



## Effect of short-term bee bread on testicular cell development and testosterone level in male Sprague Dawley rats

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### **ABSTRACT**

Bee bread has been widely traditionally used for male fertility enrichment although limited scientific data are available on its effectiveness. Hence, the aim of this study was to determine the effect of bee bread on testicular cell development and testosterone level in male Sprague Dawley rats. In this study, 24 adult male Sprague-Dawley rats were selected and divided equally into four treatment groups ( $n = 6/\text{group}$ ) which were Control (C: 0 g of bee bread/kg body weight), Treatment 1 (T1: 1 g of bee bread /kg body weight), Treatment 2 (T2: 2 g of bee bread /kg body weight) and Treatment 3 (T3: 3 g of bee bread /kg body weight). The bee bread was administered daily and orally according to the designated treatment groups using oral gavage for 28 days. After 28 days of treatment, rats were euthanised and testicular cell development and testosterone level were measured. Results showed that rats in the T2 group had thicker ( $P < 0.05$ ) seminiferous tubular diameter (STD) than the control group and higher ( $P < 0.05$ ) seminiferous epithelial height (SEH) compared to C and T1 groups. The testosterone level in the T1, T2 and T3 groups was significantly higher ( $P < 0.05$ ) compared to control. In conclusion, this study suggests that supplemented with 2 g of bee bread per kg body weight is likely to give a positive effect by improving testicular cells development and testosterone level in male Sprague Dawley rats.

**Keywords:** Bee bread, male fertility, testicular cells, testosterone, rat

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### **INTRODUCTION**

Lately, many studies in exploring possible alternative medicine from both natural or herbal remedies in curing and improving difficulties in infertility were done by experts (Zaid et al., 2021). Therefore, the honeybees' products including bee bread especially have been commonly used as sources of functional food that have been used in complementary medicine as well as in the enhancement of male fertility (Suleiman et al., 2021). Bee bread is an "alchemical" that consists of 25% honey or nectar and 70% bee saliva (Vialli 2014; Matt 2022) or combination of bee pollen, nectar, and digestive enzymes (Mohammad et al., 2020). It is rich with polyphenols, nutrients (vitamins and proteins), fatty acids, carbohydrates, minerals, oligo-elements, essential oils, enzymes, pigments, and other biologically active natural substances suitable for human's consumption (Barta et al., 2022; Milojkovic, 2018) making bee bread a natural healthy food that contains all important nutrients needed by

humans (Savaiano, 2014; Naseron and Teet, 2016; Milojkovic, 2018). Bakour et al. (2022) claim that bee bread has 300 or more compounds and comprises a variety of compositions including sugars, organic acids, vitamins, free amino acids, fatty acids, polyphenols, minerals, microbes, and minerals.

Previous findings shown that bee bread can be considered as a food supplement due to its nutrients that provide health benefits. For example, bee bread has antioxidant, anticancer, antihypertensive, antimicrobial, neuroprotective, and anti-inflammatory properties (Bakour et al., 2017; Sobral et al., 2017; Kowalski and Makarewicz, 2017; Khalifa et al., 2020). According to study by Kowalski and Makarewicz (2017), both bee bread and propolis have the same antibacterial and antioxidant qualities as honey. These properties boost immunity in the body to fight bacteria and to repair body tissue (Mărgăoan et al., 2019). In addition, bee bread is also capable of treating hyperglycaemia, hyperlipidemia, inflammation, and is also a source of antioxidants to fight free radicals and scavenge reactive oxygen species (Bakour et al., 2022). Bee bread is also known to improve athletes' running performance due to its high antioxidants (Ping et al., 2018). Consumption of this ingredient is also good as it is reputed to have a positive impact on water intake and glucose metabolism in diabetic rats, offering potential for the treatment of hyperglycemia and other diabetes-related condition according to research by Li et al. (2019). Furthermore, supplementation of bee bread increased sperm morphology in adult male rats (Zakaria & Haron, 2020).

However, lack of information and study were done by using bee bread particularly on the effect of bee bread supplementation towards testicular cells development and hormonal changes. This study aimed to determine the effect of short-term bee bread supplementation on histological changes in seminiferous tubules and testosterone level in male Sprague Dawley rats.

