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Optimizing Reproductive Outcomes of Peranakan Etawa Goat with Microalgae Diet by Determining Ovarian Activity, Estradiol 17β Levels and Serum Malondialdehyde

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ABSTRACT

This study aimed to identify Spirulina (*Athrospira sp*.) supplementation to examine the effects on the blood estrogen and malondialdehyde (MDA) levels and the ovarian activity of Peranakan Etawa (PE) goats during anestrous conditions using ultrasound. A completely randomized design (CRD) was employed in the study, with three treatments totaling six replications (6 individuals) in each group. Group 1 (K-) consisted of untreated normal PE goats; Group 2 (K+) treated anestrus-prone PE goats with prostaglandin (PGF2α) and Gonadotropin-releasing hormone (GnRH) hormones; Group 3 (KP) treated anestrus-prone PE goats with PGF2α and GnRH hormones with super-antioxidant feed at a dose of 0.5% body weight for 21 days. The data observed included the onset of estrus, ultrasound picture of the ovaries, serum estrogen levels using the Enzyme-linked Immunosorbent Assay (ELISA) method, and MDA levels using the Thiobarbituric Acid (TBA) test method. ANOVA (Analysis of variances) post hoc LSD test was used to examine the collected data, and a significance value of $P<0.05$ was set. The findings indicated no significant difference between the goats in the KP and K-control groups ($P<0.05$) regarding estradiol 17β levels in those goats experiencing post-partum anestrus. Moreover, significantly lower serum MDA levels ($P<0.05$) were seen in the KP group. The ultrasound observations' outcomes further demonstrated the presence of a dominating follicle image, which suggested ovarian activity following microalgae feeding. In goats undergoing post-partum anestrus, administering two injections of PGF2α in conjunction with microalgae supplementation was found to dramatically raise serum estradiol 17β levels.

Key words: Anestrus, Estrogen, Goat, MDA, Microalgae

INTRODUCTION

Reduced ovarian function in cattle causes a condition known as ovarian hypofunction, which can result in infertility or subfertility (Skovorodin et al. 2020). Numerous variables, including nutritional inadequacies, hormonal imbalances, genetic predispositions, viral infections, and environmental conditions, can be potential causes of ovarian hypofunction (Salman et al. 2021). The primary symptom of numerous additional conditions impacting the estrous cycle in livestock is failure to enter the estrous or anestrus (Masruro et al. 2020). According to Hermadi et al. (2017), declined stimulation related to hypothalamic-pituitary-ovarian function, which will cause decreased secretion of gonadotropins, changes in the quantity and quality of hormonal secretions, and a failure of follicular cells to respond to hormonal stimulation, are all common causes of anestrus due to ovarian hypofunction. A comprehensive understanding of ovarian hypofunction in goats is crucial for effectively diagnosing and managing this condition.

Livestock health status can be estimated and assessed using blood biochemistry (Mohammed et al. 2016). Measuring particular indicators is necessary to identify different clinical, physiological, and metabolic issues in infertile goats. Oxidative stress conditions have been extensively studied in veterinary medicine over the past ten years, and it is believed that they have a tight correlation with the patho-mechanism of a number of disorders, including infertility. Oxidative stress is brought on by an imbalance in the body's system for producing excessive amounts of antioxidants, a deficiency in antioxidants as a defense, and the generation of free radicals (Fujii et al. 2005).

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The result of lipid peroxidation in cell membranes is MDA, which functions as a biological biomarker of lipid peroxidation and assesses the level of oxidative stress (Singh et al. 2020). It can also be used as an indicator to evaluate oxidative damage to an organ resulting from an increase in free radicals in the body. Elevated MDA values suggest elevated quantities of free radicals within the body and insufficient activity of antioxidant enzymes. The body produces more radicals and causes more extensive tissue damage in proportion to the MDA level (Lu et al. 2018).

