: Despite the important role of progesterone in egg production, the information on its impact on serum biochemistry is still scanty. This study was therefore carried out to identify and assess the impact of various doses of crystalline progesterone (CP) on serum biochemical parameters in Lohmann Brown layers over six weeks. A completely randomized design was used, with each treatment (0, 5, 10, 15, 20, and 25 mg per bird) of crystalline progesterone being administered intramuscularly via the breast muscle and replicated thrice. Data were analyzed using the GraphPad InStat®package. Results indicated significant changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (10 mg vs. 25 mg = 294 µmol/L vs. 152 µmol/L), non-significant (P>0.05) effect of CP serum glucose, blood urea nitrogen, and total protein. There was significant (p<0.05) effect of CP on median serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, calcium (5 mg vs. 20 mg = 3.6 mmol/L vs. 2.9 mmol/L), phosphorus (20 mg vs. 25 mg = 1.6 mmol/L vs. 2.8 mmol/L), cholesterol (0 mg vs. 25 mg = 3.1 mmol/L vs. 1.7 mmol/L) and albumin (20 mg vs. 25 mg = 22 g/L vs. 20 g/L) levels. Thus, these findings suggest potential implications in reproductive health and metabolic functions in poultry as CP administration diminishes the synthesis of calcium used in egg shell formation, which is a great loss for producers' income.

Keywords: Biochemistry, crystalline progesterone, impact, layers.

INTRODUCTION

Serum constitutes an important panel in assessing the health status, physiological, and reproductive conditions of animals (Gbolabo *et al*., 2015). It comprises proteins, enzymes, hormones, vitamins, and minerals. Fractions of serum, such as calcium and phosphorus, are essential for reproduction, and they are required throughout the lifetime of animals (Spears, 1999; Schroeder, 2004). Andrieu (2008) and Lopez-Alonso (2012) highlighted the role of minerals in the animal body, especially in reproduction and immunity, and their analyses assist in determining the health status of the animal. In order to diagnose tissue and liver damage, the liver function enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are much more important in diagnosing tissue and liver damage (Senanayake *et al*., 2015).

According to Hodgson *et al*. (2000), hormones are chemicals released into the bloodstream by ductless glands that modify the activity of their target cells. Hormones are regarded as integral components of reproduction in farm animals (Elnagar *et al*., 2002). Numerous hormones, including luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2), testosterone, and progesterone P4, are involved in reproductive function in chickens (Bigsby *et al*., 2005; Evans, 2007). Synthetic hormones have been used in animal agriculture to improve reproduction and performance (Qaid and Abdoun, 2022).

The primary steroid hormone in avian species, progesterone (P4), is produced by the granulosa cells of the giant ovarian hierarchical follicle (F1) in laying hens (Navara, 2013). Progesterone activity induces luteinizing hormone (LH) surge for ovulation (Nie *et al.*, 2024). Progesterone induces the production of the protein avidin, which is associated with deposition of egg white in the

poultry oviduct (Elnagar *et al.,* 2002). Progesterone is reported to affect the regulation of calcium metabolism, egg shell formation, and enhance growth in laying hens (Lu *et al.,* 2024).

Evaluation of immune responses of the poultry has become possible by analysing serum biochemical and haematological measures that have been shown to offer important insights into the birds' immunological status (Kral and Suchy, 2000). Proper understanding of the metabolic profile of the avian species, including serum mineral and biochemical indicators, is very important in understanding the health status and physiology of animals (Harr, 2002). This study hypothesises that varying doses of Crystalline Progesterone will significantly alter serum biochemical parameters in Lohmann Brown layers. The specific objective of this study was to examine dynamics in serum biochemistry of Lohmann Brown laying hens following exogenous administration of progesterone.

MATERIALS AND METHODS

Husbandry of birds

A total of eighteen birds of 24 weeks old Lohmann brown strain of layers, weighing an average of 1.8 kg, were purchased from Sovet International Farm Limited, Tarauni, Kano. Two weeks before the purchase of the layers, the poultry house and associated facilities were inspected, cleaned, and sanitised. The birds were managed at the Poultry Unit of the Teaching and Research Farm, Department of Animal Science, Faculty of Agriculture, Bayero University Kano (GPS Coordinates: 11.97643°N, 008.42995°E). In order to prevent stress and secondary bacterial infections, the hens were given water containing multivitamins (Anupco Vitalyte Extra®, Anglian Products Company, UK) at a rate of 0.5 g per litre for five days and Oxytetracycline Hydrochloride powder (Oxywin®, Sellwell Pharmaceuticals Ltd, India) at a rate of 1 g per litre for three days. Before the trial started, the birds were treated with a topical spray of cypermethrin butylcarbityl 6-properonol (Zed on®, Dappo Limited, Farm Centre, Kano) to protect them from external parasites. The birds were kept for two weeks acclimatization prior to data collection. Throughout the period of acclimatisation and experimentation, the experimental birds were fed layer mash (Super Layer®), which included 16.0% crude protein, 5.0% fat, 6.0% fibre, 3.5% calcium, 0.4% phosphorus, and 2600 kcal/kg energy. Water was also given *ad libitum*. Water was periodically changed, and drinkers were cleaned.