## **MATERIALS AND METHODS**

### **Experimental animals**

In this study, 24 adult male Sprague-Dawley rats (age 8 - 10 weeks old, weight 200 – 250 g) were used as animal model. Rats were maintained as per standard national guidelines and protocols (OECD, 1995). All rats were kept in clean cages and maintained in a controlled and well-ventilated animal room at  $25 \pm 2$  °C with 12-h light/12-h dark cycles. Food pellets and water were supplied to all rats' *ad libitum*. The rats were equally assigned into 4 groups (n=6/group), which were Control (C: 0 g of bee bread/kg body weight), Treatment 1 (T1: 1 g of bee bread /kg body weight), Treatment 2 (T2: 2 g of bee bread /kg body weight) and Treatment 3 (T3: 3 g of bee bread /kg body weight). Bee bread consumption in this study was taken into consideration according to the previous study (Zakaria & Haron, 2018). The rats were administered with the bee bread daily by force feeding based on their treatment groups using a syringe and oral gavage needle for 28 days of treatment (OECD, 1995). This study protocol was approved by the UniSZA Animal and Plant Research Ethics Committee (UAPREC/04/042).

### **Preparation of bee bread**

The bee bread from stingless bee (*Heterotrigona itama*) samples used in this experiment were procured locally from a bee farm in Kelantan, Malaysia similar samples as reported by Othman et al. (2019). For the feeding process of the bee bread to experimental animals, the bee bread was prepared in accordance with the recommended dosage and was pre-diluted with 1.0 ml of distilled water to ease the administration. The method used for the oral administration of bee bread is by force-feeding to the rats of control and treatment groups.

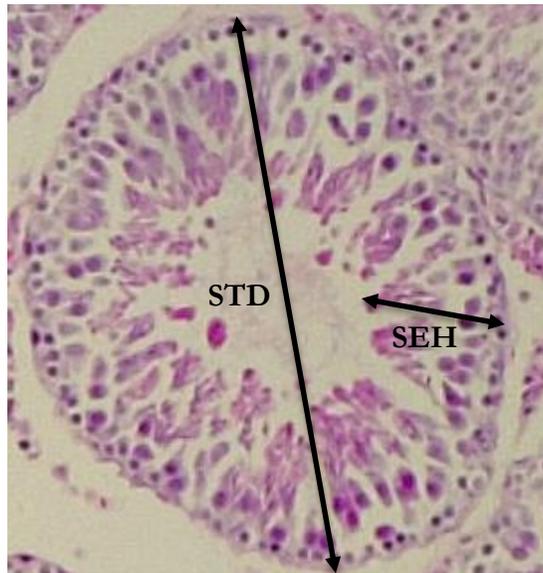
### **Blood and serum collection**

For blood collection, the rat first sacrificed and dissected. The blood was taken using needle with a syringe (5 ml/cc) which carefully inserted into the inferior vena cava located just above the junction of the common iliac veins near the heart for hormone tests. The plunger is gently drawn back to generate a negative pressure within

the syringe, enabling the collection of the desired amount of blood. The drawn blood was placed into a blood collection tube with a clot activator (red cap). Before spinning the blood, it should rest for around 30 minutes at room temperature (20-25°C). The Eppendorf Centrifuge 5810 R were used to spin blood for 15 minutes at 4°C and 2500 rpm. After centrifuging, serum could be extracted using a pipette. The serum was refrigerated at -20°C for further analysis (Haron, 2014).

### Histological assessment of seminiferous tubule

After 28 days of treatment, all rats were sacrificed by cervical dislocation. Testes were removed and weighted. The testicles were submerged in 10% formalin and fixed. Each testis was sliced and placed inside a histology cassette. Before dehydrated into ascending grades of ethanol and embedded in paraffin, the solidified paraffin-embedded tissues were cut into slice of 5 µm before the sections were stained with haematoxylin and eosin (Rolls, 2020; Sampias & Rolls, 2020). The slides then mounted with dibutylphthalate polystyrene xylene (DPX) and a coverslip was applied to protect and preserve the section (NSH, 2001). The slides were examined for any morphological differences in the seminiferous tubule of the testis. The thickness of seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) was measured using LAZ X image analyser. STD was measured at 2 sites in 20 transversely cut tubules per slide from which the average diameter was calculated (McLachlan et al., 2007). SEH was measured from the basement membrane to the surface of the epithelium at two different regions and expressed as the mean of the two measurements (Nieschlag et al., 2010)



**Fig. 1.** Photomicrograph of rat testicular histology. a) Seminiferous tubular diameter (STD); b) Seminiferous epithelial height (SEH) (400× magnification).