There have been numerous reports of treatments to restore ovarian activity in situations of postpartum anestrus (ovarian hypofunction), including injecting gonadotropin hormones into cows (Long et al. 2021). Additionally, it has been noted that FSH and LH can be released in beef cattle with GnRH injection (Amin et al. 2023). But as farmers know from experience, hormone medication is not always the best solution, particularly when it comes to difficult-to-diagnose cases of postpartum anestrus in PE goats. According to Ciccioli et al. (2003), cows who eat feed that is greater in quantity and quality will go back into estrus faster than those who eat feed that is lower in quality. This suggests that the nutritional conditions of cows play a significant role in their estrus cycles and overall reproductive performance and also suggests that the main factor in enhancing postpartum anestrus conditions is feed management.

Numerous studies have been conducted on the administration of Spirulina microalgae (*Athrospira sp.*). For example, Wang et al. (2023) examined increases in body score condition (BCS), body weight, and reproductive performance by supplementing Spirulina sp. Mansour and Zeitoun (2023) examined at growth performance, puberty, and blood metabolism in goats given Spirulina supplements; and Eldaim et al. (2018) gave a combination of *Spirulina sp.* and Vitamin A supplements to goats in the late gestation phase, and the result was a decrease in fetal serum Tumor Necrosis Factor-alpha (TNFα).

Following the above description, studies investigating the effects of administering super-antioxidants derived from the microalga Spirulina (*Athrospira sp.*) to PE goats experiencing post-partum anestrus with or without hormone therapy are required. Applied research in the field, which begins with comparing and assessing how well it is delivered in terms of the post-partum estrus response, is the basis of this study.

MATERIALS AND METHODS

Ethical approval

This research was approved by the research ethics commission of Brawijaya University with ethics number No. 968-KEP-UB.

Sampling

18 PE female goats, ages 2-3 years, weighing 35±5kg and having undergone parturition, were used in the study, which adopted a completely randomized design (CRD). The eighteen goats were then split up into three groups: K-, which included six goats that had delivered normally and had shown the first estrus on the 30–60day postpartum period; K+ and KP, which each included six goats that had delivered normally and had not shown estrus until the day >60 postpartum period. The following treatments in group K+ and KP are shown in Fig. 1.

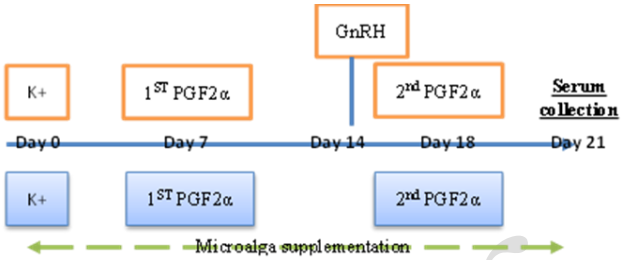

Fig. 1: Treatment administered to the K+ and KP groups during the study

Post-Parturition examination of female goat condition

PE goats were investigated to determine their reproductive history after giving birth. Estrous symptoms are regarded as normal if they develop 30 to 50 days after parturition; if they do not develop 60 days later, the reproductive status was assessed using ultrasound imaging. Using ultrasound imaging, an abdominal probe was used to measure ovarian activity. If ovarian activity (follicle development) was absent and a corpus luteum cyst was seen, the condition was labeled as anestrus.

Estrus response examination and serum sample collection

After the hormone therapy was finished, a buck (teaser) was placed into the cage of the female to observe the estrus response in both treatment groups every three hours. The jugular vein was used to obtain serum samples under the following circumstances:

1. K-: when the female displayed an estrous reaction.
2. K+ group: 3rd day following the second PGF2α injection.
3. KP group: 3rd day following the second PGF2α injection.

