Effect of different rumen protected fat from palm oil on testosterone level and testicular traits in Malin rams

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Abstract
Rumen protected fat (RPF) have been used to increase the energy density of the animal feed and most RPF product used in animal feed come from palm oil. RPF are widely used in dairy cows because it can compensate for the energy deficiency during lactation. However there is lack of knowledge on the effect of RPF on testosterone level and testicular traits in Malin rams. Most common RPF type used in animal feed is prilled fat and calcium soap of fatty acid (Casa). Both prilled fat and Casa have been chemically treated to be protected from hydrolysis and biohydrogenation in the rumen. The objective of the study was to evaluate the effect of RPF on testosterone level and testicular traits in Malin rams. Twenty adult Malin rams were randomly and equally assigned to four treatments: T1: Control, T2: 2% prilled fat, T3: 2% calcium soap fatty acid and T4: 2% canola oil. There was no significant difference (P>0.05) in plasma testosterone level of rams among the different treatment groups. Mean scrotal circumference and total weight of testis were also not significantly different (P>0.05) among treatments. Seminiferous tubule score, Leydig cell score and seminiferous tubule diameter were also not significantly (P>0.05) affected by fat supplement studied. In conclusion, supplementation of rumen protected fat from palm oil to Malin rams does not improve their testosterone level and measurements of testicular traits.

Keywords: Rumen protected fat, testosterone level, testicular traits, Malin rams

Introduction

Nutrient shortage can lead to poor reproductive performance in rams, thus decreasing animal productivity in the farm. Traditionally, concentrate or grain-based diet has been used as a solution to increase the energy density in the diet of sheep. However, there are limits on how much the concentrate and grain can be included in the diet due to high cost of the feed ingredients, limited supply and the risk of acidosis. Underfeeding in adult rams in 65 d can reduce spermatogenesis and more likely to cause sperm DNA damage (Guan et al., 2014). In mature rams, nutrition has major effects on testicular mass. The change in testicular mass reflects changes in the amount of seminiferous tissue which could result in reduced spermatogenesis capacity (Martin et al., 2010). Zamiri et al. (2010) found that testicular circumference was positively correlated with testosterone concentration, percentage live/normal sperm and semen concentration. Testosterone is important in the production of sperm and sperm maturation in testis, for the function of accessory sex glands and development of the male sexual reproductive behavior (Parkinson, 2009). Elevated testosterone

concentration was shown to increase reproductive effort which could cause an increase in energy demand by increasing metabolic rate and fat reserve loss, therefore males will significantly lose their body mass because less time is spent foraging or hunting for food (Longpre et al., 2016).

Testosterone level is correlated positively with sperm motility and progressive motility and negatively with fat measurement and ejaculation latency time (Swelum et al., 2016). A study by Ghorbankhani et al. (2015) indicated that all semen variables (except percentage of abnormal sperm and semen pH), serum testosterone concentration and testicular circumference were positively influenced by nutritional state. Therefore, it is important to gather more information whether feeding any supplement can overcome the problem. Fat supplement to the animals can be the solution as fat can give 2.24 times more energy than carbohydrate (NRC, 2001). Lipid is not just important in energy storage but also in other aspects such as hormone synthesis, composition of plasma membrane and function in sperm motility. Fat has been used to increase the energy density of animal feed. Lack of energy in ruminants can be due to low quality of the roughages offered or reduced feed intake by the animals. Usage of fat supplementation has become a common practice to increase the energy density of ruminant diet. In Malaysia, palm oil based feed products have been used in the livestock industry because they do not have the negative effect on rumen fermentation as with unsaturated oils (Manso et al., 2009). Several studies have shown that utilization of lipid supplement could increase milk yield (Alstrup et al., 2015), body condition (Bhatt et al., 2013), conception rate in dairy cows (McNamara et al., 2003) and increase semen concentration in male animals (Fair et al., 2014).

Different types of lipid supplement have different composition of fatty acids. Fatty acid composition may be related to different effects on production level of the animals. The abundance of long-chain fatty acids alters rumen fermentation, coating the fiber and depressing fiber digestion. Thus, it is recommended that lipid supplement in the animal diet should not be more than 6-7% in dietary dry matter because it can be harmful to rumen microbial fermentation.