### Determination of testosterone levels

Serum testosterone level was determined by using enzyme-linked immunosorbent assay (ELISA) commercial kit from Elabscience, USA according to the manufacturer's instructions. The assay procedure was based on the user manual provided by the manufacturer of the Elabscience Rat T (Testosterone) ELISA kit (Catalog No.: E-EL-R0155) with testosterone standards (Testosterone: 0, 0.31, 0.62, 1.25, 2.5, 5, 10 and 20 ng/mL). 50 µl standard and samples were added to each well and 50 µl of Biotinylated Detection Ab were immediately added to each well. Then, the well was incubated for 45 minutes at 37°C before aspirated and wash each well. Then, 10 µl of HRP conjugated were added to each well and incubated for 30 minutes at 37°C. Each well is then aspirated again and washed 5 times. 90 µl Substrate Reagent was added to each well and incubated for 15 minutes at 37°C. Then, 50µl stop solution was added before the optical density (OD) of each well was determined, using microplate reader set at 450 nm (Le et al., 2009).

## **Data analysis**

Computerised statistical analysis was performed using IBM SPSS Statistics for Windows, Version 19. Experimental data were statistically analysed using One Way ANOVA and expressed as mean  $\pm$  SEM. Statistical significance was accepted at  $P < 0.05$ .

