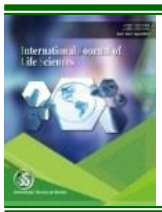

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Effect of Cod Liver Oil Supplementation in Commercial Feed on Reproductive Organs Weight and Ovarian Follicles Size of Local Rabbits

Ni Gusti Ayu Manik Ermayanti [a], Ida Bagus Made Suaskara [b], Iriani Setyawati [c], Ni Made Rai Suarni [d], I Gusti Ayu Manik Widhyastini [e]

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Correspondence Author [a]

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Abstract

This study aimed to determine the effect of cod liver oil supplementation in commercial feed on reproductive organs weight and ovarian follicles size of local rabbits. The experimental design used was Completely Randomized Design with four feed treatments, namely commercial feed without supplementation of cod liver oil (CLO) as control (P0), commercial feed supplemented by 3% CLO (P1), 4.5% CLO (P2), and 6% CLO (P3). Each treatment consisted of ten rabbits as replication and treatment were given to rabbits from aged 4 to 6 months. The parameters observed were the weight of the reproductive organs, namely the ovaries, fallopian tubes, uterus and the size of the ovarian follicles, namely primary, secondary, tertiary and preovulatory follicles of local rabbits. The results showed that various levels of cod liver oil supplementation in commercial feed differed significantly ($P<0.05$) to the weight of the reproductive organs and the size of the ovarian follicles of local rabbits. It can be concluded that cod liver oil supplementation at 4.5% level in commercial feed capable increase the reproductive organs weight and ovarian follicles size of local rabbits.

[a] Udayana University, Denpasar, Indonesia
[b] Udayana University, Denpasar, Indonesia
[c] Udayana University, Denpasar, Indonesia
[d] Udayana University, Denpasar, Indonesia
[e] Nusa Bangsa University, Bogor, Indonesia

Contents

1. Introduction

In the female rabbit, the ovaries, oviducts, and the uterus are paired organs, similar to the case in other placental mammal species. The ovaries of rabbits are small, flattened ovoid organs, lying in the right and left lateral pelvic cavity (Popesko *et al*., 1990; Capello, 2005). The surface of rabbit ovary is covered by a single layer of epithelium. A substantial basement membrane (tunica albuginea) separates the surface cells from the underlying ovarian tissue divided into the inner medulla and outer cortex, which consists of follicles and stroma (Zitny *et al*., 2004). Beneath the ovaries is the oviduct, made up of the duct, the ampulla and the isthmus (Lebas *et al*., 1997). The uterus is a complete paired organ (not partially paired as in most of the other placental mammal species). It is bicornuate with 2 cervixes, which open directly and separately into an elongated vagina. The mesometrium (broad uterine ligament) is usually filled with fat, especially in overweight or obese rabbits (Popesko *et al*., 1990; Capello, 2005). Female rabbits are classified as induced or reflex ovulatory because ovulation takes place after mating (Heape, 1905; Friedman, 1929; Spies *et al*., 1997; Harkness *et al*., 2010); hence, rabbits do not have a regular estrous cycle (Harkness *et al*., 2010). Ovulation occurs 10–13 hours after mating then there is no estrus cycle, but rather a period of receptivity occurring every 5–6 days (Popesko *et al*., 1990; Capello, 2005).

