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THE EFFECT OF EXTRACT YELLOW PUMPKINS SEED (*CUCURBITA MOSCHATA)* ON HISTOLOGY OF ENDOMETRIAL GLANDS IN OVARIECTOMIZED RATS

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Abstract

Menopausal symptoms turn out to be a health problem threatening the quality of women's life. These symptoms are triggered by the decrease of estrogen produced by ovaries. Treatment using phytoestrogens could be expected to reduce the symptoms. Pumpkin seeds (*Cucurbita moschata*) contain secondary metabolites isoflavones that have a structure similar to endogenous estrogen and it can occupy estrogen receptor. The purpose of this study was to analyze the effects of *C. moschata* seeds in ovariectomized rats on the histology of endometrial glands. An experimental study in vivo with only post-test with control group design was conducted using 30 female Spraque-Dawley rats, 8 weeks old, weight 148-280 g. The rats were divided into 6 groups: control group (Normal), only ovariectomized group (OVX), ovariectomized rats given the extract of *C.moschata* 100, CM 200 and CM 400 mg / kg body weight for 30 days. (OVX-CM100; OVX-CM200; OVX-CM400), and ovariectomized rats given estradiol 2 μg/kg as a positive control (OVX-E). Rats were sacrificed on day 31st. Uterine was removed and the number of endometrial glands, diameter of lumen glands and the thickness of the endometrial glandular epithelium was analized. Statistical analysis was using One Way ANOVA. *Cucurbita moschata* extract was significantly increase the number of glands and diameter of lumen glands ($p<0,05$).

Keywords: menopause, *Cucurbita moschata*, histology of the endometrium, endometrial glands.

1. Introduction

Menopause usually occurs around age 45 to 55 years old at 60-70% of women. The average age in the western population is around the age of 50 years. Women in developing countries usually reach menopause earlier compared to women in Western populations. Menopause is a condition of the ovary failure to produce estrogen. 50-80% of women experience symptoms of menopause, include hot flashes, night sweat, vaginal dryness, insomnia, mood swings and depression. The increasing number of women, especially of elder age, presents a new problem. Symptoms of menopause incite discomfort and decrease the quality of the woman's lives (Savitri et al., 2009).

Hormone Replacement Therapy (HRT) is commonly given to women suffering from to overcome the symptoms of post-menopause. Unfortunately, women exposed to long-term use of HRT are also exposed to its identified malicious side effect, the increasing risk of ovarian and endometrial malignancies (Manson & Martin, 2001). It is advised for menopausal women to wisely resorting to an existing alternative treatment, phytoestrogen therapy, as it is proven to be safer [Rimoldi et al., 2007]. Phytoestrogens are substances of plant origin that are structurally and functionally similar to the estrogens. Phytoestrogens are proposed to replace the function of estradiol as body's natural estrogen. Phytoestrogens are also promoted as being an estrogen agonist, because they can occupy estrogen receptors (Savitri et al, 2009). There are four main types of groups of phytoestrogen in plants such as flavonoids, coumestan, lignans, and stilbene. Isoflavonoids are one class of phytoestrogens. Isoflavonoids are divided into three groups: isoflavones, isoflavan, and coumestan (Whitten &Pattisaul, 2001 *cit*. Sitasiwi, 2009).

Pumpkin (*Cucurbita moschata*), is a fruit with complete nutritional content. Types of pumpkins that grew in Indonesia are varieties of *C. moschata* and *C. pepo* (Sushanty, 2013). In Indonesia, pumpkins have been used to produce many kind of traditional food, but the pumpkin seeds are not widely used. According to Li et al. (2009), glycoside phenolic compounds found in the seeds of *C. moschata* are phenylcarbinyl 5-O-(4-hydroxy) benzoyl-beta-D-apiofuranosyl (1-->2)-beta-D-glucopyranoside. Glycosides phenolic compounds in the seeds of *C. moschata* are one of isoflavone derivates (Koike et el.,2005). Phillips et al. (2005) mention that the pumpkin seeds contain phytoestrogen 265 mg in 100 grams of seeds. Pumpkin seeds also contain secoisolariciresinol which is a lignin compound (Sicilia et al., 2003). Lignin is one of the main classes of phytoestrogens agent (Cornwell et al., 2004). This background leads the researchers to hypothesize that pumpkin seed extract (*C. moschata*) has the estrogenic effect on reproductive organs, especially on endometrial glands.

