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A High Resolution SEM Study of the Effects Supplementation Different Ratio Of *n-6:n-3* on the Endometrial Pregnant Rat

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Abstract

This study, therefore, aims to investigate the effects of dietary fatty acids on endometrial pinopode expression. Twenty eight rats were randomly divided into four groups (seven rat per treatment).Treatment diets were rat chow only control diet (con), rat chow containing 0.9 ml soybean oil (SO) + 0.9 ml cod liver oil (CLO) the ratio 1:1, rat chow added with 1.5 ml SO + 0 .3 ml CLO the ratio 6:1 and rat chow added with 1.74 ml SO + 0.06 ml CLO the ratio 30:1 ,feeds of 15g/ day were given and water was available *ad libitum* .. The endometrial samples were evaluated under SEM. developing pinopodes were seen during increasing progesterone concentrations especially in Diet 1:1 group, and regressing pinopodes were found with decreasing progesterone in Diet 30:1 groups. In both Diet 1:1 treated and control animals, on days 15 of pregnancy, scanning electron micros copy revealed the microvilli of the uterine epithelial cells in the treated animals were more dense than those in the Diet 30:1 treated animals.

Keywords: Endometrium, pinopodes, progesterone , scanning electron microscopy.

1. Introduction

Uterus is the major female reproductive organ of most mammals, including human. On one end, the cervix opens into the vagina, while on the other is connected to both sides of the fallopian tubes. The main function of uterus is to accept a fertilized ovum which then will be implanted into the endometrium, and derive nourishment from blood vessels which are developed exclusively for this purpose (Fawcett and Jensh, 2002). The rat has a duplex uterus: the uterine horns lie in the dorsal abdominal cavity, the uterine body and vagina lie between the dorsally located rectum and ventrally located urinary bladder. The wall is composed of mucosa (endometrium), two smooth muscle layers (myometrium) and adventitia. The single row of columnar epithelium lining the mucosa forms uterine glands projecting into the endometrial propria (stroma) (Krinke, 2000).

The mammalian uterus is a very dynamic organ which is capable of considerable physical and morphological changes in response to the changing in reproductive cycle. In some species, for example, in the rat and primates including humans, the interaction of the blastocyst with the uterine epithelium initiates decidualization of the endometrium which is characterized by increased vascular permeability and a highly ordered wave of stromal cell differentiation . The myometrium is maintained in a quiescent state until the onset of labor (Jain *et al*., 2000).

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 *et al.,* 1989; Keyes and Wiltbank, 1988). However, there is

a considerable interspecies variation in the source of this hormone throughout gestation. In the rat, progesterone secretion is primarily ovarian, but is supplemented by the placenta in late gestation (Thorburn and Challis, 1979). The aim of the current study was to assess the expression of endometrial pinopodes by scanning electron microscopy may reflect in hormone-controlled.

2. Materials and Methods

2.1 Animals

The study was approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM/FPV/PS/3.2.1.551/AUP-R23).. Twenty eight female, two month old Sprague Dawley (240±20 g) rats were used in this experiment. Animals were maintained in temperature controlled quarters at 23 ℃, with a 12-h light–dark cycle and received rat pellet and water ad libitum. Daily vaginal smears of each animal were used to confirm normal cycling and for the identification of the pro-oestrous stage of the oestrous cycle, for purposes of mating.. Pregnancy was achieved by overnight mating of pro-oestrous females with males of proven fertility. The presence of a vaginal plug, as well as sperm in the vaginal smear taken the following morning, was used to confirm successful mating and this was designated as day 0 of pregnancy.

2.2 Treatment Regime

In order to examine changes in uterine progesterone effects in response to the supplementation unsaturated fatty acids to the diet , The four treatment groups included the rats fed with standard chow diet supplemented with 50% w/w soybean oil and 50% w/w cod liver oil (1:1) (diet 1), rats fed with standard chow diet supplemented with 84% w/w soybean oil and 16% w/w cod liver oil (6:1) (diet 2), rats fed with standard chow diet supplemented with 96% w/w soybean oil and 4% w/w cod liver oil (30:1) (diet 3), and rats fed with only standard rat chow diet and acted as the control (diet 4) . Tissue was taken on day 15 of pregnancy.

