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Reproductive Hormones, Leydig and Sertoli Cells Responses to Dietary Protein in Rabbits Exposed to Transient Neonatal Goitrogen

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ABSTRACT

This experiment was conducted to determine the reproductive hormones as well as responses of Leydig and Sertoli cells to dietary crude protein (CP) in rabbit bucks exposed to transient neonatal goitrogen. Sixty three (63) male rabbit neonates were transiently treated with goitrogen through suckling of their mothers' milk. The fryers were weaned after 5 weeks and 21 of them were randomly allocated to each of three dietary treatments (T1-16% CP (Control); T2-14% CP (Low) and T3-18% CP (High) in a Completely Randomized Design. The feeding trial ran from the 5th to 13th week of age. At weeks 7, 8, 9, 10, 11, 12 and 13 blood samples were collected for Testosterone and Follicle stimulating hormone (FSH) assay and three animals per treatment were slaughtered and their testes were harvested for Leydig and Sertoli cells population counts. Effect of age which also indicates the duration of feeding on the reproductive hormones, Leydig and Sertoli cells was also determined. Data for hormonal assay and the stereological estimates were analyzed using factorial analysis of variance. Low crude protein diet decreased ($p<0.05$) testosterone level (from 6.53 to 6.15 mIU/ml), while high dietary crude protein level increased ($p<0.05$) FSH production (from 5.66 to 6.04 mIU/ml). Both testosterone (5.98-6.08) and FSH (2.66-10.98 mIU/ml) levels increased significantly ($p<0.05$) with advancing age in all dietary treatments. The Leydig and Sertoli cell counts were significantly ($p<0.05$) increased with increasing dietary crude protein and advancing age. It was concluded from this study that high CP (18%) increased reproductive hormones, Leydig and Sertoli cell counts with advancing age in growing male rabbits exposed to transient neonatal goitrogen and fed with 18% CP diet, indicating a potential for enhanced sperm production.

INTRODUCTION

All living cells synthesize proteins for part or whole of their life. Lower dietary protein reduces the productive and reproductive performance in rabbits (Lei *et al.,* 2004). In mammalian testis, Sertoli cells denote the main structural component of seminiferous epithelium playing a key role in the origination and maintenance of spermatogenesis (Maeda *et al*., 2002). Leydig cells are crucial source of androgens in the mammalian male. They reside in the testis interstitium. Androgen produced by the Leydig cells are vital for proper functioning of reproductive and accessory reproductive organs as well as non-reproductive tissues such as muscle, skin, liver, haemopoetic organs and bone (Mendis-Handagama and Siril-Ariyaratne, 2005). Sertoli cell proliferation and Leydig cell multiplication occur during a restricted period of time in the middle of sexual differentiation and puberty. As Leydig-Sertoli cells differentiate to functional adult cells, they lose capacity of proliferation (Orth *et al*., 1984; Russell *et al*., 1990, Chubb, 1992 and Sharpe *et al*., 2003).

Goitrogen is a chemical substance, may be a drug or food that interferes with the production of thyroid hormones by disrupting iodine uptake in the thyroid gland. This triggers the pituitary gland to release thyroid stimulating hormone (TSH), which then enhances the growth of thyroid tissue, consequently leading to goiter (Singh *et al*., 2020).

It has been proven that transient neonatal goitrogen treatment causes hypothyroidism but enhances proliferation of Sertoli cells and multiplication of Leydig cells in adult testis (Meisami *et al*., 1992). This goitrogen treatment is effective only when begun during neonatal life, suggesting that the treatment alters an early postnatal process to eventually produce the observed increase in testis size and sperm production (Cooke *et al.,* 1991, Meisami *et al.,* 1992 and Picut *et al*., 2015).