2. Material and Methods

2.1 Plant and Chemical Materials

Seeds of pumpkin (*C. moschata*) used in the present study were collected from the commercial market in Kulonprogo Yogyakarta and authenticated at the Laboratory of Plant Taxonomy, the Faculty of Biology, GadjahMada University, Yogyakarta, Indonesia. Concentrated nitric acid solution, perchlorate acid solution, distilled water and calcium carbonate were obtained from LPPT GadjahMada University, Yogyakarta, Indonesia

2.2 The Preparation of Ethanolic Extract of Pumpkin Seeds (C. moschata)

The dried powders of seeds of pumpkin (*C. moschata*) were extracted by maceration using 70% ethanol. The ethanolic extracts were evaporated to obtain the concentrated extract.

2.3. Animals

Female Sprague–Dawley rats, aged 8 week, were purchased from Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. The animals were grouped and maintained under standard laboratory conditions with dark and light cycle (12/12 h) and allowed free access to commercial pellet diet and water ad libitum.

2.4. Administration Procedure

Rats were acclimatized to laboratory condition for 1 week before the commencement of the experiment. The ovariectomy was performed to all experimental rats except to those belonging to the normal group. Animals were assigned to the experimental groups, normal, OVX, OVX-E, OVX-ECM100 mg/kg BW, OVX-ECM 200 mg/kg BW, OVX-ECM400 mg/kg BW, with five animals per group, per experiment. Twenty days after the rats were ovariectomized, all of the rats were allowed to the controlled access to a commercial standard pellet and to deionized water ad libitum for 20 days. The extract of *C. moschata* tuber was given orally once a day during 30 days and the doses were 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW to the experimental group of OVX-ECM. The group of OVX-E was given estradiol with the dose of 2 μg/day, and the control groups and OVX did not get any treatment. On the 31st day, the rats were sacrificed and their uterine were taken to histological analysis.

2.5. Uterine Histological Analysis

The uterine horns were immediately removed and fixed in 4% para-formaldehyde (PFD) at 4oC for 4 to 5 hours. The tissues were then processed through different grades of ethanol, incubated overnight in chloroform, transferred into paraffin wax for 3 hours, placed into the molds prefilled with melted wax, and cooled immediately at -60 o C to harden the wax. Tissues were then cut into 5 μm sections and mounted onto glass slides. The slides were then stained with hematoxylin and eosin (H&E), visualized under a light microscope under magnifications of 4, 20 and 40 times. The diameter, the epithelial height and number of uterine glandular lumen were measured.

3. Results and Discussion

Table 1. The average of number, diameter of lumen glands and epithelial height of endometrial glands.
Analyses of morphology of the endometrial glands in the uterine section following treatment with different doses of extract of *C. moschata* seeds (ECM): Normal , OVX (ovariectomized), OVX-E (ovariectomized + estradiol), OVX-ECM : ovariectomized + exctract of C.Moschata seeds 100 (100 mg/kg/day), 200 (200 mg/kg/day), 400 (400 mg/kg/day). Values represent mean ± SEM with n=5 per group. The average number of glands per cross section increased with increasing doses of genistein treatment. * (p<0.05 as compared to control Normal), † (p<0.05 as compared to control positive OVX-E).

The group of treatment		Diameter of lumen's glands (mean ± SEM)	Epithelial height of endometrial glands (mean ± SEM)	Number of endometrial glands (mean ± SEM)
1.	Normal	43,27 ± 7,40	13,90± 1,58	4,70± 2,21 †
2.	OVX	28,82 ± 8,05 *	11,45± 3,36	1,35± 1,36 *†
3.	OVX-E	46,27 ± 11,98	14,03± 0,67	13,04± 4,51*
4.	OVX-ECM 100	41,66 ± 5,85	14,42± 1,91	6,2± 1,20 *†
5.	OVX-ECM 200	46,06 ± 1,84	14,85± 1,49	5,65± 1,73 *†
6.	OVX-ECM 400	46,27 ± 11,98	14,77± 1,71	9,75± 2,28 *

In table 1, the lowest number of the endometrial glands is the negative control group (OVX), while the highest number is the positive control group (OVX-E). The number the endometrial glands increased following treatment with extract *C. moschata*. A dose-dependent increase in the number of endometrial gland was observed with increasing doses of orally extract *C. moschata* treatment. Treatment with 400 mg kg/day *C. moschata* resulted in significant increase in the number of gland compared with normal control but not significantly to the positive control group (OVX-E).