Rumen protected fat has been treated through several chemical methods. Rumen protected fat is manufactured to bypass the rumen metabolism, therefore avoid toxicity of the fat to rumen microorganisms and improving energy efficiency (Ganjkhanlou et al., 2009; Sanz et al., 2002) compared to unprotected fat. The protected fat is digested in the abomasum where the pH is more acidic than that of the rumen. By using protected fat, the amount given to the animals could safely be used up to 7.5% without any adverse effect on dry matter intake and rumen fermentation (Sirohi et al., 2001), although Ramana et al. (2003) showed the safe inclusion of protected fat with calcium up to 10%. Increasing the percentage of inclusion means more energy are made available to the animals and using locally produced oil palm based product could save on feed cost. This practice will be more affordable to the producers compared to total concentrate or grain feeding and safer since it will not disturb the rumen microbes and metabolism. Therefore, the present study was conducted to evaluate the effect of rumen protected fat on testosterone level and testicular traits in Malin rams.

Materials and Methods

The experiment was carried at the National Institute Veterinary Biodiversity, Jerantut, Pahang. Twenty male Malin sheep between 10-14 mo old with average body weight 36.6 ± 5.57 kg were used. Before the

start of the experiment the animals were fed daily with fresh chopped *Bracharia humidicola* grass and commercial sheep pellet. For the experiment, the animals were housed individually with feed trough and water drinker in a raised-floor housing. Prior to the experiment, the rams were trained for semen collection with an artificial vagina and teaser ewes. Each ram received its daily diet in two parts at 0900 fed concentrate and 1100 given grass. The rumen protected fat and canola oil were mixed with the concentrate before feeding to the rams. The rams have free access to water. Table 1 shows the chemical composition of grass, concentrate and lipid supplement.

Table 1: Chemical composition of feed materials

Parameter	Concentrate	*Brachiaria humidicola*	Prilled	Casa	Canola oil
DM	88	18.8	--	--	--
CP	16.0	10.2	--	--	--
CF	10	30.4	--	--	--
EE	6.0	3.2	98	84.5	99.9
Ash	9.3	7.2	--	15.5	--
ME (Kcal/kg)	2886	1873	7130	6270	7070

Table 2: Ingredient composition of diet in Control, Prilled, Casa and Canola oils

Ingredients	Feed Formulation			
	Control	Prilled	Casa	Canola oil
Bracharia grass	66	76	75	77
Commercial pellet	34	22	23	21
Rumen Protected Fat A (Without calcium)	--	2	--	--
Rumen Protected Fat B (With calcium)	--	--	2	--
Canola oil	--	--	--	2
Total	100	100	100	100
Calculated analysis				
ME (kcal/kg)	2217	2211	2214	2210
Crude protein (%)	12.2	11.3	11.4	11.3

The animals were randomly and equally assigned to four treatments: T1, control with basal diet consisted of *Brachiaria humidicola* grass and commercial sheep pellet, T2, basal diet supplemented with rumen protected fat (RPF) as Prilled fat from palm oil at 2% per day of the total diet, T3, basal diet with 2% RPF as calcium soap (Casa) from palm oil and T4, basal diet with 2% Canola oil. The experimental diet (Table 2) was formulated to be isocaloric and isonitrogenous for all groups according to ICAR-NIANP (2013). The experiment lasted for 12 wk. The animals were placed in an adaptation period of 2 wk and treated against endo- and ecto-parasites prior to the start of the experiment.

Determination of plasma testosterone level

Blood samplings were done in the morning before the feed was offered to the rams. Samples of blood were acquired via the jugular vein using hypodermic needles and heparinized vacutainers. The blood samples were placed immediately on ice and transported to laboratory. The blood plasma was separated by centrifuging the blood at 3000 rpm for 10 min at 4^0C. Blood plasma was collected and stored at -20^0C until assayed (Jafaroghli et al., 2014). Concentration of testosterone in plasma of the rams was evaluated using ELISA kit (Enzolifesciences).

Slaughtering, sample collection and histology procedures.