There is paucity of information on the manipulation of Leydig-Sertoli cells multiplication to enhance sperm production rates in farm animals. One of the interventions for such manipulation is to vary dietary crude protein in the diet of animal, as dietary protein plays a significant role on cellular growth and reproductive development of animals, rabbit inclusive. (Reeds *et al*., 2000, FAO, 2013). This experiment was conducted to establish a relationship between crude protein level in rabbit feeds and Leydig-Sertoli cell proliferation/multiplication in buck rabbits exposed to transient neonatal goitrogen. It was also an attempt to provide baseline technical information on the effect of dietary crude protein on Leydig-Sertoli cells proliferation in rabbits exposed to transient neonatal goitrogen.

MATERIALS AND METHOD

Experimental site

The experiment was carried out at the Rabbit Production and Research unit, Teaching and Research Farm of the Ladoke Akintola University of Technology, Ogbomoso. Ogbomoso is situated in a derived savannah zone, southwest of Nigeria and lies on latitude 8° 8' 31.7940" N and longitude 4° 14' 42.6696" E. The altitude is between 300 m and 600 m above the sea level while the mean temperature and annual rainfalls are 27 ^{0}C and 1247 mm respectively (https://geohack.toolforge.org).

Animals and Management

Forty five (45) mature mongrel rabbit does (with average age of 9 months) were housed separately in wooden hutches and dewormed using Ivermectin injection (administered subcutaneously at 0.15 ml per kilogram body weight) against potential ecto and endo-parasites. The does were mated twice a day at a mating ratio of 1buck to 3 does using sexually mature bucks.

Animals were fed with concentrate diet appropriate for pregnant does (i.e. diet containing 18% crude protein and 2500 kcal/kg metabolizable energy in the morning, while forage comprising *Tridax procumbens* was offered in the evening until kindling. After first week of kindling, a goitrogenic compound (Carbimazole) was orally administered to forty two of the does for 21 days at a dosage of 5mg/1kg bodyweight /day (from the 7th to 28th day of lactation) while the remaining three does were not introduced to goitrogen treatment. Kits were sexed and the experimental protocol proceeded on the male kits.

Sixty-three (63) weaned male rabbits exposed to goitrogen were selected and randomly divided into 3 groups of twenty one (21) rabbits each in a completely randomized design. Group A (T1, control) was fed pelletized diet containing 16% crude protein. Group B (T2), Group C (T3) were fed pelletized diets containing 14 and 18% crude protein (CP) respectively. The 3 experimental diets were restricted to 100 g/rabbit/ day which was supplemented with *Tridax procumbens*. Table 1 shows the gross composition of experimental diets.

Table 1: Gross Composition of experimental diet

Feed Ingredients	T1 Control-16% CP	T2 14% CP	T3 18% CP
Maize	48.61	54.70	42.55
Soybean Meal	16.39	10.34	22.46
Brewers' Dried Grain	15.00	15.00	15.00
Rice husk	15.00	15.00	15.00
Fish meal (72%)	2.00	2.00	2.00
Oyster shell	2.00	2.00	2.00
Bone meal	0.25	0.25	0.25
Vitamin premix*	0.25	0.25	0.25
Salt	0.25	0.25	0.25
Lysine	0.15	0.15	0.15
Methionine	0.10	0.10	0.10
TOTAL	**100.00**	**100.00**	**100.00**
Calculated Nutrients	**T1**	**T2**	**T3**
CP (%)	16.00	14.00	18.00
Metabolizable Energy (Kcal/kg)	3190.83	3236.58	3146.59
CF (%)	9.74	9.47	10.01
Determined CP (%)	16.02	14.04	18.01

Vitamin Premix: Supply per kg diet: 2 000 000 iu vit. A; 400 000 iu D_3; 8.0 g vit. E; 4 g vit. b_1; 1.0 g vit. B_2; 0.6 g vit.; 0.4 mg vit. B_{12}; 24.0 g Niacin; 0.2 g Folic acid; 8.0 g Biotin; 48.0 g Choline; 320.0 g BHT; 16.0 g Manganese; 8.0 g iron; 7.2 g] Zinc; 0.32 copper; 0.25 iodine; 36. 0 mg cobalt; 16.0 mg selenium.