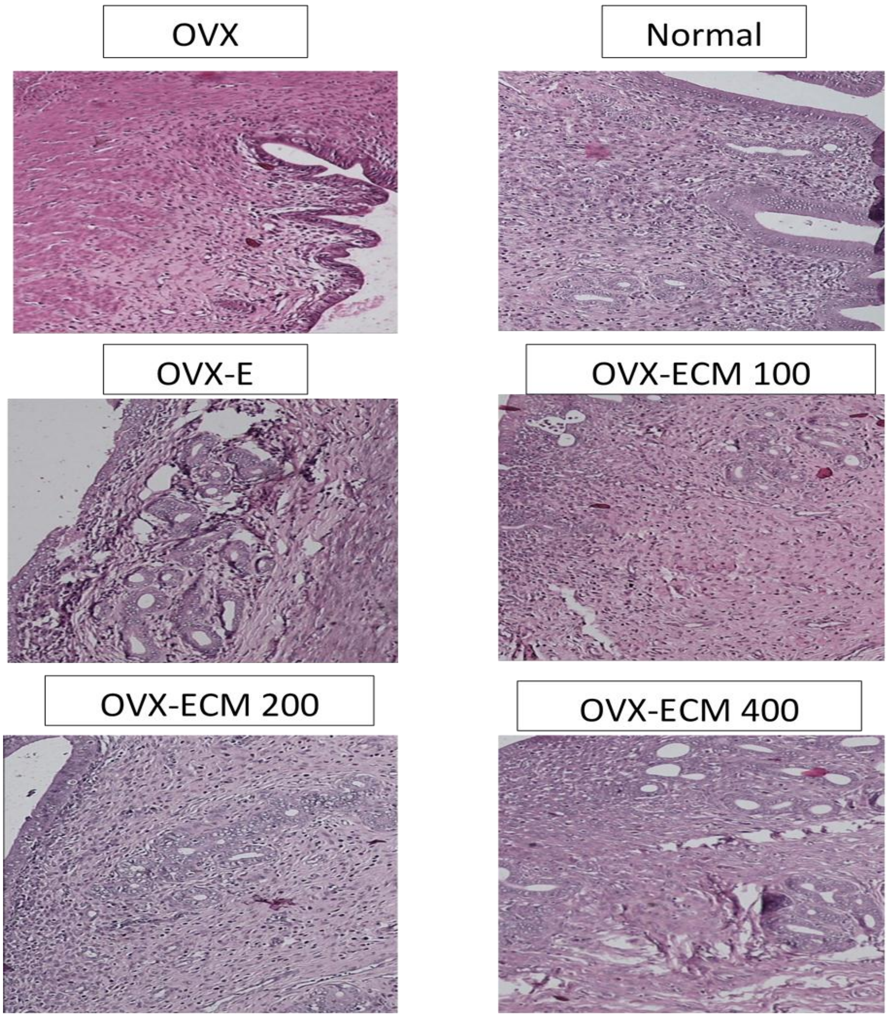

Figure 1. The morphology of endometrial glands among the groups. a. OVX (only ovariectomized); b. Normal (without ovariectomized and no treatment); c. OVX-E (ovariectomized + estradiol); d. OVX-ECM 100 (ovariectomized + ECM 100); OVX-ECM 200 (ovariectomized + ECM 200) and e. OVX-ECM 400 (ovariectomized + ECM 400). Sections were stained with H&E, magnification 40x.

Table 1 shows that the smallest gland lumen diameters belong to the negative control (OVX), while the values of the sizes of diameter among the normal control group, positive control, and treatment groups is significant differences. One Way ANOVA statistical test showed that there are significant differences in the number of endometrial glands among the six treatment groups ($p < 0.05$). Post hoc Tukey HSD test performed afterwards showed that the result from the negative control group (OVX) was significantly different with other groups. Observation on endometrial glandular epithelial shows thickness are low in the negative control group (OVX) and the highest in the positive control group (OVX-E). In the experimental group, ECM increased endometrial glandular epithelial thickness, and this increase is in line with the increasing dosage, but statistically there are no significant differences among all groups.