At 12^{th} wk of the treatment period, four out of five rams from each treatment group were slaughtered for sampling in accordance with the procedures outlined in MS1500:2009 (Department of Standards Malaysia, 2009) which allow animals to be slaughtered by severing the jugular vein with a razor sharp knife, without being stunned. In this study the slaughter was performed by a certified and highly experienced technician with a sharp knife. Sustained absence of corneal reflex and rhythmic breathing were strictly monitored and checked to ensure that each individual ram was dead prior to further processing and sampling. Testes were collected and weighed and fixed with Bouin's solution for 12 h and then rinsed with 70% alcohol for 3 times for every 2 h. The testis samples were then dehydrated for 16 h in automated tissues processor (Leica ASP 3000) and later embedded in paraffin wax using a paraffin embedded system (Leica RM 2155) to form paraffin blocks. Tissue sections were serially cut at 4 µm thickness from the paraffin blocks using a microtome machine (Leica RM2155) and then the tissue section was mounted on slides heated at 57℃ and allowed to dry. The slides were then stained with hematoxylin-eosin and examined under the light microscope and digitally photographed.

Testis histological evaluation

Two slides from each testis sample were observed using normal light microscope under x400 magnification. Three areas of seminiferous tubules were randomly selected from each slide and evaluated for spermatogenesis using Yoshida scoring method (Yoshida et al., 1997). Yoshida scoring method (Table 3) gives a score between 1 (total absence of cells within seminiferous tubules) to 12 (many late spermatids and spermatozoa). Late spermatids are situated towards the adluminal of the seminiferous tubules while spermatozoa have their heads embedded in the cytoplasm of Sertoli cells. Spermatocytes have darker nuclei and located away from the periphery of the tubule. Spermatogonia are round and smaller, located near the basement membrane of the seminiferous tubule (Yaakub et al., 2009). Leydig cell density was evaluated using the method of Donovan et al. (2013) which gives scores: 0 (no cells present), 1 (scattered/few cells present), 2 (moderate cells presents) and 3 (densely packed within the interstitial). A camera fixed to the eye piece of a normal light microscope was used to digitally photograph the seminiferous tubules and together with Dinocapture software version 1.4.1, Anmo Electronic Corporation so as to determine the diameter of the seminiferous tubules.

Table 3: Scores in seminiferous tubule[1]

Score	Criteria
12	Many late spermatids or spermatozoa (≥10)
11	Only a few spermatids or spermatozoa (<10) present
10	No spermatozoa and no late spermatids, but many round spermatids (≥10) present
9	No spermatozoa and no late spermatids, but only a few round spermatids (<10) are present
8	No spermatozoa and no spermatids, but many secondary spermatocytes (≥10)
7	No spermatozoa and no spermatids, but only a few secondary spermatocytes (<10) are present
6	No spermatozoa, no spermatids, no secondary spermatocytes, but many primary spermatocytes (≥10)
5	No spermatozoa, no spermatid, no secondary spermatocytes, but only a few primary spermatocytes (<10) are present
4	No spermatozoa, no spermatid, no secondary spermatocytes, many spermatogonia (≥10) are present
3	Only germ cells present are a few spermatogonia (<10)
2	Absence of germ cells, but Sertoli cells are present
1	Total absence of cell in tubular section

[1]Adapted from Yoshida et al. (1997) as described from Donovan et al. (2013)

Statistical analysis

All statistical analyses were completed using Statistical Analysis Software (SAS,Version 9.4). General Linear Model (GLM) was used to analyse the data and mean of treatments were compared by Duncan's Multiple Range Test (DMRT) at $P<0.05$ for significant value.

Results and Discussion

Plasma testosterone level

Table 4 presents the plasma testosterone level of the rams in the treatment groups. No significant difference ($P>0.05$) was observed in plasma testosterone level at the end of experiment among the treatment groups. However, at wk 7, significant difference ($P<0.05$) was noted in plasma testosterone level in the Control group compared to the Canola group. The plasma testosterone in the Control was not significantly different than Prilled and Casa groups. Interestingly, at wk 9, the plasma testosterone level in Canola group was significantly higher ($P<0.05$) than the Casa group. However, the plasma testosterone level in Canola group was not significantly different than those of the Control and Prilled groups.