Progesterone administration experimental design

Crystalline progesterone (Gesteron-25®) was purchased in 1 ml ampoules of 25 mg per ml from Wellcare Pharmaceuticals, Kano. The treatment groups received Crystalline Progesterone injections intramuscularly via the breast muscle at doses of 0, 5, 10, 15, 20, and 25 mg/bird. The control group, three birds designated treatment (A) were injected with a placebo, 1 ml of normal saline. Injections were given twice per week at an interval of two days apart in the morning (between 10.00 am and 11:00 am) throughout the experimental period of six weeks. The experiment was laid out in a completely randomised design.

Blood sample collection, serum harvesting, and serum biochemistry

At 30 weeks of age, 5 ml of blood samples were collected from the wing vein as described by Bermudez and Stewart-Brown (2003), using a sterile syringe. The spot where blood is to be collected was swabbed thoroughly with a clean cotton wool dipped in methylated spirit. The blood vessel was engorged by gentle tapping, after which the sterile needle was inserted into the vein. Afterwards, 3 ml of the collected blood was transferred into sterile EDTA universal bottles for haematological analysis, while 2 ml was transferred into a plain bottle without EDTA for serum biochemical analysis. Blood sample was transported to the laboratory on ice pack in a Styrofoam box for further processing.

In order to allow for clotting, blood samples were left at room temperature for two hours. A blunt wooden stick was used to dislodge the clot. The remaining solution was centrifuged (Centrifuge 800D®, Techmel & Techmel, USA) for 30 minutes at 4000 revolutions per minute to recover the serum. The serum was transferred into labelled plain tubes using a dropping pipette. The harvested serum was stored at -20ºC until further analysis.

Serum stored at -20°C was allowed to thaw at room temperature. The samples were incubated using a laboratory incubator (DIGITAL TT9052®, Techmel & Techmel, USA). The results of serum analytes were read using a colorimeter (Colorimeter 257, CIBA CORNING®) at a wavelength of 546 nm using principles, methods and reagents based on the instructions of the manufacturers of the respective commercial kits. Globulin concentration in serum was derived from the difference between total protein and albumin.

Data analysis

Data were analysed with GraphPad InStat Statistical Package (GraphPad InStat®, version 3.05, 32-bit for Win 95/NT, GraphPad Software Inc., 2000), using the Kruskal-Wallis test followed by Dunn’s Multiple Comparisons test to identify significant differences between treatment groups. The completely randomised design was chosen to minimise variability and ensure robust results.

Table 1. Selected Pairs Dunn's multiple comparisons of serum biochemical across crystalline progesterone treatment levels in Lohmann Brown.

Parametres	Comparisons	Mean rank difference	Median concentrations	level of significance
Alanine Aminotranferase (U/L)	00 mg *vs*. 25 mg	10.667	3.0 (U/L) *vs.*10.0 (U/L)	*
	10 mg *vs*.15 mg	-11.250	3.0 (U/L) *vs.*10.5 (U/L)	*
	10 mg *vs*. 25 mg	-10.667	3.0 (U/L) *vs.*10.0 (U/L)	*
Aspartate Aminotranseferase (U/L)	05 mg *vs.* 25 mg	-12.000	36.0 (U/L) *vs.* 59.0 (U/L)	*
	10 mg *vs*. 25 mg	-13.000	10.0 (U/L)*vs.* 59.0 (U/L)	*
Alkaline Phosphatase (U/L)	00mg *vs.* 20 mg	13.333	64.0 (U/L) *vs.* 17. (U/L)	*
Creatinine (µmol/L)	10 mg *vs.* 25 mg	12.500	294.0 (µmol/L) *vs.* 152.0 (µmol/L)	*
Calcium (mmol/L)	05 mg *vs.* 20 mg	13.667	3.6 (µmol/L) *vs.* 2.9 (µmol/L)	**
	00 mg *vs.* 5 mg	9.167	2.8 (µmol/L)*vs.* 1.6 (µmol/L)	*
Phosphorus (mmol/L)	00 mg *vs.* 20 mg	9.833	2.8 (µmol/L) *vs.* 1.6 (µmol/L)	*
	20 mg *vs.* 25 mg	-10.667	1.6 (µmol/L) *vs.* 2.8 (µmol/L)	**
Cholesterol (mmol/L)	00 mg *vs.* 25 mg	10.667	3.1 (µmol/L) *vs.* 1.7 (µmol/L)	*
Serum Glucose (mmol/L)	00 mg *vs.* all	-	-	*ns*
Blood Urea Nitrogen (mmol/L)	00 mg *vs*. all	-	-	*ns*
Total Protein(mmol/L)	00 mg *vs*. all	-	-	*ns*
Globulin (g/L)	00 mg *vs*. all	-	-	*ns*