Observation of estrogen levels using the ELISA method

All reagents were stored at room temperature, between 18° and 25°C before use. The samples' estrogen levels were determined via the enzyme-linked immunosorbent assay (ELISA) method. Using a 50mL micropipette, the sample was transferred into the microplate. After adding 100µL of estrogen enzyme conjugate to each microplate, it was shaken for 30 seconds. At 37°C, the solution was incubated for one hour. Once the incubation period was up, the solution was taken out of the microplate, and it was washed five times, shaking for three minutes each time, using 250–300µL of the washing solution. Once cleaned, it was turned over, and the paper was gently pressed with a tissue until it dried. Each microplate was filled with 100µL of TBM substrate solution as instructed by the sequence. Ten minutes after letting the tube sit at room temperature, it was wrapped in aluminum foil and covered with window film. To stop the reaction, 50µL of stop solution was gently added into each microplate, and the mixture was shaken for five seconds. Subsequently, the microplate was inserted into the ELISA reader (iMark™ Microplate Absorbance Reader, USA), observed, and the results were documented. Next, the data on estrogen levels were assessed using IBM SPSS for Windows version 25 (Laksmi and Trilaksana 2020).

Measurement of serum MDA levels

100µL of the goat blood serum was poured into the microtube. 550µL of pure water was added to the microtube. Then, 100µL of 10% TCA was inserted into the microtube and thoroughly mixed using a vortex. Subsequently, 250µL of 1N HCl was added to the microtube, followed by the introduction of 100µL of Na-thio 1%. The contents of the microtube were mixed using a vortex. The microtube was then centrifuged at 4°C and 500rpm for ten minutes. The supernatant was transferred to a new microtube. The microtube was delicately wrapped in parafilm. It was placed on a hotplate inside a glass beaker and heated to 100°C for 20 minutes before being allowed to cool to room temperature. According to Mansour and Zeitoun (2023), the absorbance value of MDA was determined at 530nm.

RESULTS AND DISCUSSION

Ovarian activity

Ultrasound examination was used to identify postpartum goat samples which have tested positive or negative for anestrus (Melia et al. 2021). According to Sayuti et al. (2019), hair must be shaved, and the orientation area must be selected in advance in order to acquire an accurate ultrasound image. Images can be taken in either lateral or dorsal recumbency. The operator's arm is positioned to the left of the ultrasound machine in the first stage, while the recumbent goat is positioned to the right. The ultrasound image is composed of three elements: gray (hypoechoic), white (hyperechoic), and black (anechoic). The ovaries are clearly delineated and look gray (hypoechoic) on an ultrasonography monitor, but the corpus luteum appears hyperechoic (Pertiwi et al. 2018). The results of an ultrasound examination used to identify anestrus and normal are shown in Fig. 2.

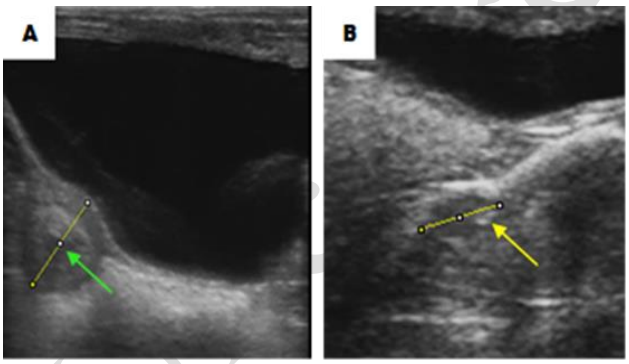

Fig. 2: An ultrasound image of the ovaries used to examine the corpus luteum and follicles. A corpus luteum is indicated by yellow arrows, whereas green arrows show the existence of a dominating follicle.

Fig. 2A displays a dominant follicle (green arrow) indicating that the animal is capable of resuming estrous after giving birth, while Fig. 2B illustrates the absence of follicles but the presence of a corpus luteum in the ovary. In addition to the ultrasound results, the samples' examination results for lust symptoms are also included. These data serve as the foundation for classifying the goats into two groups: those in good health, or negative controls, and those in post-partum anestrus.