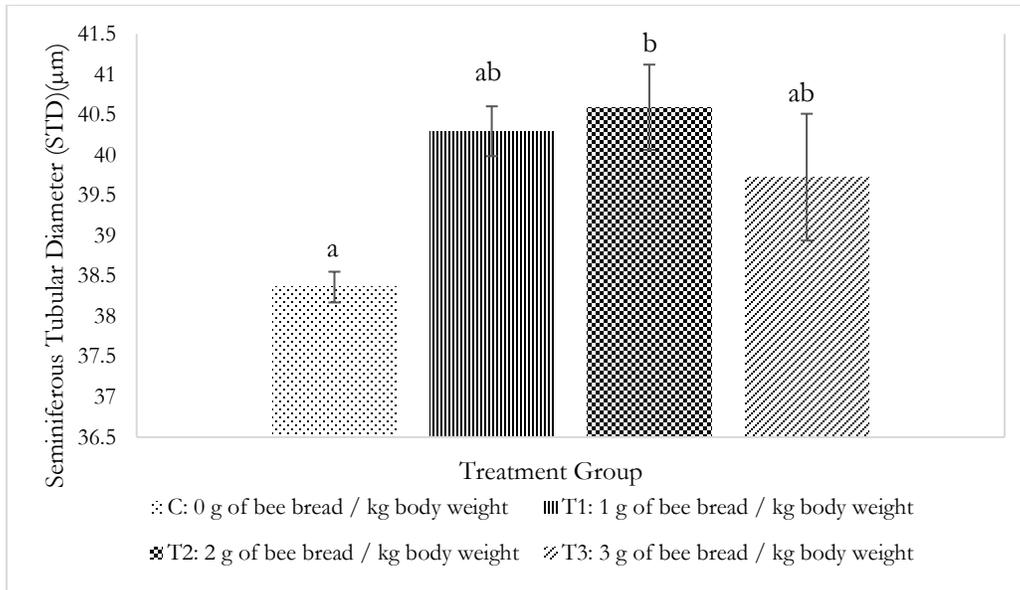
## **RESULTS AND DISCUSSION**

### **Seminiferous tubule of testis**

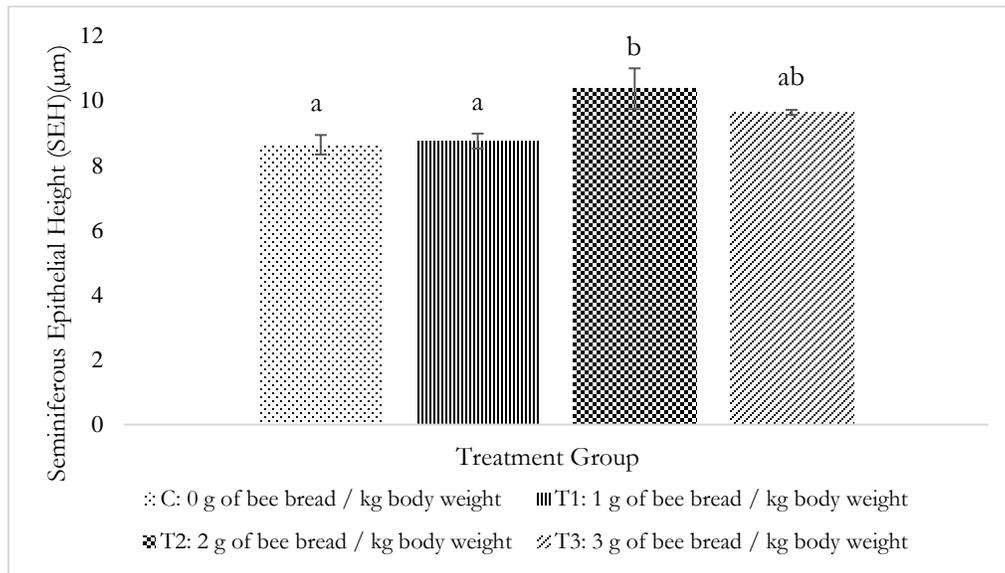
There is scarce of information on the effect bee bread on male reproduction because it is not well studied or explored. In contrast to bee bread, most studies reported have been focussing on other bee products such as bee pollen and honey due to the high volume produced and easy to be collected. Moreover, as bee bread is composed of approximately 25% honey or nectar, 70% pollen and bee saliva (Vialli, 2014), most of the findings in this study can be accounted by citing research on other bee products including honey and bee pollen.

The findings on testicular cells development (i.e., thickness of seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH)) are presented in Figures 2 to 7. The thickness of STD was significantly higher ( $P < 0.05$ ) in T2 group, compared to Control and T1 groups (Figure 2). Whilst, the thickness of SEH was significantly higher ( $P < 0.05$ ) in T2, compared to Control and T1 groups (Figure 3). The current result is supported by Mehraban et al. (2014) and Syazana et al. (2011) which showed that supplementation of honey and pollen increased the length of seminiferous tubular diameter (STD).

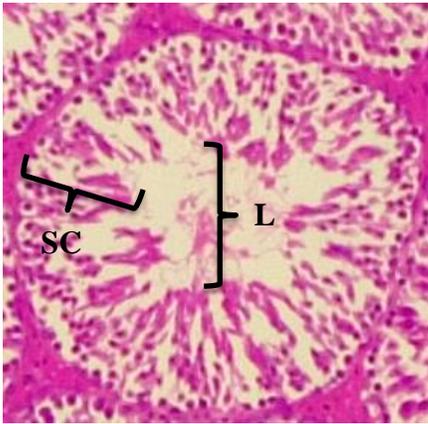
Moreover, honey enhances spermatogenesis by interacting with Sertoli cells (Syazana et al., 2011) known as testis' somatic cells which are important in controlling spermatogenesis and altering sperm production rates (Fetouh and Azab, 2014). Sertoli cells function as a regulator in seminiferous tubules by assisting in the conversion of germ cells into spermatozoa (Schulster et al., 2016). This finding suggests that Sertoli cells and bee bread had interacted to enhance spermatogenesis by revealing the development of many spermatogenic cells in T2 group. This can be supported from the present result that the increase of sperm counts in T1, T2 and T3 groups compared with Control group is related with spermatogenesis although not significantly difference (Zakaria & Haron, 2020). Moreover, T3 group does not show better and increase in thickness of SEH and STD results compared to T2. However, there were no significant difference in the thickness of SEH and STD in T3 group compared to other groups.



**Fig. 2.** Thickness of seminiferous tubular diameter (STD) (mean  $\pm$  SEM) of male Sprague Dawley rats' in four different treatment groups. Different superscript (a, b) indicates a significant difference ( $P < 0.05$ ).



