Testicular histology and blood testosterone levels of male rabbit after given concentrated diets containing calliandra leaf meal and pineapple peels

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Abstract. *Calliandra calothyrsus* Meissn is one of a highly protein source of forage, however, it is not widely used for non ruminant feed because it contains antinutritional substances in the form of condensed tannin. Tannin can bind proteins, this tannin-protein complexes are difficult to be digested in the gastrointestinal tract, then will be defecated outside the body. To optimize the utilization of calliandra in the diet, pineapple peels were added as a source of protease (bromelain). Beside of waste utilization, it is expected that bromelain can degrade the tannin-protein complexes thereby reducing the negative effects of tannins. This research is a feeding experiment on male rabbit (*Lepus* sp), five weeks old. The diet formulation is prepared according to the standardized diet of the local rabbit. This experiment used a Completely Randomized Design (CRD) with four treatments i.e. control group which were only given commercial feed (R0), commercial feed contained 15% of Calliandra leaf meal and 30% of pineapple peel (R1), commercial feed contained 30% of Calliandra leaf meal and 30% of pineapple peel (R2), and commercial feed contained 45% of Calliandra and 30% of pineapple peel (R3). The treatment was done for three months. The results of this study on the reproduction of male rabbits showed that the increase of calliandra leaf meal level in a diet containing 30% of pineapple peels affect the testicular histology, and also decreased the diameter of seminiferous tubule and blood testosterone levels of male rabbits.

1. Introduction

As a forage, *Calliandra calothyrsus* Meissn. has quite high protein content (20-25%), but it is still limited to be used as non ruminant feed i.e. rabbits, pigs and chickens. *C. calothyrsus* leaves contain condensed tannins, due to their interaction with proteins, show antinutrition effects. Tannins are naturally a plant polyphenolic compound which can strongly bind protein molecules. Tannins form complexes with carbohydrates, polysaccharides, cellulose, minerals, bacterial cell membranes, and enzymes involved in the digestion of proteins and carbohydrates [1].

Tannins in the diet can reduce protein digestibility of non-ruminants. Tannin-rich feed decrease digestibility of proteins and dry matter due to the tannins inhibitory effect on trypsin and other digestive enzymes activity in the intestines of rat and chicken [2]. In poultry, 7% of tannins will result in death
and 0.5-2.0% of tannins decrease the rate of animal growth and egg production. High levels of tannins cause abnormalities of chicken limb development (in starter phase) [3].

The combination of tannin and protein causes protein resistance to the proteolytic enzymes (tannins act as protease inhibitors). Tannins generally inhibit trypsin, which is the activator of all enzymes released by the pancreas (zymogen). The antinutritional effects of tannins can be reduced by the addition of exogenous proteases, such as bromelain derived from pineapple peel waste. Contrary to the action of tannins as protease inhibitors, the bromelain (a class of protease cysteine enzymes), contains in all parts of the pineapple plant including the peel.

Bromelain, a proteolytic exogenous enzyme, has the ability to hydrolyze peptide bonds on proteins or polypeptides into smaller molecules or amino acids, so it is very useful to help protein digestion. The combination of pineapple peel waste with a high-protein plants such as C. calothyrsus and concentrate feed is expected to reduce the cost of non-ruminant production.

Good reproductive performance can optimize rabbit production and it is very necessary to reduce the cost of feed. So, it is important to study the effect of C. calothyrsus leaf meal addition on feed containing pineapple peel on testicular histology and blood testosterone level of local rabbits (Lepus sp).

2. Methods

2.1. Animal feed
Leaves of C. calothyrsus are hand-picked in the area of Mekarsari, Baturiti, Tabanan, Bali. The leaves were wilted for 6 days, blended and sieved. Pineapple (Ananas comosus) peels were collected freshly from Badung Market (Denpasar, Bali) and blended. This study used rabbit commercial feed which were mashed into flour. All of the ingredients were mixed homogeneously by a mixer and put into a pelleting machine. The pellet were dried with a freeze dryer for 8 hours. Feed were stored in a cool, dry place.

2.2. Treatments
The research was conducted on weaned rabbits (Lepus sp), five weeks old. This experiment used four treatments i.e.control group which were only given commercial feed (R0), commercial feed contained 15% of Calliandra leaf meal (CLM) and 30% of pineapple peel (PP) (R1), commercial feed contained 30% of CLM and 30% of PP (R2), and commercial feed contained 45% of CLM and 30% of PP (R3). Each treatment was repeated 6 times.

Before treatments, each rabbit was injected with Ivomek 0.2 mL to prevent parasite attacks. The weaned rabbits were adapted to housing and feeding for one week. The rabbits were housed individually under a rabbit standard laboratory conditions (23-25°C temperature, 50-60% relative humidity, and 12 hours light/dark cycle). The first week was the period of feed adaptation so that all rabbits were able to consume their feed up to 100% without residual feed and without decreasing the consumption and body weight. The feed was given 150 g/day (twice a day, in the morning and evening). Rabbits were given drinking water ad libitum. The experiment was approved by The Animal Ethical Committee, Faculty of Veterinary Medicine, Udayana University.