Nutrition is one of the environmental factors that influence hormonal profile and consequently, reproductive and productive performance of animals (Meshreky & Metry, 2000; Chiericato *et al*., 2001; Meshreky & Shaheed, 2003; Meshreky *et al*., 2007). Fish oil is a source of long-chain polyunsaturated fatty acids (PUFA). PUFA supplements (particularly n-3 PUFAs in fish oil) are promoted for general health reasons (Wathes *et al*., 2007). PUFAs must be provided by the diet, since it in vivo synthesis is not possible due to the absence of proper enzymes. PUFA a-linolenic acid (ALA) is necessary for numerous processes, including growth, reproduction, vision, and brain development (Gurr *et al*., 2002; Ermayanti *et al*., 2016; Nuriyasa *et al*., 2018)**.** The result of a study reporting the benefits of fish oil on male and female reproductive performance has been largely undertaken and obtained highly variable results. Cod liver oil supplementation up to 4.5% could increase testosterone levels and the quality of spermatozoa of local rabbit epididymis (Ermayanti *et al*., 2016), fish oil diet positively affect testes developments and spermatogenesis in the goat (Adibmoradi *et al*., 2012), fed a diet supplemented with LNA (n-3) can increase estradiol during the follicular phase in cows (Robinson *et al*., 2002), supplementation of n-3 PUFA rich fish oil significantly increased the number of pre-ovulatory follicles and ovulation rate in goat (Mahla *et al*., 2017), dietary PUFAs are known to mediate a broad range of actions in reproductive tissues including effect on membrane fluidity, intra-cellular cell-signalling cascades and susceptibility to oxidative injury (Wathes *et al*., 2007), change in the composition of dietary fatty acids not only modifies fatty acid composition in the blood plasma but also of the reproductive tissues including, follicular fluid, cumulus cells and the oocytes (Ferguson & Leese, 1999; Zeron *et al*., 2002; Bilby *et al*., 2006; Childs *et al*., 2008; Fouladi-Nashta *et al*., 2009; Wonnacott *et al*., 2010), which can directly influence the competence of oocytes for further development and/or fertility (Wonnacott *et al*., 2010; Petit *et al*., 2001). Fatty acids are also precursors for prostaglandins and progesterone synthesis and therefore play an important role in the regulation of normal reproductive function (Abayasekara & Wathes, 1999; Mattos *et al*., 2000). Polyunsaturated fatty acids (PUFAs) play a significant role in increasing the number (Lucy *et al*., 1991) and size of ovarian follicles (Zeron *et al*., 2002), level of LH (Lucy *et al*., 1991), and progesterone in follicular fluid (Ryan *et al*., 1992), regulation of ovulation, CL function (Abayasekara & Wathes, 1999; Mattos *et al*., 2000) and pregnancy rate (Bellows *et al*., 1999).

Nutrition is related to fertility and fertility is an important factor for successful fertilization. Many studies have reported the effect of fish oil on fertility in several animal models. Furthermore, whether cod liver oil supplementation is able to increase fertility in rabbits before mating, a study is conducted that aims to determine the effects of cod liver oil supplementation on the weight of reproductive organs weight and ovarian follicles size in local rabbits.

2. Materials and Methods

Animal

The animal used is 40 female local rabbits aged 4 months with an average body weight of 2000.60 to 2100.15 g. Rabbits were obtained from Riang Gede Village, Tabanan Regency, Bali. Animals were kept in enclosures of individual battery systems. They were kept under a controlled light-darkness cycle (12 h light; 12 h darkness). Air temperature in the cage was 27.05^{0}C and the air humidity in the enclosure was 75.4.

Administration of feed

The feed used is commercial feed pellets for rabbits. Commercial feed constituents are yellow corn, bran, soybean meal, molasses, and palm oil. Forty mature female local rabbits divided into four feeding treatments, i.e. commercial feed without supplementation by cod liver oil (P0) as control, commercial feed supplemented by 3% (P1), 4.5 % (P2), and 6% (P3) of cod liver oil. Each treatment consisted of ten rabbits as replication and the treatment is given for two months (from the age of rabbit 4 to 6 months).

Reproductive organs weight

At the end of the study, all the rabbits were killed and then dissected for reproductive organs collection, namely the ovaries, fallopian tubes, and uterus. Before being weighed, the ovaries were washed with 0.9% NaCl then dried with filter paper.