2.3 Tissue Preparation for Scanning Electron Microscopy (SEM)

The microvilli density, small secretion and pinopodes cell morphology in the endometrium were examined using scanning electron microscopy (SEM). Samples of uterine tissue were fixed in 4% glutaraldehyde buffered solution (PH 7.3). The samples were then washed 3 times in 0.1 M sodium cacodylate buffer for 10 minutes each and then post fixed for 2 hours with 1% osmium tetroxide (OsO4). The fixed samples were rinsed and dehydrated in a graded acetone series. The samples were then dried by critical point dryer (CPD 030 Bal-TEC, Swaziland) and were coated with gold (SEM Coating Unit E 5100, Parlon Equipment England). The samples were then viewed under JSM 6400 scanning electron microscope.

2.4 Data Collection

Digital images were collected of secondary electron microscopy images using magnifications: ×2000.

2.5 Morphological Analysis of Tissue

Table 1 shows the three luminal epithelial surface characteristics and their descriptions used in the morphological analysis of the fatty acids treated and normal pregnant uterine tissue. For analysis, the most representative area of each tissue sample was photographed and studied. Three representative micrographs were taken from each animal's tissue sample. For the morphological analysis of the secretions and Pinopodes, the ×20,000 micrographs from each animal were used.

Table 1. Description of the scoring system used to analyse the surface epithelial features from SEM micrographs of the luminal epithelium of the endometrium at day 15 of pregnancy of the control animals and animals treated with different ratio n-6:n3 fatty acids at day 15 of pregnancy .

Epithelial characteristics		**Scoring system**
1	Microvilli density	The relative number of microvilli seen per cell (1, sparse; 2, medium density; 3, dense)
2	Small secretions	The number of small secretory droplets seen within the field (0, none; 1, some; 2, many)
3	Large surface protrusions	The number of large protrusions seen on the cell surface (0, none; 1, some; 2, many)

Seven uterine epithelial cell characteristics were analysed and scored according to the scoring system described.

2.6 Statistical Analysis

Once the data was scored according to the scoring system (Adams et al., 2004) summarised in Table 1, descriptive statistical analysis (Huck et al., 1974) was performed using JMP 5.0.1 statistical program (version 3.1.6.2; SAS Institute, Cary, NC). The median and quartile (25% and 75%) values were obtained and data expressed as median ± quartiles.

For each section, 7 fields were taken, and the measurements for each field were averaged to give a mean score and standard deviation for that section. Kruskal–Wallis non-parametric one-way analysis variance (ANOVA) was used

.

3. Results

Table 2 provides an overview of the results obtained from the morphological analysis of the electron micrographs with regards to each of the 3 characteristics used for comparison.

Table 2. The results from the control and treated animals, killed on days 15 of pregnancy.

Treatment	Microvilli density	Small secretions	Cell surface protrusions
Diet 1:1	Medium/dense	Some/many	Many
Diet 6:1	Medium	Some/many	Some
Diet 30:1	Sparse/medium	Some	None/some
Diet control	Medium/dense	Some/many	Many

The scanning electron micrographs were morphologically analysed by a scoring system described in Table 1. The average score was taken for each group of animals and then matched to the scoring system in Table 1 to define each observed characteristic.

The microvilli of endometrium in both the control group (Figure 1) and Diet 6:1 group (Figure 1) rats on day 15 of pregnancy were generally short, while not much variation was seen in the microvilli length for Diet 6:1 group and Diet 30:1group (Figure 2). However, variation was observed in the microvilli density (Figure 1). In the control group, the microvilli were observed to have medium to dense (Figure 1), while in the Diet 30:1group, the microvilli were sparse with low density and the epithelial surfaces were flat with very low number of microvilli (Figures 2). It appeared that low ratio of *n6:n3* (Diet 1:1) increased the density of the microvilli of the uterine epithelial cells.

The most interesting results found in this experiment were the small, round and smooth secretory droplets that were seen near the periphery of the uterine epithelial cells in the Diet 1:1 and Diet 6:1 group on day 15 of pregnancy , respectively. On the same day of pregnancy, in the treated animals of Diet 6:1 group and control group , many of these small secretions were observed in the uterine epithelial cells , respectively.

