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IMPACT OF CRYSTALLINE PROGESTERONE ON SPLEEN HISTOMORPHOMETRY AND SHELL GLAND HISTOLOGY IN LOHMANN BROWN LAYERS

[1]Ahmed, B. and [2]Yusuf, A.

[1]Department of Animal Science, Umaru Musa Yar'adua University P.M.B 2218, Katsina State

[2]Department of Animal Science, Federal University, Dutsin-Ma Katsina State

Corresponding Author: bishirahmed944@gmail.com,+2347030286505

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ABSTRACT

The experiment determined the effect of Crystalline Progesterone (CP) on spleen histomorphometry and shell gland histology in Lohmann Brown layers. Completely randomized design was used with each treatment (0, 5, 10, 15, 20 and 25 mg per bird) administered intramuscularly via the breast muscle and replicated thrice for six weeks. The birds were managed on battery cage, fed commercial diet and water was given *ad libitum* Data were analyzed using GraphPad InStat® package. Results revealed there were significant difference (P<0.05) in proportion of spleen red pulp (0 mg vs. 15 mg = 80% vs. 50%; 5 mg vs. 15 mg = 80% vs. 50% and 5 mg vs. 15 mg= 80% vs. 50%) and ellipsoids and peri-arteriolar lymphoid sheath (PALS) (0 mg vs. 15 mg = 10% vs. 50%; 5 mg vs. 15 mg = 10% vs. 50% and 10 mg vs. 15 mg = 15% vs. 50%). Whereas, (P>0.05) effect of CP proportion of spleen vascular skeleton and B follicles Histological investigation conferred similar magnitude (>10 fat cell aggregates) of shell gland fat infiltration between 10 and 15 mg CP treatment groups. It also showed non atrophic change at all doses. Beyond 15 mg CP, the magnitude of shell gland fat infiltration decreased to 6-10 fat cell aggregates and subsequently remained the same. Shell gland in the 5 mg CP group had no fat infiltration when compared to those in the control group which had 1-3 fat cell aggregates. It has been concluded that, CP affected proportion of spleen red pulp and ellipsoids and PALS., In the same vein affected, shell gland fat infiltration.

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INTRODUCTION

Productivity is the key to growth and reproduction status of our farm animals (Verma *et al*., 2012). Sexual maturity and egg production in birds is controlled by many factors including hormones especially oestrogen that plays important role in reproductive performance while progesterone is related to ovulation process (Rozenboim *et al*., 2004; Rozenboim *at al*., 2007). Synthetic hormones have been used in animal agriculture to improve reproduction and performance (Ledda *et al*., 1999). Spleen in avian species is a round or oval structure lying dorsal to and on the left side of the proventriculus (Payne, 1971). Eerola, et al. (1987) observed that splenic development occurs after hatching, following exposure to antigens. Spleen is a principal organ of systemic immunity and its importance in disease resistance is accentuated by the scarcity of avian lymph nodes (John, 1994). It also used as primary organ of systemic body defense and plays important role against invasion of pathogenic organisms into the animal's body (Kannan *et al.,* 2015). The main function of the spleen is filtering of blood and production of immunoglobulins and lymphocytes (T-help) involved in body defense (Kopp, 1990). It does not function as a reservoir of blood as in mammals and its function is not oriented towards supply of oxygen (Jeurissen, 1991). Spleen in birds has attracted the interest of many scientists (Corbin *et al*., 2008). Thus, the spleen is a greater asset in the study of immune response as producer and a store for lymphocytes (Smith & Hunt, 2004).

Shell gland is the segment of the oviduct immediately succeeding the isthmus and of similar diameter but after a short course, expands to form a pouch in which the egg is retained during the entire period of shell formation (Mohammadpour *et al.,* 2012). The shell gland is

involved in deposition of albumen which occurs within 19 to 20 hours before oviposition and strengthening of shell membrane (inner layer) of the egg (Nys & Guyot, 2011). The hard shell is formed in the shell gland 1.5 to 2.0 hours before oviposition (Nys & Guyot, 2011). Despite the important role of progesterone in egg production, the information on its impact on spleen histomorphometry and gland histology is still scanty. The specific objective of the study is to evaluate spleen histomorphometry and assess changes in shell gland histology induced by Crystalline Progesterone administration in Lohmann Brown laying hens.

