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Effect of soybean enriched diet on plasma oestradiol and progesterone concentration in layer chicken

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Abstract
Phytoestrogens are naturally occurring heterogenous group of herbal substances that are parts of plant's defence mechanism. Legume beans like soybeans are a good source of these phytoestrogens and are nonsteroidal with a spatial conformation similar to oestradiol 17 β. Under the influence of gonadotropins, granulosa cells of chicken ovarian follicles synthesise progesterone, which primarily diffuses into theca cells and gets converted to testosterone and oestrogen. Therefore, phytoestrogens can act either as oestrogen agonists or antagonists depending on endogenous oestrogen levels and exert various effects on the physiology of mammals.
This work had been assigned to assess the influence of dietary soy based is flavones on the level of plasma oestradiol 17β and progesterone in Gramasree layer chicken. At the end of the experimental period of 56 days, the plasma oestradiol concentration was found to be significantly higher ($p<0.05$) in birds fed a standard layer diet without soybean supplementation (304.41±1.01 pg/mL) and it was significantly lower (86.09±1.82 pg/mL) in birds fed with is flavone enriched diet. The progesterone concentration was not significantly different among the treatment groups and ranged between 1.92±0.06 to 1.98±0.03 ng/mL.

Keywords: Phytoestrogens, is flavones, soybeans, oestradiol, progesterone

1. Introduction
Legume beans are good source of flavonoids and is flavones are a subclass of flavonoids and are the major phytoestrogens naturally found in plants. Soy supplements contain concentrated amounts of is flavones like genistein and daidzein which are natural plant glucosides that have weak oestrogenic activity by their chemical structure (Woclawek-Potocka *et al*., 2013) [15]. Soybean meal is the main source of plant derived protein and animal protein substitute incorporated in animal/poultry feeds up to 25 per cent by weight.
Oestradiol 17β (E_2) regulates the integration and coordination of folliculogenesis, accumulation of yolk in the follicles, facilitates luteinising hormone (LH) induced ovulation and development of oviducts. Oestradiol also induces oviduct AL and uterine glandular development and expression of the genes responsible for egg white protein formation. Progesterone (P_4) along with LH induces the ovulation of yolk from the ovary, the development of oviduct AL glands and stimulates the development of secondary sexual characteristics. On the other hand, intake of higher doses of is flavones could be detrimental. Infertility problems have been encountered especially in animals fed with high phytoestrogens.

2. Materials and Methods
The study was conducted in thirty-six numbers of 28 weeks old Gramasree layer birds which was approved by the Institutional Animal Ethics Committee (IAEC/22/16 dated 06/04/2022). The birds were randomly distributed in a completely randomised experimental design and placed into three treatment groups, each with 12 birds. Three is caloric and is nitrogenous experimental feeds, with different is flavone levels, were formulated and provided to laying hens. Birds in group I were fed with standard layer diet containing soybean meal (BIS, 2007) [17]. Group II birds were fed with standard layer diet without soybean meal (BIS, 2007) [17]. Soy is flavones enhanced diet (0.5g of dried hypocotyl sprout of soybean/100g feed) in addition to standard layer diet with soybean meal (BIS, 2007) [17] was fed to birds in group III.

2.1 Estimation of circulating plasma oestradiol 17β and progesterone concentration by radio immune assay (RIA): Radio immune assay (RIA) was carried out as per the manufacturer's protocol (M/s Beckman Coulter, California, USA) for the *in vitro* determination of oestradiol 17β and progesterone in plasma. Samples and calibrators were incubated with ^{125}I labelled oestradiol/ progesterone as the tracer, in antibody-coated tubes. After incubation, the content of tubes was aspirated and bound radioactivity was measured.

2.2 Oestradiol 17β analysis: Calibrator, control and test plasma samples (100 µL for oestradiol and 50 µL for progesterone) were added to the antibody (anti-oestradiol or anti-progesterone) coated labelled tubes. Tracer (500 µL, diluted as per manufacturer's instruction) was added to each tube and mixed well by vortexing. An exact 500 µL of tracer was added to two new tubes was used to obtain total counts per minute (CPM). The tubes were incubated for 2 h at 18-25 °C with continuous shaking in an orbital shaker at 350 rpm. After incubation, the contents in the tubes were aspirated (except the two tubes for total CPM). The tubes were placed in a gamma counter set for Iodine- 125 (^{125}I) and radioactivity was estimated in CPM (one minute per tube). Results were obtained from the standard curve interpolation for both oestradiol and progesterone (Fig 1 and Fig 2).