Histological ovary

The ovary is fixed in NBF solution and histological sections (5 µm) were serially mounted and stained with hematoxylin and eosin (Alturkistani *et al*., 2016). The processing of the ovary was made at Veterinary Research Center Denpasar Bali. Furthermore, the histology observations were conducted at the Faculty of Mathematics and Natural Sciences of Udayana University Denpasar Bali. All sections were observed. The ovarian follicles were measured with ocular micrometer calibrated on the binocular microscope. The initiation of primordial follicles growth starts a series of morphological changes leading to subsequent stages of follicular development – the primary and secondary follicles (preantral), tertiary and finally, the preovulatory follicles (antral) (Ross *et al*., 1995).

Data Analysis

The data obtained was to analyze statistically using one-way ANOVA and Duncan's Multiple Range Test (DMRT) was used to analyze statistical differences between groups.

3. Results and Discussions

The results of the statistical analysis of the effect of cod liver oil in commercial feed on reproductive organs weight and size ovarian follicles size of local rabbits can be seen in Table 1 and Figure 1.

Table 1
The effect of cod liver oil in commercial feed on reproductive organs weight and ovarian follicles size of local rabbits

	P0	P1	P2	P3
Body weight (g)	2515.00a	2515.02a	2811.13b	2811.12b
Reproductive organs weight (g)				
Ovaries	0.13a	0.14a	0.18b	0.17b
Fallopian tubes	0.25a	0.25a	0.30b	0.30b
Uterus	4.62a	4.63a	4.71b	4.70b
Ovarian follicles size (μm)				
Primary	7.30a	7.32a	12.73b	12.71b
Secondary	25.25a	25.26a	33.23b	33.22b
Tertiary	50.41a	50.42a	87.08b	192.12b
Preovulatory	100.00a	100.02a	192.10b	192.10b

The values followed by different letters in the same row show significantly different results (P<0.05), P0=0% (control), P1=3% CLO, P2=4.5% CLO, P3=6% CLO

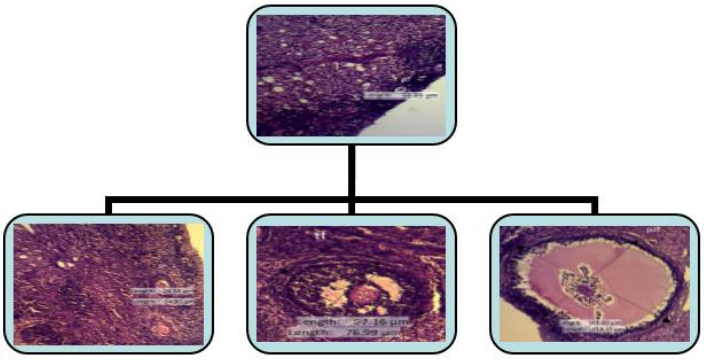

Figure 1. Histological ovary of local rabbits (4.5% CLO), primary follicles (pf), secondary follicles (sf), tertiary follicles (tf), preovulatory follicles (pof). Stained by H&E technique. x10.

The effect of cod liver oil supplementation in commercial feed on reproductive organs weight of local rabbits (Table 1). The weight of the reproductive organs is significantly different (P<0.05) to various levels of fish oil supplementation in commercial feed. The rabbit in the group P1 (3% CLO) were not significantly different (P>0.05) from the group without CLO (control). The rabbit in the P2 (4.5% CLO) and P3 (6% CLO) groups was significantly different (P<0.05) from the group without CLO (control). However, the rabbit in the group P2 (4.5% CLO) were not significantly different (P>0.05) from group P3 (6% CLO). This shows that the higher the level of supplementation of fish oil in commercial feed can no longer increase the weight of the reproductive organs. The highest of reproductive organs weight was found in the group P2 (4.5% CLO). This indicated that rabbits in the group P2 (4.5% CLO) had the most influence on the weight of the reproductive organs of local rabbits.

Increasing growth of local female rabbits will be followed by increasing body weight and weight of the reproductive organs, namely the ovary, fallopian tube, and uterus. The occurrence of an increase in the weight of ovary in this study, probably caused by increased estrogen secretion by ovarian follicles. In this study, the rabbits in the group P2 (4.5% CLO) produced the highest increase in body weight and reproductive organs weight. This show, rabbits in the group P2 (4.5% CLO) had reached puberty with the presence of estrus and mating behavior. Therefore rabbits in group P2 (4.5% CLO) are ready to mate first and are likely to have the best fertility compared to other groups.

