Full Length Research Paper

Histological changes in the endometrial of pregnant Sprague-Dawley rats under supplementation levels of *n-6:n-3* fatty acid ratio

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This study describes a changed uterine morphometry and its application to the endometrial structure of a pregnant rat. The number and the size of uterine gland and blood vessels changed during the pregnancy period of the rat. This effect on day 15 was significantly changed in the different groups. When the endometrial morphology was related to the circulating progesterone concentrations on day 15, it was observed that relationships were found on day 15 and a high progesterone concentration in the Diet 1:1 group was associated with an increased number of the uterine gland and blood vessel. Furthermore, animals in the Diet 1:30 group were slaughtered on day 15 and a low progesterone concentration on that same day was associated with the decreased uterine gland size, though a simultaneous decrease was found in the number of endometrial gland. In contrast, the number of implanted embryos was significantly lower in the Diet 3 group at 15 days of gestation The results suggest that the endometrial glands do not only grow and regress during the supplementation of high ratio *n-6:n-3* fatty acids, but the number and size of the endometrial glands in the endometrial area are controlled by progesterone, which leads to changes in the structure and maintenance of the uterine during the pregnancy period.

Key words: *n-6:n-3* Fatty acid ratio, progesterone, blood vessel, uterine gland, pregnant rat.

INTRODUCTION

The uterus is the major female reproductive organ of most mammals, including humans. One end, which is the cervix, opens into the vagina, while the other is connected on both sides to the fallopian tubes. Maintenance of pregnancy throughout gestation is hormonally regulated by the coordinated actions of estrogen and progesterone. Progesterone maintains the uterus in a quiescent state and appears to be the principal hormone required for maintenance of a conceptus supportive environment in all species (Bazer and First, 1983; Bazer et al., 1989; Keyes and Wiltbank, 1988; Thatcher et al., 1986). In the rat, progesterone secretion is primarily ovarian, but is supplemented by the placenta in late gestation (Thorburn and Challis, 1979). The objective of this study is to determine the effect of different dietary ratio *n-6:n-3* fatty acid supplementation on the changes in the morphological structure of the pregnant rats' uterine. Morphometry has been successfully used to monitor pathological changes in the bovine endometrial (Gonzalez et al., 1985) and to study the effects of drugs and hormones on human endometrial (Johannisson et al., 1991).

Furthermore, in human endometrial, glandular diameter during the proliferative and secretory phases has been correlated with estrogen and progesterone levels,

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Abbreviations: SBO, Soybean oil; **CLO,** code liver oil; **P,** progesterone; **UG,** uterine gland; **BV,** blood vessel; **CL,** corpus luteum.

whereas morphometry has also been applied to the analysis of gland morphogenesis in animals depleted of uterine glands (Gray et al., 2001; Carpenter et al., 2003). The aims of the present study are to analyze the endometrial structure, to assess the perimeter of the gland as a novel parameter for morphometric evaluation of changes in the endometrial gland structure, to clarify the morphological changes in the endometrial glands during the supplementation of different ratio *n-6:n-3* to pregnant rats and to investigate their association with the circulating progesterone levels.

MATERIALS AND METHODS

Experimental animals

Twenty eight females of Sprague Dawley rats (240 ± 20 g) that are two months old comprised the experimental animals. After 2 weeks of adaptation, the rats were randomly divided into four treatment groups consisting of seven rats in each group. The study was reviewed and approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM/FPV/PS/3.2.1.551/AUP-R23). The treatment groups included rats fed with normal rat chow diet [control (C) group], rats fed with chow diet supplemented with 5% w/w soybean oil (SBO) and 5% w/w cod liver oil (CLO) (Diet 1:1 group), rats fed with chow diet supplemented with 8.4% w/w SBO and 1.6% w/w COL (Diet 6:1 group), and rats fed with chow diet supplemented with 9.6% w/w SBO and 0.4% w/w COL (Diet 30:1 group). This study was reviewed and approved by the Animal Care and Use Committee in the Faculty of Veterinary Medicine (ACUC). After two months feeding, daily vaginal smears were taken to determine the estrous cycle of each rat. Pregnancy was induced overnight by caging a proestrus female with a male of proven fertility. The next day, the presence of a vaginal plug or spermatozoa in the vaginal smear was termed as day 0 of pregnancy. Pregnant females were separated from male rats on the day of pregnancy. On day 14 prior to sacrifice, food was withdrawn at 9:00 A.M. and the animals were sacrificed at 9:00 A.M the following morning on day 15 of pregnancy. After 10 weeks (8 weeks + 15 days) of feeding, rats were anesthetized with an intraperitoneal injection of 60 mg/kg body weight ketamine + 8 mg/kg body weight xylazine. The blood were collected into ethylenediaminetetraacetic acid (EDTA) coated tube and kept at 4°C. Subsequently, the tube was centrifuge at 3000 g for 10 min in order to collect plasma from it, and was later stored at -80°C until progesterone (P) was confirmed by radioimmunoassay (RIA).