Pinopodes were observed covering, developing and bulges without or with only some wrinkles in the surface of the luminal epithelium of the endometrium in the rats of control group and Diet 6:1 group as shown Figures 1, respectively. However, they became slightly less, regressing and started to disappear in the Diet 30:1group (Figure 2). Developed pinopodes were larger, bulges without wrinkles in the rats of Diet 6:1 group (Figure 2). The results showed that the appearance of these pinopodes was directly related to the different ratio of *n6:n3* fatty acids supplementation diets

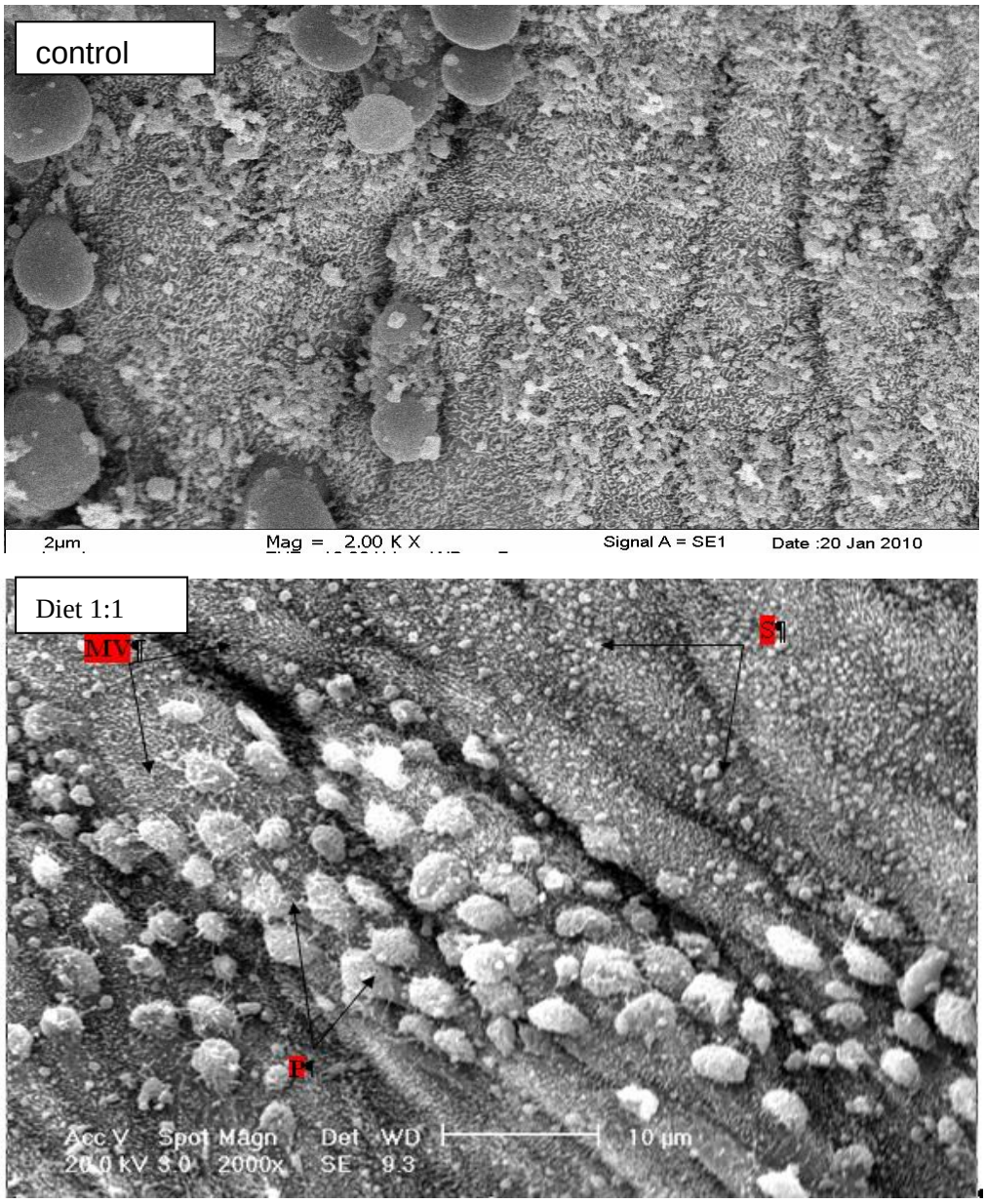

Figure 1. Scanning electron micrograph of the uterine epithelial surface on day 15 of gestation in rat of control group. Note that microvilli (MV) of endometrium are become generally short and clearly seen with medium to dense. The small secretions (S) appear small, round and smooth secretory droplets and the pinopodes (P) are observed covering, developing and bulges without or with some wrinkles in the surface of the luminal epithelium of the endometrium. And the Diet 1:1 group. Note that the MV of endometrium are generally short and clearly seen with high density and numerous in number. The small secretions are small, round and smooth and the (P) are observed covering, developing and bulges without or with some wrinkles on the surface of luminal epithelium of the endometrium. x2000