The ovary is the main reproductive organ in female animals, because it functions as an exocrine organ that produces eggs/ovum and as endocrine organs that secrete female sex hormones, namely estrogen, and progesterone. An estrogen is a group of steroid compounds, named for their importance in the estrous cycle and functioning as the primary female sex hormone. It is also a main reproductive hormone affecting growth, development, maturation, and functioning of the reproductive tract as well as the sexual differentiation and the behavior (Balthazart *et al*., 2009). Progesterone plays an important role in the regulation of normal reproductive function (Abayasekara & Wathes, 1999; Mattos *et al*., 2000). In cows, fed n-3 can increase estradiol concentrations in the follicular phase (Robinson *et al*., 2002). Supplementation of 4% fish oil in feed can increase the weight of young rabbits (Kowalska & Bielanski, 2007). In general, female rabbits reach puberty (i.e. the onset of sexual receptivity and ovulation) at around 14 weeks of age (Hulot *et al*., 1982; Rommers *et al*., 2001), although the age at first mating depends upon the breed of rabbit (Harkness *et al*., 2010). Different feeding systems (*ad libitum* vs restrictive) will affect reproductive performance in rabbit (Rommers *et al*., 2001) and some research recommended that breeding begin when the female rabbit reaches about 75% of its adult weight (Gosalves *et al*., 1994).

The effect of cod liver oil supplementation in commercial feed on the size of ovarian follicles of local rabbits (Table 1). The size of the ovarian follicles is significantly different ($P<0.05$) to various levels of fish oil supplementation in commercial feed. The rabbit in the group P1 (3% CLO) were not significantly different ($P>0.05$) from the group without CLO (control). The rabbit in the P2 (4.5% CLO) and P3 (6% CLO) groups was significantly different ($P<0.05$) from the group without CLO (control). However, the rabbit in the group P2 (4.5% CLO) were not significantly different ($P>0.05$) from group P3 (6% CLO). This shows that the higher the level of supplementation of fish oil in commercial feed can no longer increase the size of the ovarian follicles. The highest of ovarian follicles size was found in the group P2 (4.5% CLO). This indicated that rabbits in the group P2 (4.5% CLO) had the most influence on the size of the ovarian follicles of local rabbits. The effect of cod liver oil supplementation in commercial feed on the histological ovary of local rabbits (Figure 1). Figure 1 taken is the most representative image for group P2 (4.5% CLO). The size of ovarian follicles from primary follicles to preovulatory follicles in group P2 (4.5 CLO) is larger than P0 (control), P1 (3% CLO) and P3 (6% CLO) groups.

In this study, rabbits in the group P2 produced the highest ovarian weight, so it was the possibility of rabbits in the group P2 had the highest follicular size as well compared to other groups. The size of the follicles in the group P2 is primary (12.73 µm), secondary (33.23 µm), tertiary (87.08 µm), and preovulatory (192.10 µm) follicles. This show, in the group P2 occurs the development of follicles in the ovary. Folliculogenesis reaches its peak in the form of mature and ovulating preovulatory follicles. This only takes place after the animal reaches puberty. Without mature follicles, signs of estrus will not be seen. This is related to harmonious interactions between the ovaries, pituitary, and hypothalamus.