Preparation of the histological sections

Pregnant rats were killed 15 days after mating and the tissue samples from the uterine horn were fixed in 10% (v/v) formalin in a phosphate buffered saline (PBS) for 24 h. The tissue was processed for paraffin embedding and sectioned at 4 µm thickness using Leica 2045, stained with hematoxylin and eosin, and was examined using light microscopy (Olympous BX51, Japan) equipped wit an image analyzer (Analysis LS Research). Each uterine cross section, analyzed with the endometrial gland and blood vessels, were counted in perimeter (µm), diameter (µm) and area (µm 2).

Biochemical analysis

Plasma progesterone was assayed by radio-immunoassay (RIA,

Perkin-Elmer® 1470 Wizard Automatic Gamma Counter, and Waltham, MA, USA) using commercial radioimmunoassay kit (Coat-A-Count, Siemens Medical Solution, Los Angeles, CA).

Statistical analysis

All data were presented as means ± S.E.M. Consequently, the endometrial characteristics on day 15 were compared by the one way analysis variance (ANOVA). Relationships between progesterone concentrations and endometrial characteristics were analyzed by linear regression analysis. However, all statistical tests were carried out using the General Linear Model of SAS (Statistical Analysis Systems Institute Inc., 1992).

RESULTS

There were significant differences in the uterine horn morphology among the treatment groups. The mean uterine gland (UG) and blood vessels (BV) area for Diet 1:1 and Diet 6:1 group was significantly higher than the Diet 30:1 group as shown in Figures 1 and 2, although the mean values are shown in Table 1. The perimeter of glands increased in Diet 1:1 group in response to α-linolinic acid (n-3). However, there was no apparent increase in the gland perimeter in Diet 30:1 group after feeding, in that high ratio *n-6:n-3* was used in this study. The mean of the UG and BV diameter for high ratio n6:n3 in Diet 30:1 group were significantly lower than that in Diet 1:1 and Diet 6:1 group. The mean of the BV perimeter was significantly higher on low ratio *n-6:n-3* in Diet 1:1 group compared to high ratio *n-6:n-3* in Diet 30:1 group; as such, there were no significant differences for the UG and BV area and diameter on Diet 1:1 group compared to the control group.

Table 3 shows the effect of feeding on different *n-6:n-3* fatty acid ratio supplementation on the plasma levels of progesterone hormone in pregnant rats. Throughout the 15 days of gestation, progesterone was significantly lower in rats fed high ratio of *n-6:n-3* fatty acid in Diet 30:1 supplemented with LA (*n-6*), in comparison with the control (P < 0.05) group, but there was no difference in progesterone between the control rat and the rat fed a low ratio of *n-6:n-3* fatty acid in Diet 1:1 supplemented with LNA (*n-3*).