Table 4. Effect of dietary treatments on plasma testosterone level (mean±S.E)

Plasma testosterone (ng/ml)	Control	Prilled fat	Casa	Canola oil
Week 1	1.36±0.02	1.41±0.02	1.38±0.04	1.34±0.04
Week 3	1.47±0.03	1.44±0.04	1.39±0.04	1.42±0.04
Week 5	1.42±0.04	1.43±0.02	1.44±0.02	1.43±0.02
Week 7	1.41±0.01[a]	1.39±0.02[ab]	1.36±0.02 [ab]	1.34±0.02[b]
Week 9	1.33±0.02[ab]	1.35±0.02[ab]	1.28±0.03[b]	1.39±0.01[a]
Week 11	1.38±0.04	1.40±0.01	1.38±0.02	1.36±0.04
Average	1.39±0.03	1.40±0.02	1.37±0.03	1.38±0.03

[a,b] Means bearing different superscripts within a row differ significantly (P<0.05)

Normal testicular function is reliant on two aspects; first the endocrine hormone which is produced outside the testis and second the role played by somatic cells within the testis (Saunders, 2003). Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are endocrine hormones synthesized and secreted by the pituitary gland. While somatic cells in the testis are the Leydig and Sertoli cells. LH stimulated Leydig cells to synthesize and secrete testosterone while FSH stimulates Sertoli cells to nurse the germ cells. Without these two important pituitary hormones, it can cause loss of germ cells (McLachlan et al., 2002).

Steroid hormones such as testosterone are biosynthesized from cholesterol and most steroidogenic cholesterol come from circulating lipoproteins (Miller, 2013) and feeding diet high in fat may increase the concentration of serum cholesterol and later increase serum steroid hormone (Swecker et al., 1987). Absence of testosterone could result in an incomplete spermatogenesis process in the testis. Sertoli cells stimulated by FSH can only enable completion of spermato-cytogenesis and meiosis stages. While the last stage of spermiogenesis is incomplete because in absence of testosterone it will cause less spermatids to go through morphological changes to transform into spermatozoa (Saunders, 2003).

Upsurge of testosterone concentration has been reported to cause an increase in energy demand by increasing metabolic rate (Buchanan et al., 2001) but high testosterone can also cause decreased feed intake, body weight (Iwasa et al., 2017) and body fat (Swelum et al., 2016). As a result, males will significantly lose their body mass. In the present study, there was no significant difference in testosterone concentration among the treatment groups. However, in wk 7 of the experiment, the animals in Canola group showed significantly lower testosterone concentration compared to the Control group, yet the testosterone concentration in the Canola group showed no significant difference in the following weeks.

The finding of no significant difference in testosterone level among the treatment groups is contrary to that of Tran et al. (2016) who studied diets high in PUFA which increased testosterone production and this may be due to the different concentration and type of fat supplement. Tran et al. (2016) used a higher concentration of protected vegetable oil that was high in PUFA compared to this study which used lower concentrations of unprotected vegetable oil. Nevertheless, the findings in this study agree with El-Hamid et al. (2016) that diet supplement which was poor in PUFA did not

affect steroid hormone concentration in the blood, which might be due to feeding energy supplement high in saturated fat that may have modulated the hypothalamic and pituitary functions and therefore gonadial activity. No significant difference in testosterone level in animals among the groups may also explain why there was no significant difference in body weight and feed intake because high testosterone level may have decreased feed intake and body weight (Iwasa et al., 2017). The results of this study indicate that Calcium soap and Prilled fat from palm oil which are high in saturated and monosaturated fat do not influence the rate of testosterone synthesis.

Testicular traits and histological structures

The mean measurement values of the testicular traits and histological structures for all treatments in response to dietary fat supplement are shown in Table 5. Neither the mean of scrotal circumference nor total weight of testis was significantly different (P>0.05) among supplement dietary treatments. Mean seminiferous tubule score, Leydig score and seminiferous tubule diameter are presented in Table 5, and none of them resulted in significant difference among the fat supplement treatments studied (P>0.05). However, on closer inspection of the results the testes weight and seminiferous tubule diameter in the Canola group tended to be higher than Prilled and Casa groups.