In female animals, fertility is related to follicular development (folliculogenesis) (Leung & Adashi, 2004) and it is affected by endocrinology (Paris *et al*., 2009) and nutrition (Chavatte-Palmer *et al*., 2014). Nutritional factors influence hypothalamic-pituitary function and therefore gonadotrophin profiles, directly through effects of nutrients or metabolic hormones such as insulin acting on target organs or through changes insensitivity of these organs to estradiol, progesterone and other hormonal feedback mechanism (Rhind, 1992; Muzvondiwa *et al*., 2011). Lower planes of nutrition delayed the onset of puberty by inhibiting maturation of the endocrine system (Patterson *et al*., 1992). Follicular development is thought to be supported by the growth of 5 to 10 follicles on each ovary at any one time. Once follicles reach an ovulatory size, they secrete estrogens in increasing amounts and rabbits show sexual receptivity for a period of time. When those follicles degenerate, secretions of estrogen decline and females rabbits become non receptive (Harcout-Brown, 2002). Likewise, results of others have shown that receptive rabbits had more large follicles and a higher concentration of estradiol in the follicular fluid than those of no receptive rabbits (Lefèvre & Caillol, 1979).

Ovarian function in rabbits is not well understood. Unlike South American camelids (i.e., induced ovulating species in which follicular wave activity has been well documented), the pattern of follicle development in rabbits has not been established. Unlike most other mammals, the formation, activation, and development of ovarian follicles occurred entirely postnatally in rabbits, with primordial follicle assembly presumably completed between 2 and 4 weeks of age (Hutt *et al*., 2006). With the onset of puberty, follicles reached ovulatory status and were related to sexual behavior. For instance, some authors have indicated that follicles with a diameter >1.8 mm were present only in receptive females (Lefèvre & Caillol, 1979). However, there is no clear understanding of the relationship between follicle diameter and ovulatory capability in rabbits.

Preovulatory follicles were also referred to as those >800 to 900 um in diameter (Kranzfelder *et al*., 1984). Others have reported that preovulatory follicles are those that are >1.5 mm or > 2 mm in diameter (Parkes, 1931; Hunzicker-Dunn *et al*., 1979; Marongiu & Gulinati, 2008). Continuous growth and regression of follicles appear to occur in receptive rabbits and investigators in an early study suggested that follicles able to ovulate remain for about 7 to 10 days and then regress (Hill & White, 1933). Follicular development is thought to be supported by the growth of 5 to 10 follicles on each ovary at any one time. Once follicles reach an ovulatory size, they secrete estrogens in increasing amounts and rabbits show sexual receptivity for a period of time. When those follicles degenerate, secretions of estrogen decline and females rabbits become non receptive (Harcout-Brown, 2002). Likewise, the results of others have shown that receptive rabbits had more large follicles and a higher concentration of estradiol in the follicular fluid than those of nonreceptive rabbits (Lefèvre & Caillol, 1979). Additionally, the ability to ovulate in response to mating was suggested to differ between receptive (accepted mating) and nonreceptive rabbits (refused to mate and were subjected to assisted mating); and 12 h post-mating, ovulations were detected in 4/6 vs 0/4 rabbits, respectively (Boumahdi-Merad *et al*., 2011). Findings from others (Hulot *et al*., 1988) have suggested that failure to ovulate in rabbits may be caused by a lack of discharge of LH rather than to a lack of mature follicles in the ovaries.

Alpha-linolenic acid (ALA; C18:3) is the dietary precursor for the long-chain omega-3 PUFAs (Brenna *et al*., 2009). It plays an important role in folliculogenesis, developmental competence of oocyte (Moallem *et al*., 2013), fertilization rate and embryo quality (Thangavelu *et al*., 2007). As ALA is produced by the ovarian follicles and the amount of ALA increases as the ovarian follicles enlarge (Veshkini *et al*., 2015); a role of specific unsaturated fatty acids like ALA may be speculated in the oocyte maturation and/or follicular growth. In fact, ALA has been shown to improve the fertility rate in both cattle and sheep by improving folliculogenesis and fertilization rate *in vivo* (Moallem *et al*., 2013). When supplemented in the *in vitro* maturation medium it was reported to regulate the molecular mechanism leading to increased number of MII stage oocytes and improved their subsequent development into early embryos in both cattle (Fouladi-Nashta *et al*., 2009; Marei *et al*., 2009) and sheep (Ghaffarilaleh *et al*., 2014). The supplementation of fish oil (rich with omega 3 fatty acids) improved oocyte quality leading to enhanced blastocyst development and further supported by increased luteal function from the ovary which increased embryo survival and subsequent reproductive performance of sows (Smits, 2010).