Fetal number and implantations

Table 4 shows the fetal number at the fifteenth day of gestation. The results showed no significant difference between Diet C and Diet 1:1 groups, but showed a significant difference (P<0.05) between Diet C and Diet 3 groups. Similarly, the fetal implantations at day 15 of gestation were observed to be non-significantly different (P>0.05) in Diet 1 and Diet C groups. However, the number of implanted embryos was significantly lower in the Diet 3 group at day 15 of gestation.
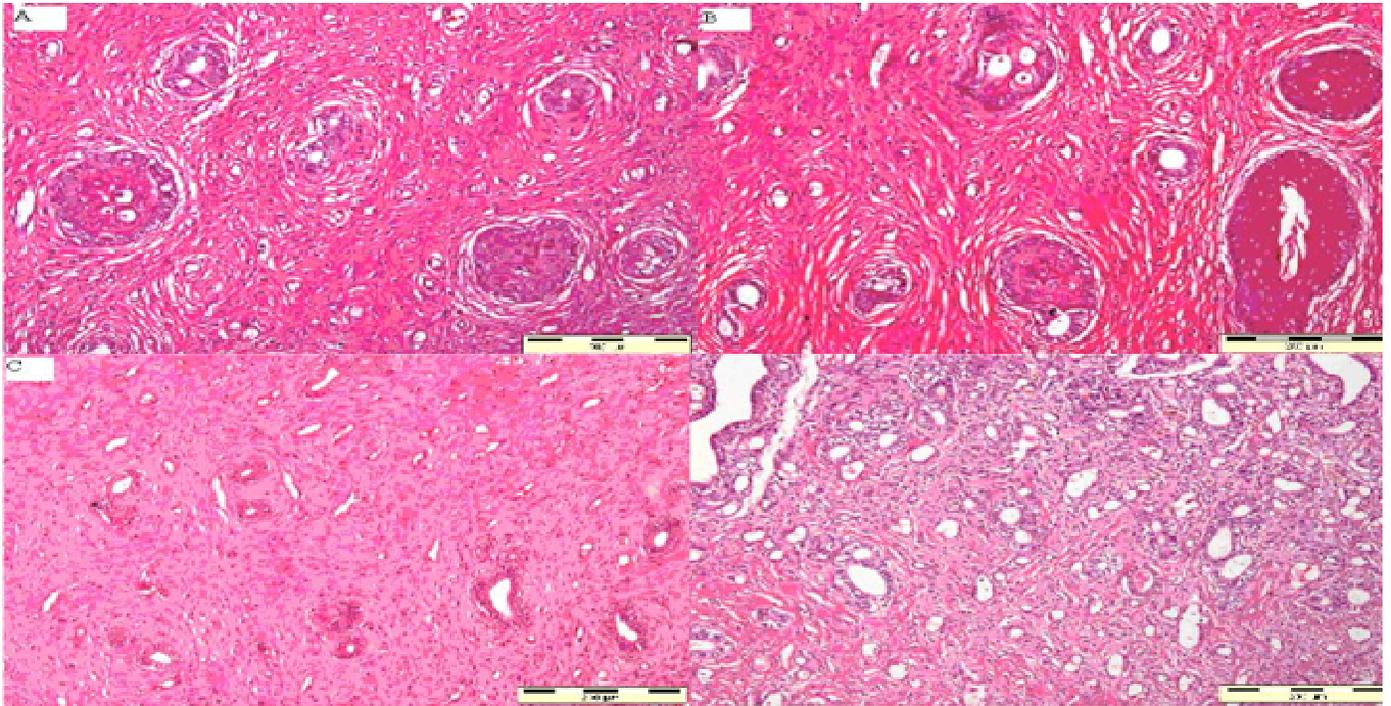
Figure 1. Panel A: The rats from Diet 1:1 group showed significantly higher uterine gland area. Panel B: The rats from Diet 6:1 group showed significantly higher uterine gland area. Panel C: The rats from Diet 30:1 group showed significantly lower uterine gland area. Panel D: The rats from Diet C group showed significantly higher uterine gland area.
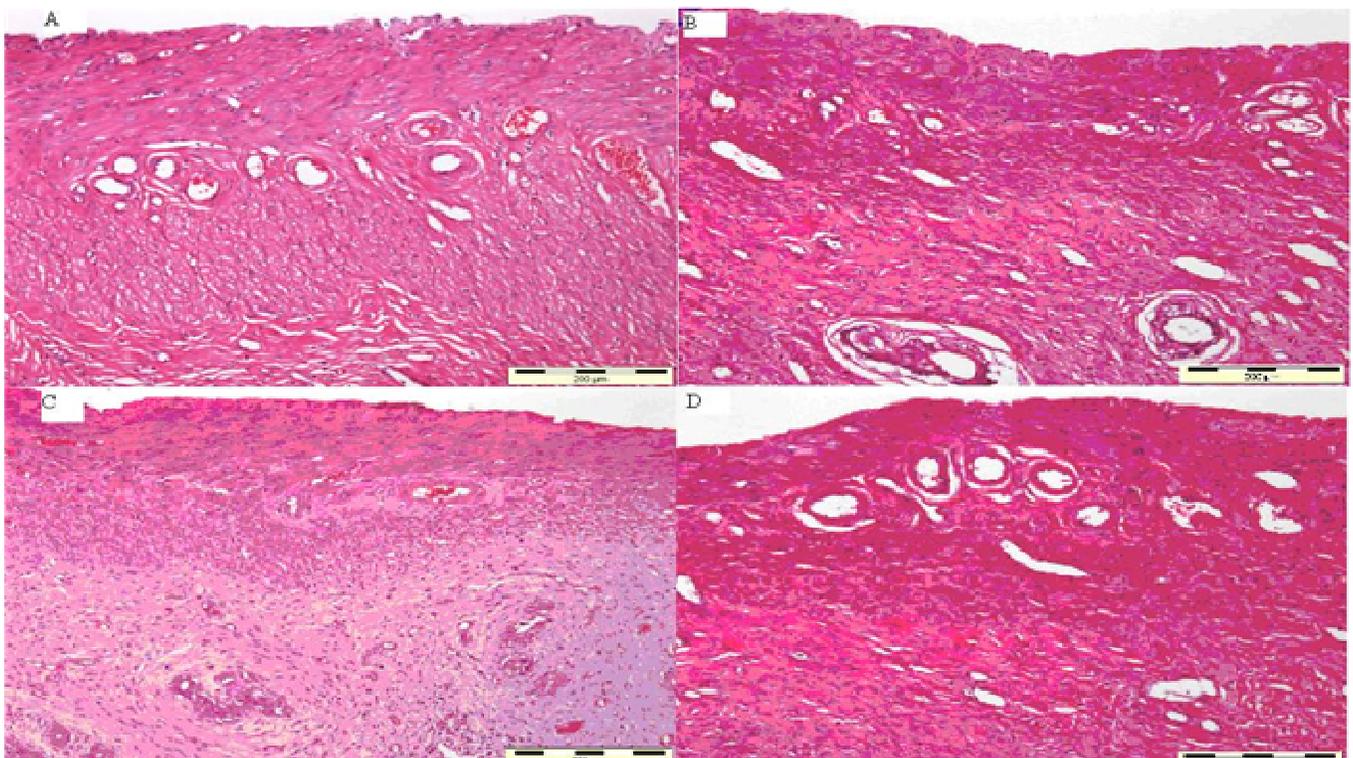
Figure 2. Panel A: The rats from Diet 1:1 group showed significantly higher blood vessels area. Panel B: The rats from Diet 6:1 group showed significantly higher blood vessels area. Panel C: The rats from Diet 30:1 group showed significantly lower blood vessels area. Panel D: The rats from Diet C group showed significantly higher blood vessels area.