MATERIALS AND METHODS

Experimental Birds and their Management

A total of eighteen 24-week old Lohmann Brown strain of layers were purchased from Sovet International Farm Limited, Tarauni, Kano. Two weeks before purchase of the layers, the poultry house and associated facilities were inspected, cleaned and sanitized. On arrival to the Poultry unit of the Teaching and Research Farm of the Department of Animal Science, Faculty of Agriculture, Bayero University Kano (GPS Coordinates: 11.97643ºN, 008.42995ºE), the hens were given water with multivitamins (Anupco Vitalyte Extra®, Anglian Products Company, UK) at 0.5 g per litre and Oxytetracycline Hydrochloride powder (Oxywin®, Sellwell Pharmaceuticals Ltd, India) at 1 g per litre as prophylactic for stress and secondary bacterial infections, respectively. Topical spray of cypermetrin butycarbityl 6-properonol (Zee on®, Dappo Limited, Farm Centre, Kano) was used on the birds against external parasites before the commencement of the study. The birds were allowed to acclimatize to the new environment for two weeks before commencement of the experiment. The experimental birds were fed layer mash (Super Layer®) which contained 16.0% crude protein, 5.0 % fat, 6.0 % fibre, 3.5 % calcium, 0.4 % phosphorus, and 2600 kcal/kg energy and water was provided *ad libitum* throughout the period of acclimatization and experimentation. Drinkers were washed and water replaced periodically.

Sample Size Determination and Experimental Design

Sample Size

The procedure of Mead, Gilmour and Mead (2012) was used to determine the sample size.

Experimental Design

The experiment was laid in a single factor completely randomized design with six treatment groups replicated three times. Six treatments were assigned as 0, 5, 10, 15, 20 and 25 mg Crystalline Progesterone levels corresponding to treatments A, B, C, D, E and F, respectively.

Progesterone Administration

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 5, 10, 15, 20 and 25 mg/bird. The control group was injected with 1 ml normal saline. Injections were being given on Mondays and Thursdays in the morning between 10.00 am and 11:00 am throughout the experimental period of six weeks.

Spleen and Shell Gland Harvesting and Histological Processing

At the end of the experiment, the birds were weighed before slaughter using weighing scale (HANA®, model J1603444602, China). After slaughter, the spleen and shell gland were harvested using the procedure of Thierry (2000) for organ harvesting in birds. After harvesting, both organs were immersed into 10% Neutral Buffered Formalin. The fixed samples were taken to laboratory for histological processing. Samples fixed in 10% Neutral Buffered Formalin were processed using standard histological techniques as described by Bancroft and Gamble (2008) and histological slides were prepared.

Spleen slides were subjected to histomorphometry while shell gland slides were observed for qualitative changes. Both procedures were carried following the method of Maas and Orthel (1976) with modifications by the use of an Olympus CX21 microscope at x100 total magnification (eyepiece + nosepiece) for visual percentage analysis. Five fields were examined and observer-dependent percent composition of each component visible on hematoxylin and eosin only stained slides were noted and the averages were recorded as percentages. Vascular skeleton was identified as medium- and small-sized arterioles with their branches visible at x100 magnification. Red pulp includes all red pulp areas including capillary and sinusoids. Ellipsoids and peri-arteriolar lymphatic sheath (PALS) were evaluated as ellipsoids merged with PALS without added histochemical staining (reticulin stain) to separate them. B follicles were identified as circular aggregates of small-sized lymphocytes.

Data Analysis

Data generated were subjected to analysis using GraphPad InStat Statistical Package (GraphPad InStat®, version 3.05, 32 bit for Win 95/NT, GraphPad Software Inc., 2000). Kruskal-Wallis test was used to determine the effect of different levels of Crystalline Progesterone on proportion

of splenic red pulp, and proportion of ellipsoids and periarteriolar lymphoid sheath. Significant differences in mean rank differences across Crystalline Progesterone treatment groups were separated using the Dunn's Multiple Comparisons test. One-Way Analysis of Variance was used to determine the effect of different levels of Crystalline Progesterone on the proportion of splenic vascular skeleton and B follicles. Significant mean differences across treatments were separated using Tukey's test.