Leydig and Sertoli Cells count

Three rabbits were randomly selected from each group on weekly basis from week 7 to 13 and sacrificed and the testes were harvested for further processing. Testis samples were fixed in Bouin's solution and processed for histological evaluation to identify the Leydig and Sertoli cells for counting. The histological slide preparation followed a standard procedure of washing, dehydration, clearing, embedding, sectioning and staining. Stained sections were examined though the optical fractionator of a light microscope and cells in each tissue were counted using a counter. The Sertoli cells were recognized in the seminiferous tubules by their pale invaginated irregular nuclei with prominent nucleoli while the Leydig cells were recognized in the interstitium as relatively large ovoid shaped cells each with an eccentric nucleus.

Hormonal assays

Blood samples were collected once per week (Week 7 to 13) from the bucks before slaughtering. Blood was collected into plastic tubes that did not contain any anticoagulant. They were centrifuged at 1000 rpm for 10 minutes to obtain the plasma. Glass beads were introduced into the plasma to coagulate the fibrinogen to give the serum used for hormonal assay.

FSH was determined by the method described in Accu-Bind ELISA (Microwells) FSH test system (product code: 425-300). The microplates were well formatted then 0.05 ml sample was pipetted into the assigned well. 0.1 ml of FSH-enzyme reagent solution was added to all the wells and the microplate was swirled gently for 30 seconds to enhance proper mixing. It was then incubated for 60 minutes at room temperature before the content of the microplate was decanted. 0.35ml of washing buffer was added and 0.1

ml of working substrate solution to all the wells. It was then incubated at room temperature for 15minutes, 0.05 ml of stop solution was added to each well and mixed gently for 20 minutes. The absorbance was then read at 450 nm. The concentration of FSH in the sample was then ascertained from dose-response curve. Testosterone was assayed using the same procedure with testosterone enzyme reagent solution.

Data analysis

Data generated were subjected to analysis of variance (ANOVA) appropriate for a 3X7 factorial for age and dietary treatment effects (SAS, 2000). Significant means were separated by Duncan's option of the same statistical package.

RESULTS AND DISCUSSION

Table 2 shows the effect of dietary crude protein on the Sertoli and Leydig cell count of rabbits exposed to transient neonatal goitrogen. The Ledig and Sertoli cell counts were significantly ($p<0.05$) affected by varying dietary crude protein. Sertoli cell count significantly ($P<0.05$) reduced at T2 while a significant ($P<0.05$) increase was observed at T3 when compared with the control treatment. The same trend was exhibited by the Leydig cell count.

The overall Sertoli and Leydig cell count of rabbits on 14% CP was the lowest and that of 18%CP was the highest suggesting that 14%CP was inadequate in maintaining the Sertoli and Leydig cell proliferation in rabbits exposed to transient neonatal goitrogen. The reason for this suboptimal Sertoli and Leydig cell proliferation in rabbits fed low dietary CP could be that the protein level was too low for the synthesis of the necessary biochemical compounds to offset the effect of transient neonatal goitrogen which is expected to optimally aid an overall Leydig and Sertoli cell proliferation (Cooke *et al*., 1991, Hess *et al.,* 1993, Jannini *et al.,* 1995*,* Jansen *et al.,* 2007). Earlier report has indicated that lower dietary protein is likely to reduce the reproductive performance of rabbits (Sanchez *et al.,* 1985).

The effect of age on Sertoli and Leydig cell counts of growing rabbits fed varying dietary crude protein after exposure to transient neonatal goitrogen is shown in Table 3. The results showed that both Sertoli and Leydig cell counts significantly ($P<0.05$) increased as the age of the rabbits increased. The significant increase in Leydig and Sertoli cells with increasing age observed in this study tend to contradict the report of Sharpe *et al.,*(2003) that Sertoli and Leydig cell proliferation occur during a limited period comprised between sexual differentiation and puberty, that as they differentiate to functional adult cells, they lose the capacity of proliferation.