Table 5. Effect of dietary treatments on the testicular traits and histological structures of testes (mean±S.E)

Parameter	Control	Prilled	Casa	Canola
Scrotal circumference (cm)	26.33±0.29	26.10±0.19	26.85±0.21	26.64±0.17
Testes total weight (g)	281.67±2.03	272.50±8.50	273.33±13.69	280.00±24.78
ST[1] score	10.73±0.24	11.17±0.24	10.83±0.24	11.08±0.26
Leydig score	1.30±0.21	1.25±0.13	1.33±0.23	1.16±0.11
ST[1] diameter(µm)	184.95±3.26	182.74±2.37	179.18±3.61	188.03±2.54

[1]ST – Seminiferous tubules

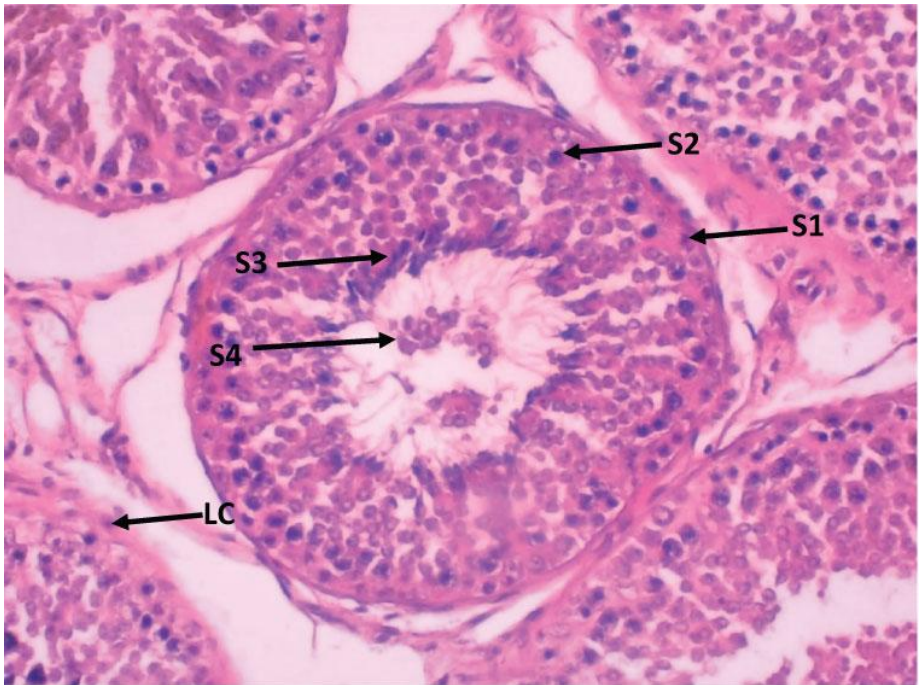

Figure 1. Cross section of testis of Malin sheep.
Normal spermatogenesis (S1 - Spermatogononia, S2 - Spermatocyte, S3 - Spermatid, S4 - Spermatozoa, LC – Leydig cells. (400x)