Six anestrous goats in the K+ group were given PGF2α injections twice, with an 11-day interval between each injection, at a dose of 1.25mg/goat in the current study. A female goat can be induced to enter the first estrus earlier by injecting PGF2α preparations directly into the uterus, which will cause uterine involution. Another method is to manipulate the uterus to increase feed quantity and quality, which will support the healthy function of the reproductive organs (Tschopp et al. 2022). After the second injection of PGF2α, the individual will undergo an evaluation of estrus symptoms, which will include the following: 1. estrogen levels using ELISA; 2. behaviors, such as acceptance when mounted by a buck; and 3. vulva observation of changes and the discharge of transparent mucus from the vulva. Blood estrogen and an ultrasound to evaluate the health and appearance of the follicles in the ovaries on the third day following injection. The quality of the estrous is assessed by measuring the amount of the hormone estradiol 17β in the blood at the time of estrous after the second PGF2α injection. Fig. 3 shows an ultrasound image of the K+ group, which received a second injection of PGF2α on the third day following the first injection.

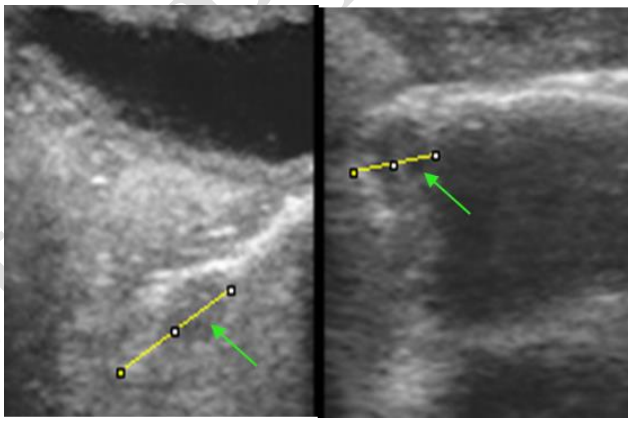

Fig. 3: Ultrasound image of the K+ group ovaries. Green arrows indicate the development of follicles, both small and dominant follicles.

PGF2α can alter the reproductive cycle by initiating follicle growth in the ovaries, which can lead to the start of a new estrous cycle. The onset of estrous symptoms precedes the follicles' ovulation, which occurs once they reach maturity (Manalu et al. 2017; Tschopp et al. 2022). PGF2α can cause the corpus luteum to shrink, which lowers progesterone secretion in the following phase of this effect.

According to Tanjung et al. (2015), low estrogen levels can affect the ovaries' capacity to generate estrogen and interfere with the appropriate function of FSH production, which can cause problems with follicle formation. This disease is influenced by gonadotropin hormone (GnRH) secretion, to which the anterior pituitary is insensitive. Low body score condition (BCS), physical condition, stress during lactation, and elevated prolactin levels are some of the factors that inhibit the release of gonadotropin hormones (Suwiti et al. 2017). Estrogen plays a key role in the ovaries' function of follicles (Xiao et al. 2022). Estrogen which is produced will be present in the antral follicle (Laksmi and Trilaksana 2020). Consequently, the blood vessels absorb the estrogen and transfer it to the targeted tissues. Reduced gonadotropin hormone levels,

especially FSH, may be the cause of postpartum ovarian hypofunction. Goats may find it difficult to display the common signs of estrus due to reduced ovarian activity brought on by a decrease in estrogen release in the body (Hermadi et al. 2017; Salman et al. 2021).

Serum estrogen levels using the ELISA method

Table 1 shows the average 17β estradiol levels as measured by ELISA. The 17β estradiol concentration significantly differed from K+ but not from KP ($P<0.05$), with the highest concentration values in the KP group and the lowest in the K+ group. Because the K-group goats were healthy without post-partum anestrus, the estrogen concentrations did not differ significantly in this study. Furthermore, the estrous symptoms have been determined before blood collection. Estrogen levels in the K+ group were considerably higher than those in the K- and KP groups three days following the second PGF2α injection. This suggests that PGF2α double injection therapy and microalgae feed additives dramatically increased estrogen levels. It is thought that this notable increase in estrogen would help to explain the emergence of main and secondary desire symptoms, improving the timeliness of AI.