*p<0.05, **p<0.01; ns = not significant >0.05

RESULTS

Serum alanine aminotransferase

The effect of crystalline progesterone on serum alanine aminotransferase concentration in Lohmann brown layers is shown in Table 1. There was a statistically significant (P<0.05; mean rank difference = -10.667; Kruskal-Wallis statistic = 13.797) difference in median serum alanine aminotransferase concentration (3.0 U/L *vs*. 10.0 U/L) between birds administered crystalline progesterone at 0 and 25 mg. Also, a significant (p<0.05; mean rank difference = -11.250; Kruskal-Wallis statistic = 13.797) difference was recorded in median serum alanine aminotransferase concentration (3.0 U/L *vs*. 10.5 U/L) between birds administered 10 and 15 mg crystalline progesterone. Similarly, a significant (p<0.05; mean rank difference = -10.667; Kruskal-Wallis statistic = 13.797) difference in serum median alanine aminotransferase concentrations (3.0 U/L *vs*. 10.0 U/L) was recorded between birds administered crystalline progesterone at 10 and 25 mg.

Serum aspartate aminotransferase

Serum aspartate aminotransferase concentration in Lohmann brown layers is highlighted in Table 1. There was a statistically significant (P<0.05; mean rank difference = -12.000; Kruskal-Wallis statistic = 14.670) difference in median serum aspartate aminotransferase concentration (13.0 U/L *vs*. 59.0 U/L) between birds administered crystalline progesterone at 5 and 25 mg as well as significant (P<0.05; mean rank difference = - 13.000; Kruskal-Wallis statistic = 14.670) median serum aspartate aminotransferase concentrations (10.0 U/L *vs*. 59.0 U/L) between birds administered crystalline progesterone at 10 and 25 mg.

Serum alkaline phosphatase

The conferred result of serum alkaline phosphatase concentration in Lohmann brown layers reveals, statistically significant (P<0.05; mean rank difference = 13.333; Kruskal-Wallis statistic = 14.054) difference was recorded in serum median alkaline phosphatase concentration (64 U/L *vs*. 17 U/L) between 0 and 20 mg crystalline progesterone dose levels. All other comparisons among crystalline progesterone doses gave statistically similar (p>0.05) alkaline phosphatase concentrations.

A statistically significant change in Serum creatinine was observed (p<0.05; mean rank difference = 12.500, Kruskal-Wallis statistic = 13.885). Every other analogy between crystalline serum creatinine concentrations was statistically equivalent (p>0.05) based on progesterone levels.

Serum calcium

The effect of crystalline progesterone on serum calcium concentration in Lohmann brown layers is statistically significant (p<0.01; mean rank difference = 13.667, Kruskal-Wallis statistic = 14.387). A difference was recorded

in serum calcium concentration (3.6 mmol/L *vs*. 2.9 mmol/L) between the 5 and 20 mg crystalline progesterone groups. All other comparisons among crystalline progesterone treatment levels gave statistically similar ($p>0.05$) serum calcium concentrations.

Serum phosphorus

The effect of crystalline progesterone on serum phosphorus concentration in Lohmann brown layers was statistically significant ($p<0.05$; mean rank difference = 9.167; Kruskal-Wallis statistic = 13.245). A difference was recorded in median serum phosphorus concentration (2.8 mmol/L *vs*. 1.60 mmol/L) between the control and birds administered 5 mg crystalline progesterone. Also, significant ($p<0.05$; mean rank difference = 9.833; Kruskal-Wallis statistic = 13.245) difference in median serum phosphorus concentration (2.80 mmol/L *vs*. 1.60 mmol/L) was recorded between the control and 20 mg crystalline progesterone treatment levels as well as significant ($p<0.01$; mean rank difference = -10.667; Kruskal-Wallis statistic = 13.245) difference in serum median phosphorus concentration (1.60 mmol/L *vs*. 2.80 mmol/L) between 20 and 25 mg crystalline progesterone treatment levels.