2.3. Sampling collection and analysis
Rabbits were sacrificed at the end of the treatments with an intramuscular ketamine injection on the hamstrings. Blood samples were taken 5 mL with an EDTA capillary tube from the right ear veins, fed into an effendorf tube and centrifuged (3000 rpm) for 10 minutes. Serum was taken 50 mL followed by deproteinase with addition of 500 μL uranil acetate, centrifuged (3000 rpm) for 5 minutes and 500 μL supernatant was taken. The procedure for determining testosterone levels was performed according to the protocol of the Total Testosterone C-A-C kit.

Rabbit testes were cleaned with 0.9% NaCl solution, dried with tissue paper, then fixed into Neutral Buffer Formalin solution. Preparation of histological slide was performed by paraffin method and Hematoxylin-Eosin staining. Testes histopathology were observed using an electrical microscope.
(magnification 100x and 400x), documented with a microscopic camera (Optilab) and measured with Image Raster software.

2.4. Data analysis
Data analysis was done statistically by SPSS program i.e. Kolmogorov-Smirnov normality test (the normality of the data), Leven’s test (the homogeneity of the intergroup variances), and One Way Anovatest followed by the Duncan posthoc test.

3. Results and discussion
The effects of feeding trials given in this study on the reproductive system of male rabbits were assessed through histopathological examination of testicular organ and blood testosterone levels.

3.1. Diameter of Testicular Seminiferous Tubules
The measurement of testicular seminiferous tubules diameter can be used to predict spermatozoa production. The combination of calliandra leaf meal and pineapple peel in concentrate feed showed a significant decrease of seminiferous tubules diameter of the rabbit testes. The highest level of calliandra leaf meal (R3 treatment) significantly decreased the diameter of the seminiferous tubules compared to the control and other treatments (R1 and R2). However, the levels of calliandra leaf meal between the R2 and R3 treatments did not show a significant difference of the testicular seminiferous tubules diameter.

Table 1. Diameter of testicular seminiferous tubules (µm) of male rabbit fed a combination of calliandra leaf meal (CLM) and pineapple peel (PP) in commercial rabbit feed

| Treatment | Diameter of testis seminiferous tubules (µm) |
|-----------|---------------------------------------------|
| R0        | 146,907 ± 21,274 a                           |
| R1        | 112,756± 18,595 b                           |
| R2        | 103,315± 10,195 b                           |
| R3        | 83,093 ± 13,007 c                           |

Different letters follow (Mean ± SD) values in the same column show significant differences (P<0.05). R0 = control group which were only given commercial feed, R1 = commercial feed contained 15% of CLM and 30% of PP, R2 = commercial feed contained 30% of CLM and 30% of PP, and R3 = commercial feed contained 45% of CLM and 30% of PP.

The results were in line with the histopathological examination of the seminiferous tubules of the control group of male rabbit testes (Fig. 1A) which were only given commercial feed without calliandra leaf meal and pineapple peel. The wall of seminiferous tubules was relatively thicker with spermatogenic cells surrounding a quite large tubular lumen, showed a normal spermatogenesis.

The higher the level of calliandra leaf meal in the feed, the diameter of the seminiferous tubules decreased significantly compared to the control (R0), however, R2 treatment did not show a significant difference with the R3. In the feed with addition of graded calliandra leaf meal levels, the diameter of testicular seminiferous tubules significantly decreased between treatments (R1, R2 and R3) compared with the control (R0) (Table 1). The decrease of testicular seminiferous tubules diameter was along with the decrease of the wall thickness of seminiferous tubules itself (Fig. 1). This phenomena illustrated the disruption of spermatogenesis.
Figure 1 The histology of male rabbits testes which were fed commercial feed containing a combination of a graded calliandra leaf meal (CLM) and 30% of (PP): (A). Control group which were only given commercial feed (R0), (B). Commercial feed contained 15% of CLM and 30% of PP (R1), (C). Commercial feed contained 30% of CLM and 30% of PP, (D). Commercial feed contained 45% of CLM and 30% of PP (R3) (magnification 400x, white line showed the diameter of testicular seminiferous tubules).

The wall of testicular seminiferous tubule is composed of spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids cells (from the outer to the inner layer, respectively). When the process is complete, the lumen of seminiferous tubules will appear to be filled with spermatozoa cells. Based on the testicular histology observations in this study, only the lumen of the control group appeared to be filled with few spermatozoa cells. This results were similar to the research on male rabbits fed with 20% leaf meals of Leucaena and Gliricidia that caused a depressive effect on the spermatozoa cells motility and concentration, the seminiferous tubules diameter and the volume of rabbit semen [4].