RESULTS AND DISCUSSIONS

Splenic red pulp

The effect of Crystalline Progesterone on the proportion of red pulp in the spleen of Lohmann Brown layers is shown in Table 1. There was a statistically significant ($P<0.05$; mean rank difference = 17.900; Kruskal-Wallis statistic = 22.708) difference in median proportion of red pulp in the spleen (80% *vs*. 40%) between birds administered Crystalline Progesterone at 0 and 15 mg. Also, a significant ($P<0.001$; mean rank difference = 22.100; Kruskal-Wallis statistic = 22.708) median proportion (80% *vs*. 40%) of red pulp in the spleen was recorded between birds administered 5 and 15 mg Crystalline Progesterone as well as significant ($P<0.05$; mean rank difference = 17.000; Kruskal-Wallis statistic= 22.708) spleen red pulp proportion (80% *vs*.50%) between birds administered 5 and 25 mg Crystalline Progesterone.

Effect of crystalline progesterone on ellipsoids and PALS

The effect of Crystalline Progesterone on proportion of ellipsoids and PALS in the spleen of Lohmann Brown layers is highlighted in Table 2. There was a statistically significant ($P<0.01$; mean rank difference = -19.700; Kruskal-Wallis statistic = 22.711) difference in median proportion of ellipsoids and PALS (10% *vs*. 50%) between birds administered Crystalline Progesterone at 0 and 15 mg. Also, a significant ($P<0.01$; mean rank difference = -19700; Kruskal-Wallis statistic = 22.711) proportion of spleen ellipsoids and PALS (10% *vs*. 50%) was recorded between birds administered 5 and 15 mg Crystalline Progesterone as well as significant ($P<0.05$; mean rank difference = -16.800; Kruskal-Wallis statistic = 22.711) median ellipsoids and PALS proportion (15% *vs*. 50%) between birds administered Crystalline Progesterone at 10 mg

Effect of crystalline progesterone on B. follicles of lohman brown hens

The effect of Crystalline Progesterone on proportion of B follicles in the spleen of Lohmann Brown layers is presented in Table 3. There was no statistically significant ($P>0.05$) difference in mean proportion (0.0, 0.0, 1.6, 0.0, 0.0 and 2.6 %) of B follicles in the spleen of Lohmann Brown layers is across respective Crystalline Progesterone treatments levels (0, 5, 10, 15, 20 and 25 mg).

Vascular skeleton

The effect of Crystalline Progesterone on the proportion of vascular skeleton in the spleen of Lohmann Brown layers is presented in Table 4. There was no statistically significant ($P>0.05$) difference in mean proportion (10.4, 6.0, 8.0, 11.0, 11.0 and 7.0 %) of vascular skeleton across respective Crystalline Progesterone treatment levels (0, 5, 10, 15, 20 and 25 mg).

Table 1: Selected Pairs Dunn's Multiple Comparisons for Proportion of Red Pulp in the Spleen across Crystalline Progesterone Treatment Levels in Lohmann Brown Hens

Crystalline Progesterone (mg)	N	Median (%)	Minimum (%)	Maximum (%)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	80	73	80	109	21.8	
5	3	80	80	85	130	26.0	
10	3	70	70	85	95	19.1	
15	2	40	40	45	19	3.9	
20	3	60	40	80	66	13.2	22.708*
25	3	50	45	60	45	9.0	

Comparison	Mean Rank Difference	Level of Significance
0 mg *vs*. 15 mg	17.900	*
5 mg *vs*. 15 mg	22.100	***
5 mg *vs*. 25 mg	17.000	*

*P<0.05; ***P<0.001

N = number of experimental animals

Table 2: Summary Statistics, KW Statistic and Selected Pairs Dunn's Multiple Comparisons on Proportion of Ellipsoids and PALS[1] across Crystalline Progesterone Treatment Levels in Lohmann Brown Hens

Crystalline Progesterone (mg)	N	Median (%)	Minimum (%)	Maximum (%)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	10	10	15	39.0	7.8	
5	3	10	10	15	39.0	7.8	
10	3	15	10	25	53.5	10.7	
15	2	50	40	50	137.5	27.5	
20	3	30	10	40	82.0	16.4	22.711*
25	3	40	35	42	114.0	22.8	

Comparison	Mean Rank Difference	Level of Significance
0 mg *vs*. 15 mg	-19.700	**
5 mg *vs*. 15 mg	-19.700	**
10 mg *vs*. 15 mg	-16.800	*

*P<0.05, **P<0.01, PALS [1]= periarteriolar lymphatic sheath N = number of experimental animals

Table 3: Effect of Crystalline Progesterone on Proportion of B Follicles in the Spleen of Lohmann Brown Hens

Crystalline Progesterone (mg)	Mean Proportion (%)	Standard Error of the Mean
0	0.0	0.000
5	0.0	0.000
10	1.6	1.030
15	0.0	0.000
20	0.0	0.000
25	2.6	1.939