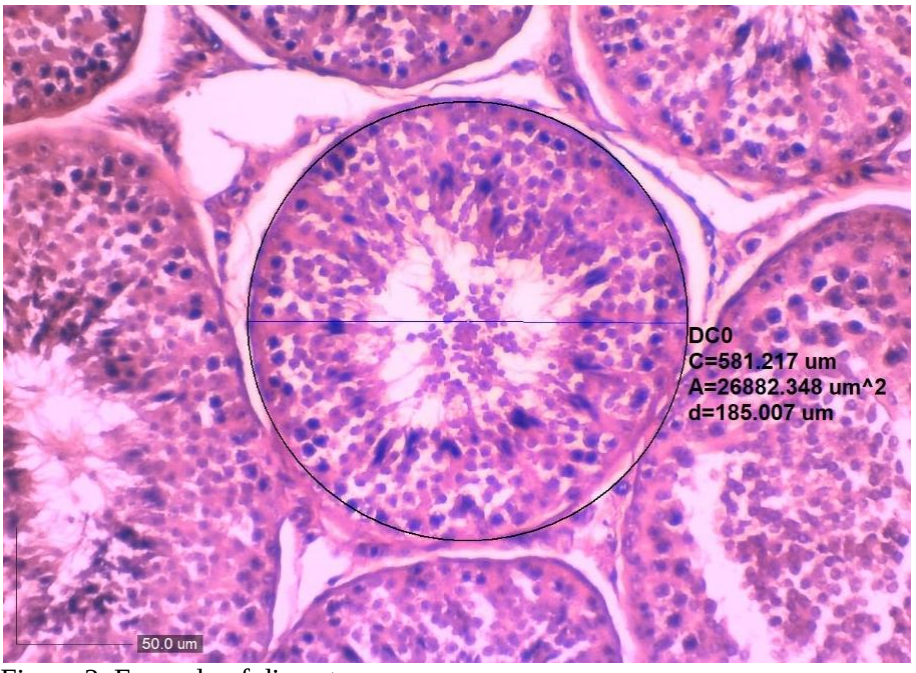

Figure 2. Example of diameter

There was no significant difference in seminiferous tubule score, Leydig score and seminiferous tubule diameter among all groups. However, testes weight and seminiferous tubules in Canola group were higher than Prilled and Casa groups. In male animals, a lot of interests were given to using fatty acids, especially polyunsaturated fatty acids (PUFA) to improve semen quality in animals. The reason is PUFAs comprise about 60% of the phospholipids that bind fatty acids of cells and in sperm (Wanhong et al., 2017).

Ruminants are highly efficient in the absorption of saturated fatty acids compared to non-ruminants. This capability is attributed to the ruminant bile which is high in taurine-conjugated bile. Taurine conjugated bile has the effect of solubilisation of fatty acids under acidic condition of the ruminant upper intestine compared to other herbivores that have excess to glycerine-conjugated bile that loses its solubilisation effect at much less acidic condition (Lock et al., 2006). Another factor that influences the efficient absorption of fatty acids is the presence of lysolecithin which promotes the formation of micelle. Lysolecithin have marked effect of micelle solubility on saturated fatty acids. Micelle is an important complex needed to move the fatty acids to be absorbed by the intestinal cells.

Upon absorption by intestinal cells, the free fatty acids are re-esterified into triglycerides, while PUFAs attach to form phospholipids rather than forming triglycerides (Woods and Fearon, 2009). In this way PUFA will not be used as a source of energy instead is incorporated into the cell membrane. Canola oil contains 30% of PUFA which is higher compared to Prilled fat and Calcium soap made from palm oil which have only 10% of PUFA (Giakoumis, 2018). Diet high in PUFA can stimulate seminiferous tubule development, increase number of Sertoli cell as well as testis weight (Li et al., 2017), which is in agreement with the present study although not significant. Dietary treatment with high PUFA increases the expression of genes involved in PUFA metabolism and steroidogenesis, thus would improve testis development. However, PUFAs are also associated with increased oxidative stress which causes detrimental effect on testes and reduces semen quality (Li et al., 2017). Unsaturated fat is more prone to oxidation as oxidation takes place at the double bond, while saturated fats have higher oxidation stability. Prilled fat and Calsium soap are from palm oil which have higher saturated fats, thus much more stable compared to Canola oil which is high in unsaturated fats.

The other reason why unsaturated fat more likely to affect spermatogenesis is due to different testicular cells which have morphologically different mitochondria causing different metabolic preferences. Spermatogonia on basal membrane are mostly reliant on glycolysis for their energy production while sperms rely on glycolysis, oxidative phosphorylation and beta oxidation for energy production. Other evidence from Lenzi et al. (2000) indicated that immature germ cells had higher saturated fatty acids, higher essential fatty acids and lower long chain PUFAs while mature spermatozoa had higher concentration of n-3 PUFA docohexaenoic acid (DHA).

This explains why fatty acid use for energy is less likely to affect spermatogenesis. As PUFAs are important for the construction of cell membrane in testicular cells, thus causing canola oil to have bigger diameter of seminiferous tubule compared to Prilled fat and Calcium soap.

Conclusion

In conclusion, supplementing the rumen with protected fat from palm oil and feed enriched with PUFA to adult male Malin sheep does not affect testosterone level. Calcium soap and prilled fat supplementation that are high in saturated and monosaturated fatty acids does not influence testosterone synthesis and testicular cell activity.

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