This research was performed to know the estrogenic activity of the pumpkin seed extract orally. To induce conditions similar to those of menopause, rats were ovariectomized and spared for 30 days. The observed parameters are the histology of endometrial glands, including the number, diameter and thickness of the endometrial glandular epithelium.

C. moschata seeds contain secoisolariciresinol, which belongs to the compound of lignans (Sicilia et al., 2003), and phenolic glycosides that are included in isoflavones (Lie et al., 2009). Lignans and isoflavones are primary classes of a phytoestrogen agent (Cornwell et al., 2004). There are four main types of phytoestrogens, namely flavonoids, coumestan, lignans and stilbene (Whitten &Pattisaul, 2001 *cit*. Sitasiwi, 2009). The phytoestrogens are supposedly working as an estrogen agonist by engaging with estrogen in a competition to occupy the estrogen receptors (Savitri et al., 2009). When estrogen levels are down, there will be an excess of estrogen receptors that does not bind to estrogen. Isoflavones, although having low affinity, can still bind the receptor (Cahyati et al., 2013).

The estrogen receptors (ER) have multiple functional domains. There are two types of ER, namely ERα and ERβ. The differences are in the C-terminal ligand-binding domain and the N-terminal transactivation domain. The estrogen receptor α (Erα) is abundant in cells in the reproductive organs such as the endometrium, breast cancer cell line, and ovarian stroma, whereas the estrogen receptor β (Erβ) is widely distributed in cells besides reproductive organs including the kidney, mucosal intestinal, lung parenchyma, bone, brain, endothelial cells, and prostate gland. Thus, the bonding estrogen-ER is an important process to causing estrogenic effects (Kuiper et al., 1998; Weirman,2007).

The estrogenic effects generated by the phytoestrogens are 10^2 to 10^3 times lower than the estrogenic effects of 17β-estradiol, which are the endogenous estrogen in the body (Benassayag et al., 2002). This is due to the affinity of phytoestrogens to the estrogen receptor which is lower than that of estrogen (Cederroth et al., 2007). The phytoestrogens are able to bind to estrogen receptors (ERα and ERβ) and functioning as the estrogen receptor antagonists or agonists (Benassayag et al., 2002).

The results of this study showed that administration of extract *C.moschata* significantly increases the number of glands and the diameter of the lumen of endometrial glands, whereas the epithelium thickness of the endometrial glands shows no significant increase. A dose-dependent increase in the number of glands was observed with increasing doses of extract of *C. moschata* given orally. In the negative control group that only performed ovariectomy, all three parameters are the lowest. The ovariectomy performed on the rats has successfully exhibit the symptoms of menopause on those rats.

The increasing number of endometrial glands is supposedly due to the proliferation and differentiation of endometrial glands through a mechanism described by Cooke, et al (1998). This mechanism commences when phytoestrogens bind to hormone receptors on the target cell, and accordingly they can transform the conformation of the hormone receptors. This leads to conformational change in which the phytoestrogens-receptor complexes become activated so they can bind with the DNA chain. Interaction between the phytoestrogens-receptor complexes with DNA causing gene expression is increased.

The endometrial glands are simple tubular glands. These glands change throughout the estrous cycle. The glands undergo repeated branching. On the observation the number of the gland transverse incision in increasing numbers. Gland development occurs because of estrogen going to cause proliferation, morphogenesis, and differentiation. Estrogen plays a role in cell proliferation, especially in the transition of cells from phase G to phase S and affects gene regulation of growth (Cooke et al., 1998).

The increasing diameter of the endometrial glands after *C.moschata* extract is given during this research indicates the occurrence of gland development. Also such an occurrence is presumably related to with increasing secretion of fluid into the lumen of the gland as described in previous studies. Salleh et al (2013) already examined the effect of genistein on the endometrial glands and they concluded the endometrial glands increase in lumen diameter were in line with the increase ofthe amount of fluid in the lumen of the gland. Increasing fluid in the gland lumen is suspected to give a compression effect on endometrial epithelial cells so that the gland epithelium endometrial thickness remains unchanged.

The conclusion of this study is *C.moschata* seed extract treatment registered orally significantly increases the number of the endometrial glands and increases the diameter of the lumen of endometrial glands, but this treatment still provides no evidence of thickening the endometrial glandular epithelium in ovariectomized rats. Further research about the effects of *C.moschata* on other organs is still required to conclude that the extracts of *C. moschata* have estrogenic effects.

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