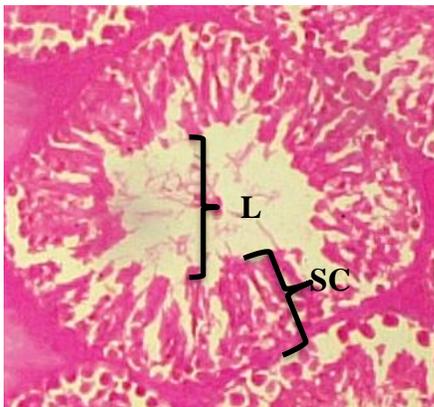
**Fig. 3.** Thickness of seminiferous epithelial height (SEH) (mean  $\pm$  SEM) of male Sprague Dawley rats' in four different treatment groups. Different superscript (a, b) indicates a significant difference ( $P < 0.05$ ).



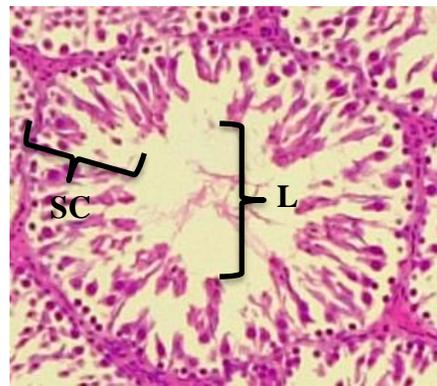
**Fig. 4.** Seminiferous tubule of C group of male Sprague Dawley rats' testis shows less dense packing of spermatogenic cell (SC) with wide emptied lumen (L). (400x magnification).



**Fig. 5.** Seminiferous tubule of T2 group of male Sprague Dawley rats' testis shows densely packed and showed small lumen (L) densely filled up with sperm tails in T2 group. (400x magnification).



**Fig. 6.** Seminiferous tubule of T1 group of male Sprague Dawley rats' testis shows less dense packing of spermatogenic cell (SC) with wide emptied lumen (L). (400x magnification).



**Fig. 7.** Seminiferous tubule of T3 group of male Sprague Dawley rats' testis shows less dense packing of spermatogenic cell (SC) with wide emptied lumen (L). (400x magnification).

### Testosterone Level

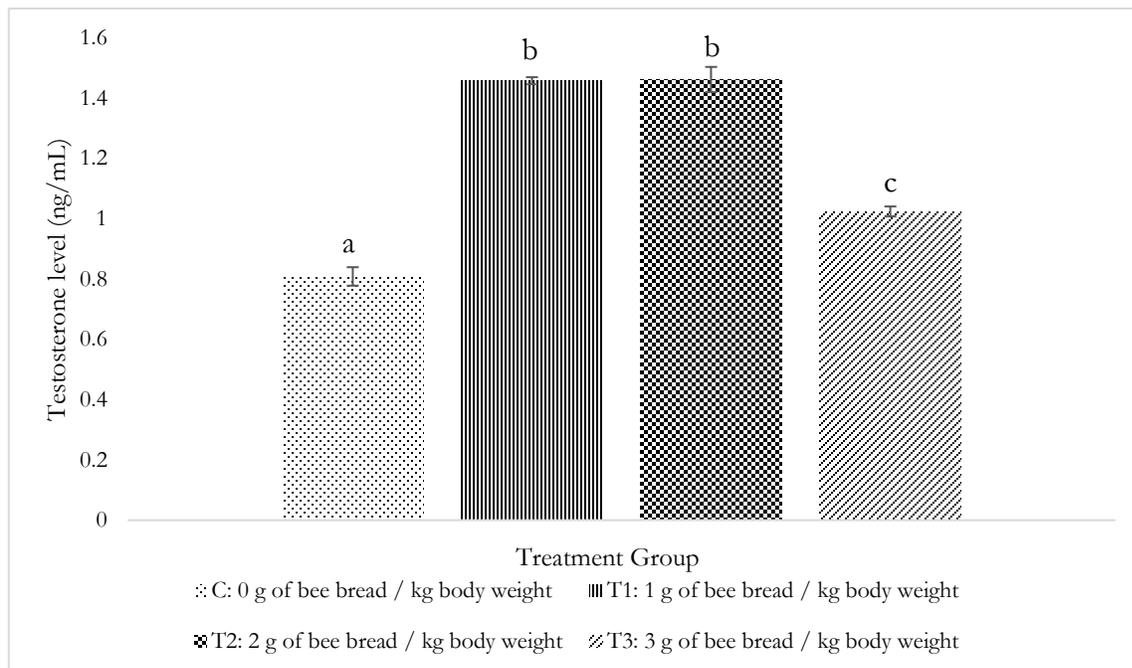
The level of testosterone was significantly higher ( $P < 0.05$ ) in T1 and T2 groups compared to C and T3 groups. The study agreed with Gholami et al. (2018), who reported that pre-treatment with honey increases FSH, LH, and testosterone levels, reduces cellular damage, and protects the testicles from chemotherapeutic damage and testicular ischemia-reperfusion (IR) injury. Furthermore, supplementation of pollen improved the testosterone level, sperm counts and daily sperm production (Selmanoglu et al., 2009). Additionally, Nasrolahi et al. (2013) revealed that administering honey at  $1.0 \text{ g kg}^{-1} \text{ day}^{-1}$  for 40 days can prevent diabetes-related oxidative damage to testicular tissues and raise testosterone levels in the blood. Furthermore, compared to the control, mature male rats treated for 13 weeks with Tualang honey at  $1.2 \text{ g}$  showed higher levels of serum testosterone (Mahaneem et al., 2011).