Serum cholesterol

The effect of crystalline progesterone on serum cholesterol concentration in Lohmann Brown layers is shown in Table 1 was statistically significant ($p<0.05$; mean rank difference = 10.667; Kruskal-Wallis statistic = 12.323). A difference was recorded in median serum cholesterol concentration (3.1 mmol/L *vs*. 1.7 mmol/L) between the 0 and 25 mg crystalline progesterone groups. All other comparisons among crystalline progesterone treatment levels gave statistically similar ($p>0.05$) of serum cholesterol concentrations.

DISCUSSION

Juvenile Budgerigars had greater plasma glucose levels than adult Budgerigars, as reported by Senanayake *et al*. (2015). Additionally, variations are caused by the time of day and the level of environmental stress, which was in agreement with the findings reported by Navara (2013). Fasting birds' plasma glucose concentrations follow a circadian cycle (Lu *et al.,* 2024). Progesterone administration in rats resulted in an increased amount of insulin concentration in plasma in response to intravenous control of glucose in the rats (Kalas *et al*., 2021). Since islets of Langerhans extracted from the pancreas of progesterone-treated rats have been shown to produce greater levels of insulin *in vitro*, this is most likely the result of enhanced pancreatic secretion of insulin (Senanayake *et al*., 2015). The experimental findings of numerous studies propose that progesterone treatment reduced insulin sensitivity (Nie *et al.,* 2024; Lu *et al.,* 2024). This could be an indirect reason for the lack of significant changes in median serum glucose level in the present study, which was within the range of glucose values (7.0-13.2 mmol/L) reported by Nanbol *et al.* (2016). Sex hormone concentration is reported to influence insulin sensitivity, which in turn affects serum glucose level (González *et al*., 2000).

Aminotransferases, such as aspartate (AST) and alanine (ALT), are a class of enzymes that catalyse the transfer of amino groups to convert amino acids and oxoacids (Navara, 2013). When paired with other more focused testing, elevated AST activity offers the most insight into liver or muscle injury (Navara, 2013; Nie *et al*., 2024; Lu *et al*., 2024). Muscle injury cannot be the reason for elevated AST activity if creatine kinase (CK) activity is present (Nie *et al*., 2024; Lu *et al.,* 2024). Median values for ALT and AST in the current Juvenile birds have significantly higher alkaline phosphatase (ALP) activities from bone growth and development than adults (as cited by Navara, 2013). In hens, activities of ALP are elevated prior to egg laying (Nie *et al.,* 2024; Lu *et al.*, 2024). Seasonal changes in ALP activities have been described in birds as cited by Navara (2013). Low ALP activities were reported in the pigeon liver, with no activity in other organs of pigeons (as cited by Navara, 2013). Similar findings have been described in chickens (Kalas *et al.,* 2021) and turkeys (Lu *et al.,* 2024). Despite the fact that a decrease in ALP was recorded in the current study, the median value in the 20 mg (CP) group was much higher than ALP values reported by Nanbol *et al*. (2016) for 16- to 52-week layers. It has been shown that in the fowl, exogenous estradiol increases serum ALP, which is reduced by concomitant administration of progesterone (Qaid and Abdoun, 2022). Most enzyme assays are used to document damage to cells, resulting in enzyme release. In contrast, plasma ALP activity is induced by increased cellular activity (increased synthesis) rather than cell damage (Kalas *et al.,* 2021. Therefore, it implies that there is a decrease in cellular activity of ALP in the 20 mg CP group in the present study, which may explain the corresponding decrease in serum ALP activity.

Because urea is found in avian plasma in trace amounts, measuring urea levels has traditionally been seen as having little significance (Kalas *et al*., 2021). Nonetheless, studies have demonstrated a strong link between elevated plasma urea levels renal illness (Sheldon *et al*., 2007). Urea can be a sensitive marker of dehydration in some bird species, but it may not be useful in identifying renal illness in other birds (Kalas *et al*., 2021). In the current study, the non-significant blood urea nitrogen level could imply that experimental birds were properly hydrated, indicating that almost all of the filtered urea is excreted (Kalas *et al*., 2021).