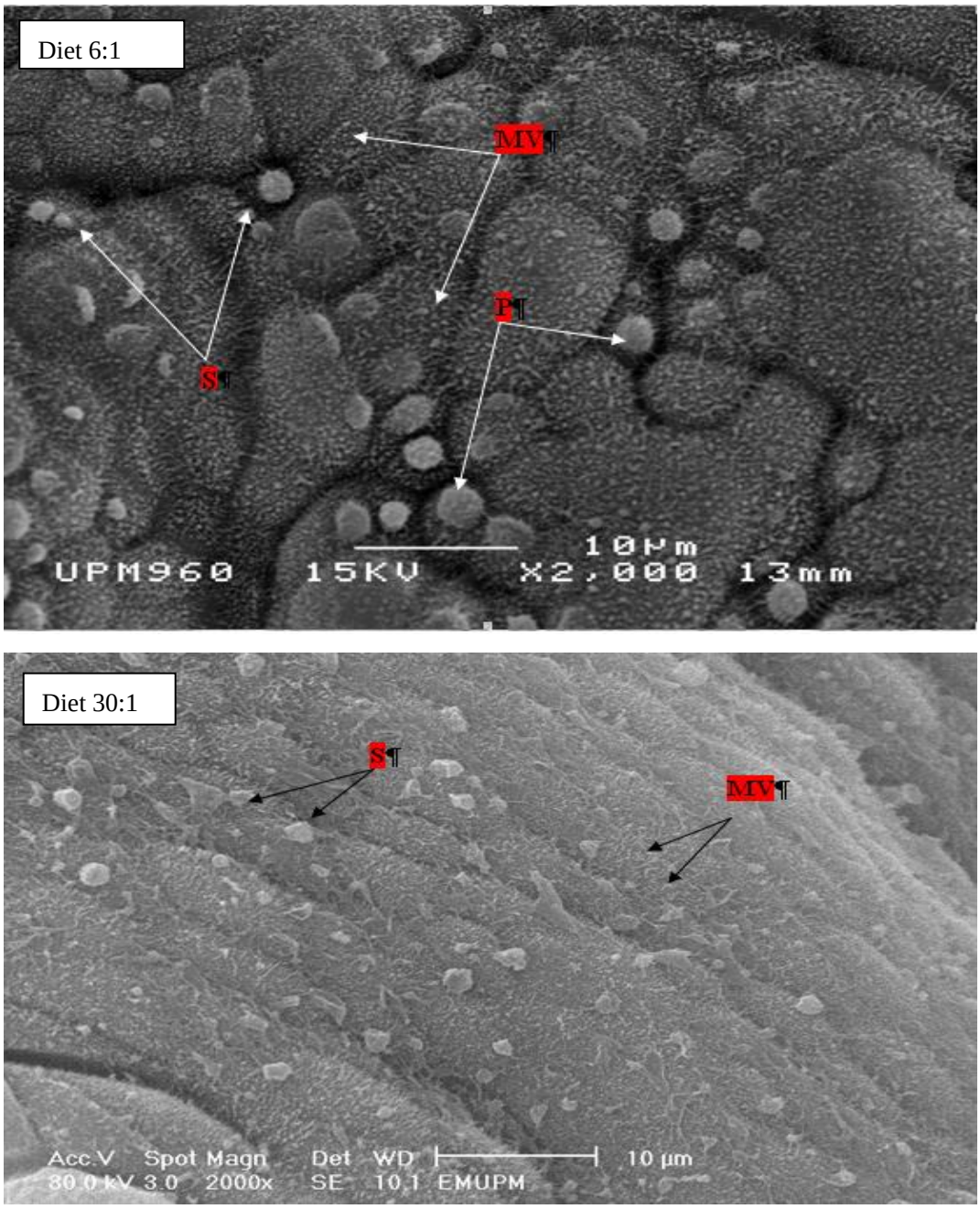

Figure 2. Scanning electron micrograph of the uterine epithelial surface on day 15 of gestation in rat of Diet 6:1 group . Note that the microvilli (MV) of endometrium are not much variation as compared to other groups. The small secretions (S) appeare small and the pinopodes (P) are developed, larger, bulges without wrinkles and less covering the surface of luminal epithelium of the endometrium.And the Diet 30:1 group. Note that the MV of endometrium are sparse and are not clearly seen with low density and very low number. The small secretions appear small and the p become slightly less, regressing and start to disappear in the surface of luminal epithelium of the endometrium. x2000