P>0.05

Table 4: Effect of Crystalline Progesterone on Proportion of Vascular Skeleton in the Spleen of Lohmann Brown Hens

Crystalline Progesterone (mg)	Mean Proportion (%)	Standard Error of the Mean
0	10.4	1.631
c5	6.0	1.000
10	8.0	1.225
15	11.0	1.000
20	11.0	2.449
25	7.0	1.225

P>0.05

Histological Responses of the Shell Gland

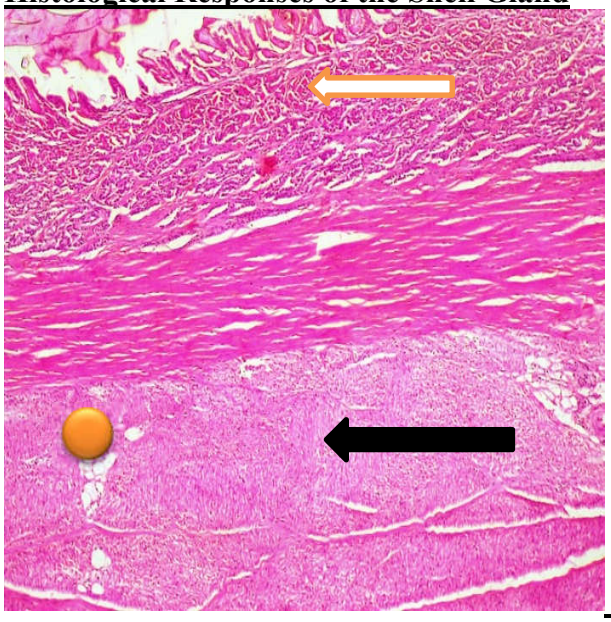

Plate 1: Shell Gland of Lohmann Brown Hens treated with Different Concentrations (A = 0 mg). White arrows show mucosal glands with mostly empty lumen; black arrows show pale staining muscularis (denoting reduced protein content). Yellow dots show fatty infiltrates.

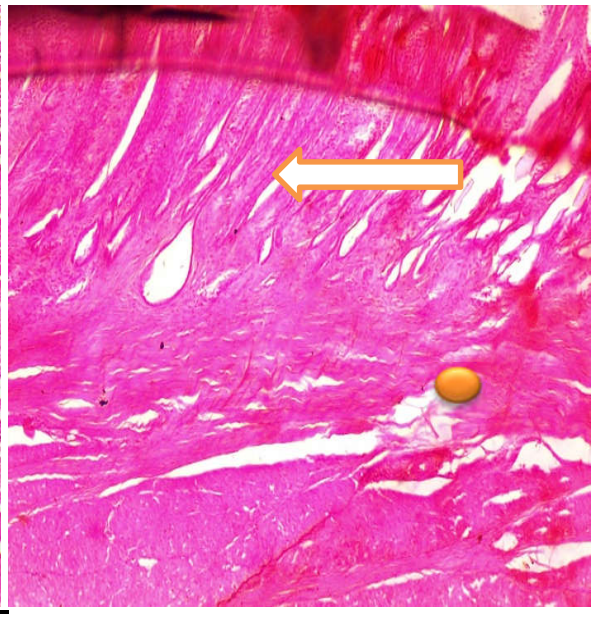

Plate 2: Shell Gland of Lohmann Brown Hens treated with Different Concentrations (B = 5 mg), of Crystalline Progesterone **B -** Mucosa: small-sized straight tubular glands lined by low cuboidal cells with abundant stroma and sparse secretions. Thickness - 0.5 mm. Muscularis: mild hypertrophy, fat infiltrates +, thickness - 10.5 mm.