Table 1: Average (mean±SD) level (ng/dL) of serum Estradiol 17β and malondialdehyde (MDA) in goats treated with hormonal therapy and microalgae

No.	Treatment	Estradiol 17β	MDA
1	K-	52.5 ± 6.302^{a}	276.50 ± 89.476^{b}
2	K+	110.4 ± 6.112^{b}	159.00 ± 52.085^{a}
3	KP	176.7 ± 13.019^{c}	106.67 ± 111.385^{a}

Superscripts in a column indicates significant differences ($P<0.05$) in each group; K-: Negative control, goats with normal postpartum estrus cycles; K+: Treatment group 1, anestrus goats treated with hormonal therapy only; KP: Treatment group 2, anestrus goats were treated with 0.5% body weight microalgae for 2 heat cycles.

The granulosa and theca cells from the de Graafian follicles in the ovaries produce estrogen, a steroid hormone (Hermadi et al. 2017; Salman et al. 2021). According to Wocławek-Potocka et al. (2013), the primary roles of the estrogen hormone are to maintain the female udder canal system, promote lust, and initiate secondary sexual characteristics. The estrogen profile in small East African (SEA) goats is 120-900pM/L during estrus and drops to 424pM/L in mid-gestation, according to research by Katangole and Gombe (2006). In a study by Akusu et al. (2006), the estrogen profile of West African Dwarfs (WAD) goats was measured at 152.62±31.6pg/mL during estrus, 131.7±4.3pg/mL on the 20th day, 309.9±27.62pg/mL24-6 hours before parturition, 191.60±58.90pg/mL during parturition, 150.30±24.30pg/mL after parturition, 109.60±34.60pg/mL 1-3 days after parturition, and on the fourth day after parturition, 92.90±48.40pg/ml. According to the previous study's results (Kumala et al. 2021), there was no significant change ($P>0.05$) in the estrogen hormone profile between the non-treated group (42.63±8.17pg/mL) and the CIDR treatment group (49.00±9.32pg/mL). In contrast to the luteal phase, the estrogen hormone profile will rise during the estrus phase (Kumala et al. 2021).

The variations in animal breeds utilized may account for the discrepancy in estrogen levels between this investigation and the one conducted by Katangole and Gombe (2006). In West African Dwarfs (WAD) goats going through estrus, Akusu et al. (2006) found a greater concentration of 152.62±31.6pg/mL. In addition to breeding, the duration of blood serum collection, the techniques utilized for evaluation, and the number of samples taken could all contribute to variations (Laksmi and Trilaksana 2020). According to another study (Socheh et al. 2019), thin-tailed goats' maximum estrogen levels throughout the estrus phase ranged from 13.03 to 15.07pg/mL.

Serum MDA levels

Oxidative stress can be caused by an animal's growing and developing reproductive system, which can lead to a compromised antioxidant defense system and increased lipid peroxidation (Sikiru et al. 2019). MDA is a biomarker for oxidative stress, which is caused by an accumulation of free radicals. The amounts of free radicals in an organ are directly correlated with its MDA level (Robbie 2020). According to Gutiérrez-Rebolledo et al. (2015), elevated MDA levels are indicative of elevated amounts of free radicals in the body, as MDA is a marker of cellular damage caused by these free radicals.

Group K- exhibited a statistically significant difference ($P<0.05$) from both P1 and P2 (Table 1), but Group P1's value demonstrated an insignificant difference from P2 ($P>0.05$). The usual MDA level value for PE goats with a normal delivery and first estrus 30–60 days postpartum without receiving PGF2a hormonal therapy or Spirulina (*Athrospira sp.*) therapy is the value of the negative control group. The MDA levels in the test results demonstrate that free radicals are still present in a healthy goat's body, which is consistent with the claim made by Zhang et al. (2022a) that the body's biochemical system (biological oxidation) can produce as many free radicals as possible as a result of metabolism. 2.5 percent of the total amount of oxygen needed, or 3.4 kg/24 hours. The MDA levels of Group P1 (PGF2a) and Group P2 (*Athrospira sp.*) were both lower. It has been demonstrated that consuming spirulina can prevent a number of diseases linked to inflammation and oxidative stress. Previous work has assessed b-glucuronidase in synovial fluid and subplantar edema, as well as different enzymes like aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase, to ascertain its anti-inflammatory activity in mouse models of chronic inflammation (Remirez et al. 2002).