The continuous increase in Sertoli and Leydig cell counts with increased age in this study suggests that transient neonatal goitrogen influenced pubertal Sertoli and Leydig cell proliferation (Cooke et al., 1991; Hess *et al.,* 1993; Jannini *et al.,* 1995; Jansen *et al.,* 2007; McKay and Smith, 2007). It was however established that neonatal hypothyroidism produces this effect by arresting Leydig cell differentiation early in life and allowing continuous proliferation of the precursor messenchymal cells that accumulate in the interstitium which would become available for differentiation later when euthyroidism is restored (Mendis-Handagama *et al.,* 1998 and Picut *et al*., 2015). Similarly it was reported that transient neonatal/prepubertal goitrogen treatment extends the length of Sertoli cell proliferation by delaying their maturation, resulting in an increased number of the cells in adult testis (DeFranca *et al.,* 1995 and Sharpe *et al*., 2003).

Moreover, high level of expression of functional T3 receptors in the proliferating Sertoli cells has been reported (Buzzard *et al.,* 2000, Jannini *et al.,* 2000). This indicates that Sertoli cells are major testicular target of thyroid hormone. It appears that thyroid hormone acts directly on Sertoli cells to inhibit proliferation while stimulating differentiation not only in rodents (Cooke *et al*., 1991, Kirby *et al.,* 1993) but also in other vertebrate species (Jannini *et al.,* 2000, Jansen *et al.,* 2007).

The interaction effects of the dietary crude protein and age (weeks) on the Leydig and Sertoli cell counts of rabbits exposed to transient neonatal goitrogen are shown in Table 4. Significant ($P<0.05$) increase was observed with increase in age for Leydig and Sertoli cells counts accross all the treatments. The significatly higher proliferation in Sertoli and Leydig cells count as the age increased in animals fed 18% dietary CP in this study corroborates the findings of Mendis-Handagama and Sharma (1994), Mckay and Smith (2007) that transient neonatal hypothyroidism increased the number of Leydig and Sertoli cells in adult rat testis and influenced pubertal Sertoli cell count. It however tends to disagree with the report of Sharpe *et al*. (2003) that Leydig and Sertoli cell proliferation occurs during a limited period between sexual differentiation and puberty as both cells lose their capacity of proliferation. The sub-optimal increase of the overall Sertoli and Leydig cell count observed in animals fed 14% dietary CP as the age increased may be linked to the report of Lei *et al*. (2004) that lower dietary protein reduces reproductive efficiency in rabbits.

The main effect of dietary crude protein on the Testosterone and Follicle stimulating hormone (FSH) is presented shown in Table 5. The testosterone and FSH concentrations were significantly ($p<0.05$) affected by dietary crude protein. Testosterone significantly ($P<0.05$) reduced by lowering the CP (T_2) while there was no significant ($P>0.05$) difference between the T3 and the control treatment**.**

The overall testosterone concentration reduction in animals fed 14%CP however agrees with the report of

Kirby *et al*. (1992) that in rodent, transient neonatal hypothyroidism suppressed the pubertal increase in serum testosterone levels. The significant elevation in serum FSH of animals fed 18%CP is however in sharp contrast to the observation in rodents by Kirby *et al.* (1992) that transient neonatal hypothyroidism suppressed the pubertal increase in serum levels of FSH. The reason for FSH elevation can be linked with the report of Raharjo *et al.* (2012) that moderately high dietary CP enhances reproductive hormones in rabbits.

Table 6 shows the effect of age on the testosterone and FSH concentrations of rabbits fed dietary crude protein after exposure to transient neonatal goitrogen. Testosterone and FSH concentrations were significantly (P<0.05) affected by age. Testosterone concentration significantly (P<0.05) increased as the age increased. Similarly, FSH concentration significantly (P<0.05) increased with age with the lowest concentration at week 7 and the highest concentration at week 13.