The reference range for creatinine in avian species has

been shown to be between 0.1 and 0.4 mg/dL (8.84 and 35.36 µmol/L), with no discernible variation among species (Kalas *et al*., 2021). Elevated creatinine levels may be a sign of severe kidney disease, particularly if the filtration rate is reduced. Because of variations in the analytical equipment or measurement techniques employed, the serum creatinine level found in this investigation was higher than that reported by Kalas *et al*. (2021). Sheldon *et al.* (2007) stated that plasma creatinine concentration is of questionable value in evaluating renal function in birds. The value of creatinine determined by conventional methods includes pseudocreatinines such as glucose, protein, ascorbic acid, and pyruvic acid and may not reflect the small quantity of creatinine generated from creatine (Sheldon *et al*., 2007; Lanje *et al*., 2010).

The amounts of calcium in ovulating chickens are substantially higher than in non-reproductive females. It was discovered that the calcium concentrations in female budgerigars were considerably higher than those in males (Kalas *et al.,* 2021). Low serum calcium levels in humans may be caused by higher progesterone than oestrogen levels during the luteal phase (Lanje *et al.,* 2010). A similar scenario may explain the decrease in median serum calcium level in the 20 mg (CP) group in the current study, since the high amount of CP mimics the luteal phase. Previous studies reported that progesterone produced an elevation in serum levels of calcium, although the effect was significantly smaller than that produced by estradiol, as reported by Preda *et al.* (2013), who found a strong correlation between the alteration in serum calcium homeostasis and parathormone, vitamin D, oestrogen, and progesterone. When progesterone induces hypocalcemia, the parathyroid glands may secrete more in response (Kalas *et al*., 2021). By implication, CP diminishes serum calcium, thus leading to the production of shell-less eggs, which affect producer income (Fu *et al*., 2024)

Certain cases of severe renal injury have elevated plasma inorganic phosphate levels (Kalas *et al*., 2021). Owing to hypervitaminosis from vitamin D (Preda *et al*., 2013), secondary hyperparathyroidism is caused by diet and low parathyroid hormone (Fu *et al*., 2024; Lu *et al.,* 2024). Fluctuation in serum phosphorus may have no severe impact on the layer, even though it is required in eggshell formation (Sinclair-Black *et al.,* 2023).

According to Preda *et al*. (2013), the Plymouth Rock hens' plasma protein levels were essentially steady, averaging 43.17 mg/ml and 46.66 mg/ml (46.66 g/L) in the Cornish hens. Median serum total protein values obtained in the present study were greater than values reported by Preda *et al*. (2013) and were within the range (40-65 g/L) reported by Nanbol *et al*. (2016) for 16- to 52-week layers. This implies that the dose of CP used in the current study did not significantly influence serum total protein levels. Preda *et al.* (2013) used plasma to determine total protein, while the present study used serum, just like that of Nanbol *et al.* (2016). Because fibrinogen is included in plasma protein values rather than serum protein, there is a slight discrepancy in the total protein concentration between the two. Plasma total protein values were on average 1.74 g higher than serum values in the pigeon (Fu *et al.,* 2024). The effect of CP on total protein could be beneficial at the early stage of growth and development to enhance hormonal signaling and liver synthesis to meet the demand in muscle development (Kasarinaite *et al.,* 2023).

Plasma albumin levels in Plymouth Rock chickens ranged from 16.0 to 20.0 mg/ml (16.0--20.0 g/L) and in Cornish hens from 19.8 to 22.8 mg/ml (19.8-22.8 g/L), with notable variations between the two groups (Preda *et al*., 2013). The serum albumin level in the current work falls within the range reported by Preda *et al.* (2013) as opposed to values reported by Nanbol *et al.* (2016), where the lowest median serum albumin level in the present work was out of their reported range of 22.0 to 40.0 g/L.

It is well known that progesterone lowers cholesterol (Amir and Fessler, 2013). The current study's findings on the lowering of serum cholesterol in birds treated with 25 mg of crystalline progesterone may be due to the blockage of the delivery of cholesterol derived from Low-Density Lipoprotein (LDL) to processing enzymes such as acetyl-Coenzyme A acetyl transferase (ACAT). According to Fu *et al.* (2024), the transfer of sterols from the plasma membrane to the endoplasmic reticulum is necessary for the synthesis of cholesterol. As a result, progesterone's inhibition of the transport of LDL-derived cholesterol also hinders the production of cholesterol (Amir and Fessler, 2013). At the organismic level, exogenous progesterone has been shown to reduce High-Density Lipoprotein (HDL) cholesterol both when administered as progestin-only oral contraceptives (Lu *et al.,* 2024) and when administered as hormone-replacement therapies (Lamon-Fava *et al*., 2006). Crystalline progesterone administration in Lohmann Brown layers significantly influenced key serum biochemical parameters, indicating its impact on liver enzymes, kidney function, and mineral metabolism.

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