Orally-treated rabbit does with sunflower oil (rich in omega 6) or linseed oil (rich in omega 3) had the similar impact of PGF2α (0.5 mg dinoprost, a synthetic analog of PGF2α) on increasing blood PG profile and decreasing blood P4 and this effect provided the opportunity to increased secretion of gonadotropin hormones from the pituitary and secretion estrogen from the ovary through inhibiting the effect of P4 on gonadotropin release (Elkomy & El-Speiy, 2015). E2 hormone is necessary for influencing the follicular growth and production of the mature ovum. Estradiol stimulates uterine secretion of PGF2α (Thatcher *et al*., 1994). Furthermore, E2 can increase the sensitivity of the CL to PGF2α, thus causing more complete regression of the CL (Howard *et al*., 1990). That dietary PUFA supplementation can modify the plasma fatty acid profile and prostanoid synthesis. The significant of these modifications vary according to the type of polyunsaturated fat and the ratio of n-6:n-3 fatty acid. On the other hand, PGF2α reduced the production of P4 because PUFA converted to PGF that caused an increase PGF concentration in the plasma with a reduction of the P4 synthesis (Amira *et al*., 2011). The follicular phase begins after luteolysis and ends at ovulation. Gonadotropins, FSH, and LH, released from the anterior pituitary, stimulate antral follicles to produce E2 (Smits, 2010).

4. Conclusion

It can be concluded that cod liver oil supplementation at 4.5% level in commercial feed capable increase the reproductive organs weight and ovarian follicles size of local rabbits.

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Biography of Authors

Ni Gusti Ayu Manik Ermayanti is a lecturer in the Biology Department, Faculty of Mathematics and Natural Sciences at Udayana University, Denpasar, Bali, Indonesia (1994-present) and lectures taught by her are Animal Physiology, Animal Reproduction, and Endocrinology. She earned a doctorate at Faculty of Husbandry at Udayana University, Denpasar, Bali-Indonesia in 2016.
Email: manikermayanti@gmail.com

Ida Bagus Made Suaskara is a lecturer in the Biology Department, Faculty of Mathematics and Natural Sciences at Udayana University, Denpasar, Bali, Indonesia (1997-present) and lectures taught by him are Animal Physiology, Animal Reproduction, and Endocrinology. He earned a master's degree at Faculty of Biology, Bogor Agricultural University, Bogor, Indonesia in 2004.
Email: made_suaskara@unud.ac.id

Iriani Setyawati is a lecturer in the Biology Department, Faculty of Mathematics and Natural Sciences at Udayana University, Denpasar, Bali, Indonesia (2000-present) and lectures taught by her are Teratology, Animal Histopathology, and Animal Comparison Anatomy. She earned a doctorate at Faculty of Husbandry at Udayana University, Denpasar, Bali-Indonesia in 2015.
Email: iriani_setyawati@unud.ac.id

Ni Made Rai Suarni is a lecturer in the Biology Department, Faculty of Mathematics and Natural Sciences at Udayana University, Denpasar, Bali, Indonesia (1994-present) and lectures taught by her are Structure and Development of Animals, Animal Reproduction and Endocrinology. She earned a doctorate at Faculty of Husbandry at Udayana University, Denpasar, Bali-Indonesia in 2016.
Email: gunggalih@ymail.com

I Gusti Ayu Manik Widhyastini is a lecturer in the Biology Department, Faculty of Mathematics and Natural Sciences at Nusa Bangsa University, Bogor, Indonesia and lectures taught by her are Animal Physiology and Cell Biology. She earned a master's degree at the Basic Medical Science Department, Faculty of Medical, Airlangga University, Surabaya, Indonesia in 2001.
Email: widhyastini2304@gmail.com