Besides, Hassan (2009) showed that supplementation of royal jelly increased the level of testosterone in rats. According to Ghazi et al. (2013), treatment with a topical propolis extract helped male rats' reproductive

parameters recover to normal levels after being adversely affected by acrylamide. These previous findings of bee products mostly showed the positive impact on male testosterone levels due to supplementation of honey products, which are relate to their contents such as antioxidant that could help to synthesis testosterone level and protect against from oxygen stress (Banihani, 2008). For example, zinc that increased testosterone and LH levels (Fallah et al., 2018), flavonoids and phenols that can scavenge free radicals and protect against the lipid peroxidation that acrylamide can cause (Yousef and Salama, 2009).

Furthermore, in the present study there is densely packed seminiferous tube and showed small lumen (L) densely filled up with sperm tails in. The improvement on the testicular structures might explain and related with our current studies which are significantly increased in testosterone level. The improvement in sperm qualities such as motility, morphology, and progressive motility in treated infertile individuals with bee pollen supplementation is caused by an increase in testosterone levels (Rasekh et al., 2015; Mohamed et al., 2018).

Moreover, it is shown that the level of testosterone of T3 is significantly lower compared to T2. According to American Society for Reproductive Medicine (2015), men with low or borderline testosterone levels can still possess enough testosterone for producing sperm. Testosterone is essential for sperm production, but the level of testosterone in the testes, where sperm is produced, is significantly higher than that found in the bloodstream. The present result can be supported from the previous study (Zakaria & Haron, 2020) by significantly increased the sperm morphology in T1, T2 and T3 groups than Control group. Hormones other than testosterone also responsible for stimulating sperm production (American Society for Reproductive Medicine, 2015). The effects of testosterone, FSH, LH, and GnRH regulate spermatogenesis, the process of producing sperm. LH increases testosterone release by acting as a tropic factor for Leydig cells. In the meantime, testosterone and FSH work together to maintain spermatogenesis in the testis. FSH is tropic to Sertoli cells. The activity of testosterone is necessary for the beginning, upkeep, and restart of sperm production (Ruwanpura et al., 2010; Chang et al., 2013). The improvement in testicular structure may be due to the current study's increased testosterone levels, which help to sustain sperm production and morphology. Thus, this observation may indicate bee bread give efficacy to the thickness of the seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) and testosterone level especially in T2 groups compared to other.



**Fig. 8.** The level of testosterone (mean  $\pm$  SEM) of male Sprague Dawley rats in four different treatment groups. Different superscript (a, b, c) indicates a significant difference ( $P < 0.05$ ).

## CONCLUSION

In conclusion, the present study showed that supplementation of 2 g of bread per kg body weight had positively affect by increasing the thickness of SEH and STD and increasing the testosterone levels in male Sprague Dawley rats. These findings suggest that bee bread has beneficial role towards male reproductive system. Although higher or lower level of bee bread supplemented (i.e. 1 g, 2 g and 3 g of bee bread per kg body weight) especially in testicular cells did not differ with non-supplemented group, there is a positive pattern where the rats supplemented with bee bread seemed to show a better development of male reproduction. Moreover, T3 group did not show better results in the thickness of SEH and STD and the testosterone levels compared to T2 group. However, further studies are warranted to investigate on concentration of bee bread in a smaller ration such 1.5 g and 2.5 g of bee bread per kg body weight.

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