Table 1. Morphometric characteristics of endometrial glands in rat endometrial during pregnancy.

Parameter	Diet 1:1	Diet 6:1	Diet 30:1	Diet C
Mean gland area (μm^2)	1454.78±170.15[a]	1467.29±307.48[a]	367.58±20.09[b]	1337.18±154.50[a]
Mean gland perimeter (μm)	412.36±13.26[a]	329.82±9.76[b]	190.21±4.10[c]	431.81±13.91[a]
Mean gland diameter (μm)	96.29±4.57[a]	89.10±7.22[a]	46.20±1.06[b]	84.30±3.82[a]

Table 2. Morphometric characteristics of blood vessels in rat endometrial during pregnancy.

Parameter	Diet 1:1	Diet 6:1	Diet 30:1	Diet C
Mean blood vessels area (μm^2)	1059.88±321.45[a]	878.28±130.31[ab]	366.49±25.96[b]	836.63±92.18[ab]
Mean blood vessels perimeter (μm)	259.72±19.73[a]	225.74±3.07[b]	162.98±2.76[c]	278.91±6.06[a]
Mean blood vessels diameter (μm)	72.84±8.49[a]	65.70±4.35[a]	48.53±1.80[b]	73.49±3.31[a]

Table 3. Plasma progesterone concentration (pg/ml) after 15 days pregnancy of rats (mean ± SE, n = 7).

Parameter	Diet 1:1	Diet 6:1	Diet 30:1	Diet C
Progesterone pg/ml	3.57±0.38[a]	2.46±0.12[b]	1.36±0.13[c]	3.38±0.27[a]

Values with different superscripts within the column differ significantly at $P < 0.05$.

Table 4. Effect of different ratio of n-6: n-3 fatty acid supplementation on fetal number and fetal implantations at 15 days of gestation in rat (mean ± SE, n = 7).

Parameter	Diet 1:1	Diet 6:1	Diet 30:1	Diet C
Fetal number	10.57±0.30[a]	5.14±0.26[b]	1.14±0.46[c]	11.00±0.44[a]
Fetal implantations	10.57±0.30[a]	10.29±0.18[a]	3.86±0.55[b]	11.00±0.44[a]

Values with different superscripts within the column differ significantly at $P < 0.05$.