The results obtained from this study suggests that the age has a direct effect on the overall concentration of both testosterone and FSH of growing rabbit bucks fed varying dietary crude protein after exposure to transient neonatal goitrogen. This contrasts the observation in rats by Kirby *et al*. (1992) that transient neonatal hypothyroidism suppressed the pubertal increase in the serum levels of FSH and delays the pubertal increase in serum testosterone levels. The dietary crude protein is suspected to be the reason for this continuous significant elevation in the concentration of these hormones with increase in age as moderately high dietary CP enhanced reproductive hormones elevation in rabbits (Raharjo *et al.,* 2012).

The interaction effect of age and dietary crude protein on testosterone and FSH concentration of rabbits exposed to transient neonatal goitrogen is shown in Table 7. Testosterone and FSH concentrations were significantly (P<0.05) affected by age. Testosterone concentration significantly (P<0.05) increased as the age increased in all the treatments. Similarly, FSH concentration significantly (P<0.05) increased with increasing age in all the treatments, with animals fed 14%CP having the lowest values compared to the control treatment.

The results obtained from this experiment indicate a continued elevation of both testosterone and FSH concentrations with advancing age of the animals. This however contradicts the report of Kriby *et al.* (1992) that in rodents, transient neonatal goitrogen suppressed the pubertal increase in the serum level of FSH and testosterone. The reason for this significant elevation in the concentration of these hormones as the age increased can be linked to dietary crude protein which is consistent with the report of Raharjo *et al*. (2012) who reported that moderately high dietary CP enhanced reproductive hormones elevation in rabbits.

Table 2: Effect of dietary crude protein on Leydig and Sertoli cell counts of rabbits exposed to transient neonatal goitrogen

Parameters (n=63)	T1Control (16% CP)	T2 (14% CP)	T3 (18% CP)	SEM
Sertoli cell count ($X10^6$)	315.43^b	161.97^c	394.33^a	5.65
Leydig cell count ($X10^6$)	112.13^b	97.2^c	266.49^a	7.41

ab: Means on same row with different superscripts differ significantly (P<0.05)
SEM: Standard Error of Mean

Table 3: Main effect of age (weeks) on the Leydig and Sertoli cell count of rabbits fed dietary CP after exposure to transient neonatal goitrogen

Age (Weeks), n=63	Sertoli cell count ($X10^6$)	Leydig cell count ($X10^6$)
7	22.19^d	8.71^d
8	29.31^d	10.48^d
9	27.48^d	13.74^d
10	62.71^c	30.48^c
11	81.56^c	44.70^c
12	416.39^b	242.97^b
13	1384.40^a	759.33^a
SEM	43.61	32.75

abcd: Means in the same column with different superscripts differ significantly (P<0.05)
SEM: Standard Error of Mean

Table 4: Effect of dietary crude protein and age on the Leydig-Sertoli cell count of rabbits exposed to transient neonatal goitrogen

Parameters	Age (Week)	T1 (16% CP- Control)	T2 (14%CP)	T3 (18%CP)	SEM
Sertoli cell count ($X10^6$)	7	46.33^{g}	6.75^{e}	13.49^{e}	0.50
	8	63.32^{f}	9.46^{e}	15.16^{e}	0.96
	9	80.74^{e}	8.48^{e}	23.22^{de}	3.06
	10	101.55^{d}	52.64^{d}	33.92^{d}	3.60
	11	123.05^{c}	76.56^{c}	45.06^{c}	3.60
	12	466.79^{b}	404.51^{b}	377.87^{b}	8.95
	13	1326.21^{a}	575.41^{a}	2251.57^{a}	35.05
	SEM	7.84	10.27	5.75	
Leydig cell count ($X10^6$)	7	13.78d	5.86^{e}	6.50^{e}	0.71
	8	14.89^{d}	6.91^{e}	9.64^{e}	0.40
	9	19.71^{cd}	7.15^{e}	14.35^{de}	1.13
	10	26.37^{c}	41.97^{d}	23.09^{cd}	2.85
	11	31.07^{b}	67.92^{c}	35.09^{c}	5.82
	12	147.23^{a}	317.07^{b}	264.60^{b}	14.62
	13	427.80^{a}	338.00^{a}	1512.19^{a}	19.06
	SEM	3.90	8.48	5.73	

abcdefg: Means on same column with different superscripts differ significantly (P<0.05)
SEM: Standard Error of Mean