3. Results and Discussion

3.1 Circulating plasma oestradiol 17 β concentration: The CPM obtained for different standards of oestradiol 17 β in the RIA kit is denoted in table 1. The average concentration of oestradiol 17 β found in the plasma of three different groups on the 56th day of the experiment is presented below (Table 2).

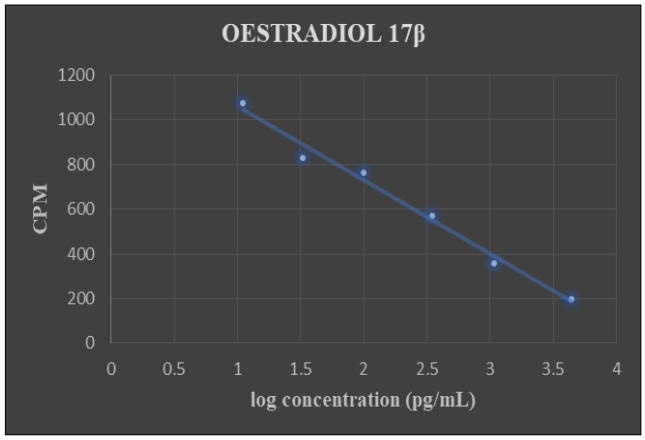

Fig 1: Calibration curve of oestradiol with different standards

The antibody used in the immunoassay was specific for oestradiol. The assay demonstrated to be linear from 8.86 to 4,216 pg/mL using plasma samples. High-concentration plasma samples were serially diluted with oestradiol diluent in the dilution test and the recovery percentages ranged from 92.5% to 119%. The LOD for oestradiol was 10.41 pg/mL, determined consistent with guidelines and LOB was found to be 4.68 pg/mL.

Table 1: Calculated concentrations and CPM of oestradiol 17β standards

Concentration of standards (pg/ mL)	CPM
Blank	1231
11	1074
33	831
99	765
350	569
1080	359
4400	198

Analytical sensitivity of the assay was 0.03 ng/mL (0.10 nmol/L). Intra-assay coefficients of variation observed was ≤ 8.15 per cent for serum samples (samples were assayed 25 times in the same series). Inter-assay coefficients of variation were found to be ≤ 8.66 per cent (samples were assayed in duplicate in 10 different series).

Table 2: Comparison of oestradiol 17β hormone level between groups

Groups	Oestradiol 17β (pg/mL)
Group I	181.91^{ab}±1.62
Group II	304.41^{a}±1.01
Group III	86.09^{b}±1.82
P-Value	0.046*

Means with different superscripts in the same column differ significantly

* Significant (p<0.05)

There was no significant difference at the beginning of the study. However, a significant difference (p<0.05) was observed in the oestradiol concentrations on 56th day of experiment between the treatment groups. The mean plasma oestradiol concentration of group I was not significantly different from that of groups II and III. Total mean plasma oestradiol level was significantly lower for group III (86.09±1.82 pg/mL) compared to group I (181.91±1.62 pg/mL) and II (304.41±1.01 pg/mL).

Phytoestrogen can act like endogenous oestrogen at low doses but block oestrogen at high doses which is in agreement with our study results. However, phytoestrogens acting as oestrogen mimics may affect the production and breakdown of oestrogen by the body, leading to lower levels of circulating oestrogen (Bibu, 2010) [2]. Phytoestrogens can competitively inhibit the production of oestradiol by aromatase which would lead to lower endogenous oestrogen levels (Jefferson *et al*., 2012) [7].

We found that feeding of dried soy hypocotyl sprout enriched diet for 56 days @ 0.5 g/100 g decreased the plasma oestradiol concentration. However, Lu *et al.* (2017) [9] found that a nominal is flavone concentration of 200 mg/kg is not expected to cause adverse effects following daily administration to laying hens for 84 days.

Mean plasma oestradiol concentration of group III birds in the present study is similar to the findings of Shodono *et al.* (1975) [13] who reported plasma oestradiol levels varying from 50 to 250 pg/mL. Graber and Nalbandov (1976) [5] found that oestrogen concentrations in plasma ranged from 25 to 600 pg/mL which is also comparable to our reports. Gjorgovska *et*

al. (2016) [4] reported that oestradiol concentration in blood of the isoflavone treated chickens was significantly higher (0.42 ng/mL) in comparison with the control group (0.31 ng/mL).
Ni *et al*. (2007) [10] measured serum oestradiol 17β levels with RIA and reported a lower serum concentrations of oestradiol 17β (160 pg/mL) for of daidzein supplemented groups compared with their control counterparts (175 pg/mL). It is in agreement with our study results, in which birds fed with is flavone enriched diet exhibited significantly lower (p<0.01) plasma oestradiol compared to the birds supplemented with standard soy based diet.