DISCUSSION

In the present study, morphometric assessment of the endometrial was performed to evaluate the histological alterations in the endometrial under the effect of different ratio n-6:n-3 fatty acid at the light microscopic level. As the main aim of the study is to observe the endometrial changes in pregnant rats at day 15, the measurement of UG area for Diet 1:1 group was significantly higher in low ratio n-6:n-3 compared with high ratio n-6:n-3 in Diet 30:1 group. Assessing the endometrial morphological parameters, including the glandular area, diameter and volume have been used to evaluate changes in the structure and function of the endometrial glands more accurately (Wahab et al., 1999). In addition, the diameter of UG and BV for low ratio n-6:n-3 in Diet 1:1 group was significantly higher when compared with high ratio n-6:n-3 in Diet 30:1 group. These findings demonstrate the effects of ovarian steroids on the endometrial, thereby provoking proliferation and consequently increasing the endometrial epithelium thickness (Aysin et al., 2004). However, as the concentration of progesterone increases, this proliferative effect seems to be higher. In the present data, the perimeter of UG and BV for high ratio n-6:n-3 in Diet 30:1 group was significantly lower when compared to low ratio n-6:n-3 in both the Diet 1:1 and control (C) groups. Under the influence of the ovarian steroids, the human uterus undergoes substantial changes in preparation for implantation. These changes have been described in the glandular epithelium (Nikas et al., 1995) and in the underlying vasculature (Roberts et al., 1992). The interactions between diet and the circulating steroid hormone concentrations were complex, in that high ratio n-6:n-3 in the Diet 30:1 group decreased the plasma prostrogen concentrations relative to other dietary treatments, while a supplementation of high ratio n-6:n-3 in the Diet 3 group increased the concentration of the circulating progesterone (Milvae et al., 1977). Secretion of progesterone is the main function of the corpus luteum (CL). Progesterone not only prepares the uterus for implantation of the embryo, but also helps in maintaining pregnancy by providing nourishment to the conceptus. Many of the losses between 25 and 55% of the mammalian embryos' death in early gestation are due to inadequate function of the luteal cells (Niswender and Nett, 1994).

However, few investigations have been carried out on the relationship between steroid hormone levels and

endometrial changes in the natural cycle, especially in ruminants. Though morphological changes in endometrial glands and stroma such as oedema and epithelial hyperplasia are considered to be controlled by steroid hormones, the relationship between cyclic endometrial changes and progesterone levels remains unclear. The present study clarifies several of such relationships between plasma progesterone levels and endometrial changes during the supplementation of different ratio n-6:n-3 on pregnant rats. On day 15, the total endometrial area as well as the endometrial gland perimeter and area were all positively correlated to the plasma progesterone concentration measured in the same animals on day 15 in Diet 30:1. This indicates that a lower concentration of progesterone on day 15 is associated with a lower total endometrial area on day 15, and it suggests that the endometrial thickness and gland size during the gestation period may be regulated by the rate at which the progesterone levels are lowered during formation of the corpus luteum. In contrast, the UG and BV diameter and area was positively associated with the progesterone level on day 15. This response probably reflects the increase in the endometrial area in response to a high level of progesterone on day 15, while the same number of gland occupies an increased area in Diet 1:1 and Diet C group. Interestingly, on day 15, the gland and blood vessels that are exposed to a low progesterone level on day 15 in the endometrial, appeared to be smaller and less condensed in the deep glands and BV than the other treatment groups. Together, these results suggest that the endometrial gland morphology is associated with circulating progesterone concentrations during pregnancy.

The cyclic changes in the total endometrial area observed here show that cyclic changes in the endo-metrial gland and blood vessels diameter and parameter may be driven by changes in the total endometrial area rather than the proliferation and regression of the glandular epithelial cells. The morphometric method described can be used to detect endometrial changes during the maid pregnancy that appear to be associated with progesterone concentration. In the case of pregnant rats, diets containing high n-6 in Diet 30:1 resulted in an abnormal development of fetuses, as evidenced by the smaller fetuses. These abnormal fetal development and fetal desorption in Diet 30:1 group might be the result of high AA (n-6) in plasma as a disturbance of the endometrial lining system which interferes with fetal development (Quackenbush et al., 1942; Leat and Northrop, 1979). These findings support the ideas that a high n-6 PUFA supplemented diet may influence fetal and placen-tal development.

Conclusion

In conclusion, the effect of different ratio n-6:n-3 signifi-cantly affects the morphology of the endometrial cells in pregnant rats. These morphological effects become more pronounced as we increase the ratio of n-6:n-3 fatty acid. Low-ratio n-6:n-3 should be preferably compared with high-ratio n-6:n-3 stimulation to minimize the unfavorable effects of progesterone on the endometrial. Furthermore, our data indicate that fatty acid, through a disturbance in the ovarian steroid, acts on some of the morphological functional features of the rat endometrial, and mainly on the success rate of the embryo implantation.

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