The testicular seminiferous tubules are lined by the seminiferous epithelium. The epithelium of testicular seminiferous tubules consists of sertoli and spermatogonia cells [5]. Histological preparation of the testicular seminiferous tubules showing the arrangement of spermatogenic cells which are more clearly and densely in the control group (Fig. 1.A), indicated a normal spermiogenesis. If compared with R0 (control) in Fig. 1.A, treatment groups which were fed with 30% of pineapple peel and graded levels of calliandra leaf meal (Fig. 1.B-D), the size of the seminiferous tubules as well as the size of the diameter of the tubules itself appeared to be smaller, and there were no appearance of spermatozoa cells within the tubular lumen.

The histological observation showed an irregular arrangement of spermatogonic cells in the rabbit testes of R1 treatment (the lowest level of calliandra leaf meal) (Fig. 1.B). Meanwhile, the histology of
rabbits testes at higher levels of calliandra treatment indicated desquamation of testes seminiferous tubules, and also spermatogenic cells disorganization and degenerative changes.

The disorderly of spermatogenic cells arrangement inside the testes seminiferous tubules (Fig. 1B) showed a disruption of Sertoli cells, so that the spermatogenic cell development was disrupted. Sertoli cells could not properly maintain and regulate the growth and development of spermatogenic cells, therefore the supply of testosterone hormone from Leydig cells was inhibited [6]. The results showed that the higher levels of calliandra leaf meal in the diets, the histology of the seminiferous tubules showed the more immature development of spermatogenic cells. Presumably tannins retarded the puberty of rabbits that were treated by tannin-rich feed before the adult period.

Rabbit puberty varied greatly, male rabbit reached their sex maturity at the age of 4-8 months, depending on the race and the feed. Motile spermatozoa cells were seen in the first ejaculate at the age of 4 months, but good fertility of spermatozoa cells were obtained at the age of 7-8 months [7]. Leaves of *C. calothyrsus* contain condensed tannin compounds which decreasing spermatozoa concentration by inhibiting cell division (mitosis) during spermatogenesis. Tannins decreased the spermatozoa concentration in line with the decrease of spermatogenic cell count if the administration of tannins compared with the administration of alkaloids + flavonoids + tannins which were obtained from *Plucheia indica* leaves [8].

Although it has not been demonstrated clearly, the mechanism or action of active substances contained in calliandra leaf meal and pineapple peels might cause the disintegration of some seminiferous tubules. The spermatocytes degeneration can be linked to the effect of calliandra tannins, as well as polyphenol compounds affected the process of DNA synthesis and cell division [4].

3.2. Testosterone hormone levels

The statistical analysis showed that R1, R2 and R3 treatments (the combination of graded levels of calliandra leaf meal and 30% of pineapple peel) have significantly differences (P<0.05) on the testosterone levels if compared with R0 (the control group) (Table 2).

| Treatment | Testosterone Hormone Level (ng/mL) |
|-----------|-----------------------------------|
| R0        | 3.621 ± 3.558a                     |
| R1        | 0.842 ± 0.405 b                    |
| R2        | 0.682 ± 0.475b                     |
| R3        | 0.755 ± 0.154b                     |

Different letters follow (Mean ± SD) values in the same column show significant differences (P<0.05). R0 = control group which were only given commercial feed. R1 = commercial feed contained 15% of CLM and 30% of PP, R2 = commercial feed contained 30% of CLM and 30% of PP, and R3 = commercial feed contained 45% of CLM and 30% of PP.

Spermatogenesis depends on testosterone hormonal action [9]. At puberty, testosterone is very necessary to initiate spermatogenesis and furthermore to maintain spermatogenesis in the adult. Testosterone is required to complete meiosis division and spermatid cells differentiation [10], and to initiate the process of meiosis of spermatocyte cells. Testosterone plays an important role in the first meiotic phase of the diakinesis stage, at the beginning of metaphase cleavage. Therefore, a decrease of testosterone level affected the testicular histological structure i.e. the seminiferous tubules diameter and spermatogenic cells development.

Several types of tannins have been shown to inhibit enzymes during protein synthesis. Tannins bind and form complexes with proteins and other polymers in the proper conditions of concentration and pH [11], then indirectly inactivate phosphat in the body. Therefore, the energy metabolism and the nutrient
qualities which were required by the spermatozoa cells will decrease. Thus tannins can disrupt the metabolism of spermatozoa cells.

In this study, there were histopathological changes and decrease of diameter of the testicular seminiferous tubules and also decrease of testosterone hormone levels of male rabbits which were given a graded level of calliandra leaf meal and 30% of pineapple peel when compared with the control group, this indicated a spermatogenesis disorder. The negative impact of calliandra tannin could not be overcome by the addition of 30% of pineapple peel because the levels of calliandra leaf meal that were used in this study were still to high.

4. Conclusion
Increasing level of calliandra leaf meal in commercial feed containing 30% of pineapple peel caused a depressive effect by decreasing the testicular seminiferous tubule diameter, as well as the decrease of testosterone hormone level of male rabbit.

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