4. Discussion

In the present study, morphometric assessment of the endometrium was performed to evaluate the histological alterations in the uterine endometrium under the influence of different ratios of *n6:n3* fatty acids supplementation at the light microscopic level. The main aim of this experiment was to determine the endometrial changes in the pregnant rats treated with different ratios of *n6:n3* fatty acids supplementation on day 15 of pregnancy. In theory, morphological alterations of the endometrium may affect the length of gestation and the growth of the embryo. Assessing the endometrial morphological parameters including glandular area, diameter and volume have been used to evaluate the changes in the structure and function of endometrial glands more accurately (Wahab *et al.*, 1999)

In rodents, the process of implantation involves complex interactions and requires a very precise coordination between the establishment of uterine receptivity and the blastocyst activation (Chakraborty *et al*., 1996). This process is primarily dependent on the concerted effects of sex steroids (Salazar and Calzada, 2006). At the beginning of pregnancy, preovulatory ovarian E2 induces epithelial cell proliferation (Given and Enders, 1980). In this study the uterine receptivity may be influenced by changes of the circulating levels of estradiol. On the other hand, progesterone is needed for the development of important cell structures, such as pinopodes and microvilli in rodent endometrial (Panzan *et al*., 2006). The scanning electron microscopic examinations showed that in comparison of apical membrane of all groups, the microvilli were present; although the number of these structures had some variation. However, in rats fed high ratio of *n-6:n-3* fatty acids supplementation (Diet 30:1), the microvilli were disappeared and in contrast, they were seen in the uterine epithelial cells in rats fed low ration of *n-6:n-3* (Diet 1:1 group). The SEM examinations also revealed that the projections on the surface were typical pinopodes, which are the implantation marker. It seems that in these groups, the microvilli decreased in number and length. Similar finding was observed by Stavreus-Evers *et al*. (2001). The estrogen causes an increase in length and number of microvilli including the amount of secretory droplets while the progesterone affects the uterine epithelial cell flattening and decrease the shortening of microvilli in bovine and other species (Fathalla, 1971). This fact may explain why there was a reduction in the number of implantations in the group of low ratio of *n-6:n-3* faty acids supplementation (Diet 30:1 group). The interactions between diet and circulating steroid hormone concentrations are complex. The high ratio of *n-6:n-3* in Diet 3 group increased plasma estrogen concentrations relative to the other dietary treatments and decreased plasma progesterone concentrations relative to the other dietary treatments. Supplementation of high ratio of *n6:n3* as in the Diet 3 group decreased concentration of circulating progesterone (Milvae *et al.*, 1986).The morphological changes in endometrial cells are controlled by steroid hormones (Rider *et al*., 1998). Thus, as the concentration of progesterone increases, this proliferative effect seems to be higher (Fazleabas *et al*., 1999).

Progesterone plays a role in preparing the female reproductive tract for conception and implantation of the embryo and in maintaining the uterine environment during growth and development of the fetus, placenta and fetal membranes until parturition. In the current study, developing pinopodes were seen during increasing progesterone concentrations especially in

Diet 1:1 group, and regressing pinopodes were found with decreasing progesterone in Diet 30:1 groups. In contrast, there are some reports on a high incidence of dysfunction of endometrium under high physiological level of estrogen and progesterone. The high level of these hormones could affect the endometrial receptivity (Kramer and De Wet, 1994; Kramer *et al*., 1993; Valbuena *et al*., 1999). Progesterone is needed for the development of pinopodes in rodent endometrial (Singtripop *et al*., 1991; Singh *et al*., 1996). Thus, embryo implantation might be related to the number of pinopodes and the circulating progesterone levels (Rossi *et al*., 2002). In this study, development of pinopodes and microvili followed the progesterone level, but absolute concentrations of progesterone and pinopode development were correlated. The presence and development of pinopodes mainly depend on the ovarian hormones, especially progesterone (Singh *et al*., 1996), while high concentration of estrogen interfere normal formation of pinopodes (Martel *et al*., 1991; Simon *et al*., 1997).

5. Conclusion

The findings of this experiment suggest that supplementation of fatty acids with different ratio of *n-6:n-3* could change the endometrial morphology during the pregnancy. The tissue morphological characteristics changes was significantly different in high ratio of *n-6:n-3* fatty acids as compared to the low ratio of *n-6:n-3* fatty acids .

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