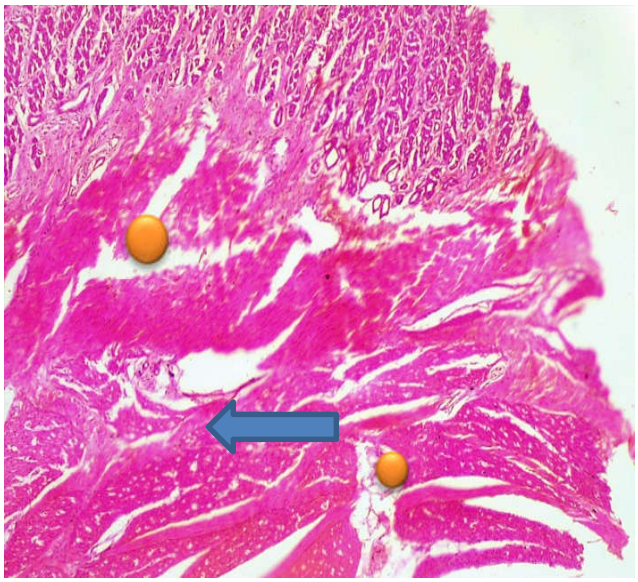

Plate 3: Shell Gland of Lohmann Brown Hens treated with Different Concentrations C = 10 mg of crystalline progesterone. Mucosa: smaller-sized straight tubular glands lined by flattened cells with some secretions, moderate fibrous stroma. Thickness- 0.5 mm. Muscularis: moderate hypertrophy, cytoplasmic vacuoles in all fields, fat infiltrates ++++, thickness - not measured. blue arrows show muscularis with deeply pink cytoplasm.

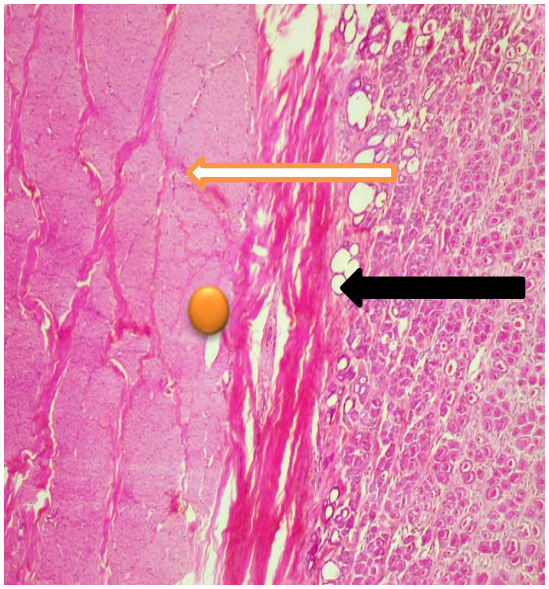

Plate 4: Shell Gland of Lohmann Brown Hens treated with Different Concentrations D = 15 mg of crystalline progesterone. **D -** Mucosa: small-sized straight tubular glands lined by cuboidal cells, abundant secretions, and fibrous stroma, thickness - 1.0 mm. Muscularis: no atrophy, cytoplasmic vacuoles in all fields, fat infiltrates ++++, thickness - not measured[2].

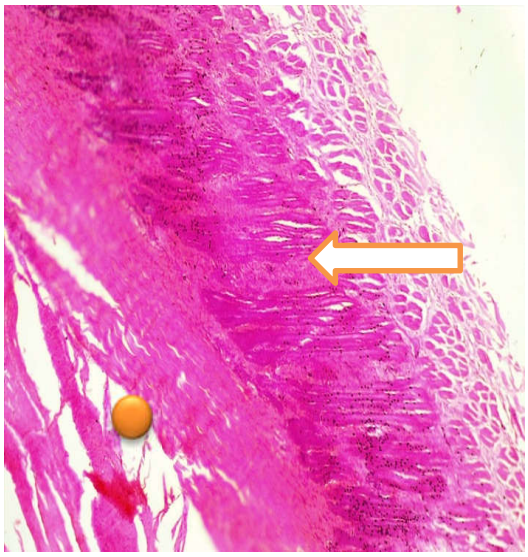

Plate 5: Shell Gland of Lohmann Brown Hens treated with Different Concentrations E = 20 mg of crystalline progesterone. **E -** Marked tissue folding obscuring slide. Mucosa: small-sized straight tubular glands lined by cuboidal cells, some secretions, abundant fibrous stroma, thickness - 0.5 mm. Muscularis: moderate hypertrophy, fat infiltrate +++, thickness - not measured.