Table 5: Effect of dietary crude protein on reproductive hormones in rabbit bucks exposed to transient neonatal goitrogen

Parameters	T1 (Control-16% CP)	T2 (14% CP)	T3 (18% CP)	SEM
Testosterone (mlU/ml)	6.53^{a}	6.15^{b}	6.46^{a}	0.10
FSH (mlU/ml)	5.66^{b}	5.74^{b}	6.04^{a}	0.03

ab: Means on same row with different superscripts differ significantly (P<0.05)
SEM: Standard Error of Mean

Table 6: Effect of age (weeks) on the Testosterone and FSH concentration of rabbit bucks fed dietary crude protein after exposure to transient neonatal goitrogen

Week	Testosterone (mlU/ml)	FSH (mlU/ml)
7	5.98^{d}	2.66^{g}
8	5.88^{d}	3.27^{f}
9	6.29^{c}	4.13^{e}
10	6.48^{bc}	4.73^{d}
11	6.56^{b}	7.03^{c}
12	6.67^{ab}	7.89^{b}
13	6.80^{a}	10.98^{a}
SEM	0.29	0.22

abcdefg: Means on same column with different superscripts differ significantly (P<0.05)
SEM: Standard Error of Mean

Table 7: Effect of age (weeks) and dietary crude protein on testosterone and FSH of rabbits exposed to transient neonatal goitrogen.

Parameters	Age (Week)	T1 (16% CPControl)	T2 (14%CP)	T3 (18%CP)	SEM
Testosterone (mLU/ml)	7	6.40[b]	6.03[b]	5.80[b]	0.08
	8	6.50[b]	5.73[b]	6.47[b]	0.14
	9	6.00[b]	5.77[b]	5.87[b]	0.12
	10	5.87[b]	6.03[b]	6.70[a]	0.08
	11	7.72[a]	5.73[b]	6.70[a]	0.19
	12	6.67[a]	6.20[a]	6.80[a]	0.09
	13	7.00[a]	6.53[a]	6.87[a]	0.05
	SEM	0.14	0.10	0.08	
Follicle stimulating hormone (mLU/ml)					
	7	2.73[g]	2.67[d]	2.57[e]	0.05
	8	3.97[f]	2.97[d]	2.87[e]	0.07
	9	3.03[e]	4.40[c]	4.97[d]	0.08
	10	4.77[d]	4.97[c]	4.47[d]	0.13
	11	5.93[c]	7.50[b]	7.67[c]	0.12
	12	6.97[b]	7.53[b]	9.17[b]	0.11
	13	12.20[a]	10.13[a]	10.60[a]	0.10
	SEM	0.08	0.10	0.08	

abcdefg: Means on same column with different superscripts differ significantly (P<0.05)
SEM: Standard Error of Mean

CONCLUSION

This study has demonstrated that 18% dietary crude protein is capable of optimally promoting Leydig and Sertoli cell proliferation, elongating the proliferation age/phase and enhancing the production and elevation of reproductive hormones (Testosterone and FSH) as age increased from 7 to 13 weeks in growing rabbits exposed to transient neonatal goitrogen. It could therefore be inferred from this study that 18% dietary crude protein significantly enhanced the physiological and reproductive characteristics of growing rabbits exposed to transient neonatal goitrogen.

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COMPETING INTERESTS

There were no conflict and/or competing interests among the authors in the course of this study.
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