3.2 Circulating plasma progesterone concentration
The CPM obtained for different standards of progesterone in the RIA kit is denoted in table 3. The average concentration of progesterone found in the plasma of three different groups are shown in Table 4. There was no significant difference between the plasma progesterone concentration of group I, II and III at the end of the experiment.

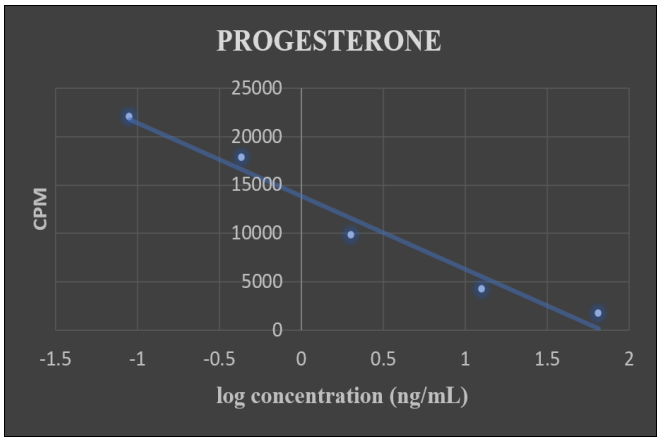

Fig 2: Calibration curve of progesterone with different standards

The antibody used in the immunoassay is specific for progesterone. The assay demonstrated to be linear from 0.02 to 76.02 ng/mL using plasma samples. The measurement range (from LOD to the highest calibrator) varied from 0.04 to approximately 50 ng/mL

Table 3: Calculated concentrations and CPM of progesterone standards

Concentration of standards (ng/ mL)	CPM
Blank	11025
0.11	9606
0.47	7277
2	5562
11.5	3170
65	1637

Table 4: Comparison of progesterone hormone level between groups

Groups	Progesterone (ng/mL)
Group I	1.98±0.02
Group II	1.92±0.06
Group III	1.98±0.03
p-Value	0.456^{ns}

Ns-non significant

For repeatability, the coefficients of variation were found below or equal to 9.48 per cent for plasma samples. For within laboratory precision, the coefficients of variations were found below or equal to 16.85 per cent for plasma samples. Low-concentration plasma samples were spiked with known quantities of progesterone. The recovery percentages obtained were between 80.5 to 98.8 per cent.
In the domestic fowl, progesterone (P_4) played a crucial role in the endocrine regulation of the hypothalamic-hypophysial-ovarian axis, with its production primarily occurring in the granulosa cells of the larger preovulatory follicles (Bahr *et al*., 1983) [1]. The production of P_4 was known to be primarily stimulated by LH (Rivas *et al*., 2016) [11].
Progesterone was also associated with functions such as avid in production, contraction of the myometrium, eggshell formation, ovulation of yolk from the ovary, and development of oviductal glands. The peak plasma concentration of the progesterone occurs 4 to 6 h before ovulation and there is no significant drop for P_4 immediately after ovulation (Shahabi *et al*. 1975) [12]. Phytoestrogens can alter the expression of receptors for progesterone (Whitten and Patisaul, 2001) [14].
Genistein can stimulate progesterone stimulation in the ovaries (Desmawati and Sulastri, 2019) [3]. Xiao *et al*. (2019) [16] found that treatment with 1 nM genistein for 48 h significantly increased P_4 secretion from granulosa cells of the follicles and the detection range of P_4 varied from 0.2 to 100 ng/mL.
Huang *et al*. (1979) [6] observed that the E_2/P_4 ratio served as a more reliable parameter for estimating egg production compared to P_4 alone. This observation aligns with the frequently observed antagonistic relationship between E_2 and P_4 in sexual physiology and metabolism, which is consistent with the studies conducted by Leszczynski *et al*. (1985) [8].

4. Conclusion
Standard layer diet without soybean meal provided to group II birds positively increased the plasma oestradiol levels. However, supplementation of soy enriched diet (0.5g died soy hypocotyl sprout/100g feed) negatively effects on reproduction traits by decreasing the plasma oestradiol 17β concentration. Hence, there is a negative correlation between is flavone content in the feed and oestradiol concentration in the plasma. Even though is flavones are weakly oestrogenic in nature they may still exert physiological effects because of their higher plasma concentration compared to endogenous oestrogens. While progesterone hormone production from the follicle granulosa cells were not affected by is flavone enrichment.

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