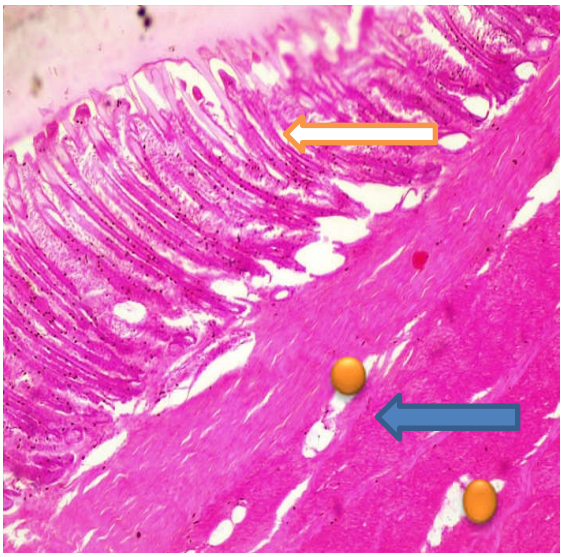

Plate 6: Shell Gland of Lohmann Brown Hens treated with Different Concentrations F = 25 mg of crystalline progesterone. **F -** Mucosa: small-sized straight tubular glands lined by cuboidal cells, some secretions, abundant fibrous stroma, thickness - 1.0 mm. Muscularis: mild hypertrophy, fat infiltrates +++, thickness - not measured.

[1] Fat infiltrate + - no fat cells present/5 (x100) fields, ++ - 1-3 fat cell aggregates/5 (x100) fields, +++ - 6-10 fat cell aggregates/5 (x100) fields, and ++++ - >10 fat cell aggregates/5 (x100) fields.
[2]No mucosa and no serosa identified on slide hence thickness is not measurable.

DISCUSSIONS

Progesterone receptor (PR) has been described in tissues where the action of progesterone is less well defined, including vascular endothelium (Perrot-Applanat *et al*., 1995) and rat thymus (Pear *et al*., 1983). Lack of well-defined action in the splenic vascular skeleton and B follicles could be responsible for the non-significant effect of Crystalline Progesterone (CP) in the present study. This trend could also be due to non-significant individual variability in hens across CP treatment groups.

Balika, et al. (1976) reported that the percentage of all splenic erythroid cells increased in newborn rats that received antenatal doses of progesterone. They also reported that the relative percentage of splenic myeloid cells in young rats fell by half under the influence of exogenous progesterone administered during the antenatal period. As the effects of progesterone are mediated by its receptor and PR is induced by estrogen in most target tissues, the delineation of specific progesterone effects, as distinct from those of estrogen, is similarly not clear (Graham & Clarke, 1997). This could be responsible for the general decrease in red pulp proportion and the corresponding increase in splenic white pulp (ellipsoid and peri-arteriolar lymphatic sheath as well as B follicles) proportion recorded in the current work. Moreover, the present experiment took place when the birds were actively ovulating; thus presenting an opportunity for adequate estrogen priming prior to administration of Crystalline Progesterone. According to Sasaki and Ito (1981), splenic white pulp showed a slight but significant increase in volume in estrogen-treated and estrogen-progesterone treated mouse. In the progesterone-treated group, however, the red and white pulps did not undergo any significant change in volume (Sasaki & Ito, 1981).

Progesterone treatment affects the differentiation of tubular gland cells (Oka & Schimke, 1969) and this depends on the stage of differentiation at which it was administered. If administered concomitantly with estrogen from inception of treatment, tubular gland cell differentiation will be abolished and by extension the growth of the oviduct (Boogaard, 1975). However, if onset of progesterone administration was delayed until when the birds are matured and actively laying as in the current study; it will not interfere with tubular gland cells because estrogen priming has already taken place (Boogaard, 1975). This sheds light on why exogenous Crystalline Progesterone (CP) in the present study showed clearly differentiated tubular glands across all CP treatment levels. The mild to moderate hypertrophy of the shell gland muscularis in the current work may point towards growth and functioning of the gland. Once the cells of the shell gland differentiate and become responsive to progesterone, they maintain this responsiveness even during the non-laying period (Yoshimura & Bahr, 1991). Progesterone receptor was reported by Yoshimura and Bahr (1991) to be present in the nuclei of the surface epithelial cells, tubular gland cells, stromal fibroblasts and smooth muscle cells in the arterial wall and myometrium of laying hens. Finally, the suggestion that estradiol and progesterone may regulate the growth of fat and fat-free tissues in female rats (Toth *et al*., 2001) may explain the pattern of shell gland fat infiltration recorded in the present study.

CONCLUSION

In conclusion, Crystalline Progesterone affected proportion of spleen red pulp and ellipsoids and peri-arteriolar lymphoid sheath. Also, the magnitude of shell gland fat infiltration was influenced by Crystalline Progesterone treatment.

RECOMMENDATION

Based on research findings, it is therefore, recommended the use of progesterone from 5 mg/bird up to 25 mg /bird as these doses meditate the activities of red pulp and differentiation of tubular gland cells.

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