Spirulina also prevents the expression of cytokines and enzymes like TNF and iNOS, as well as the activation of nuclear transcription factors that are activated by ROS (Lee et al. 2013). Cyanobacteria can prevent and delay the onset of inflammatory illnesses in this way. Because it possesses several modes of action and significant anti-inflammatory benefits, C-phycocyanin, the primary antioxidant ingredient of spirulina, has been suggested as a potent anti-inflammatory and anti-arthritis agent (Zhang et al. 2022b). According to the study findings, the control treatments had the highest MDA levels. This suggests that, compared to treated goats, PE animals lacking Spirulina algae or PGF2a hormone therapy had higher body levels of free radicals. This demonstrates that the antioxidant chemicals found in PGF2a, and Spirulina algae can suppress free radicals and lower MDA levels.

Nafiu et al. (2020) state that goats experience an average estrous cycle of 18–24 days. Estrogen levels fluctuate during the estrous and peak two days prior to ovulation (Sudrajat et al. 2021). According to Celozzi et al. (2022), this is also related to the different reproductive characteristics of goats: ovulation occurs 33 hours after the start of heat, on average between 30 and 36 hours, and the luteal phase lasts 17 days, while the follicular phase lasts 4 days. The length of a goat's heat cycle ranges from 17-24 days with an average of 20 days.

Based on the findings above, it can be concluded that providing P2 with an extra 0.5% microalgae meal can raise her 17β estradiol levels. Table 1 illustrates this point by comparing P2 to K– and P1 without receiving an additional microalgae feed. Microalgae include polyunsaturated fatty acids (PUFAs), which are involved in cellular and tissue metabolism, cell membrane fluidity and resilience, blood oxygen and electron transport, and heat adaptation (Chen et al. 2023). One of the main sources of carotenoids is microalgae, which include lutein, astaxanthin, zeaxanthin, fucoxanthin, and β-carotene among other carotenoid components. As stated by Kusnanda et al. (2021) and Sun et al. (2018), some of them are more potent antioxidants than vitamins A, C, and E. As an antioxidant source, astaxanthin is found in spirulina (*Athrospira sp.*) (Ismaiel et al. 2016), which is also utilized as a food ingredient, cosmetic, and dietary supplement. Spirulina (*Athrospira sp*.) has antioxidant activity that can boost minerals and nutrients and enhance hypothalamic function, resulting in optimal production of reproductive hormones (Gutiérrez-Rebolledo et al. 2015; Ismaiel et al. 2016). The development of ovarian follicles is influenced by optimal gonadotropin (FSH and LH) levels in the body, which might enhance the reproductive capacity of small ruminants after giving birth (Siregar et al. 2020).

Conclusion

The study's findings lead to the following conclusions: In goats undergoing post-partum anestrus, administering two injections of PGF2α in conjunction with microalgae supplementation was found to dramatically raise serum estradiol 17β levels. Additionally, the anestrus (K+) and (KP) goat groups' visual symptoms of estrus and the ultrasound results of ovarian activity demonstrated that the microalgae feed was able to enhance the condition of anestrus. The goats who were fed microalgae also had much lower MDA levels than the other groups, which further supported the use of microalgae feed supplements as a powerful antioxidant in cases of infertility in postpartum goats.

Authors' contribution

YO devised the project, the main conceptual ideas and proof outline. VFH drafted the manuscript and performed the computations. GCA and AF verified the analytical methos and contributed to the interpretation of the results. RY took part in the critical checking of this manuscript. All the authors discussed the results and contributed to the final manuscripts.

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