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THE EXPRESSION OF VEGF IN VAGINAL TISSUE OF MENOPAUSAL ANIMAL MODELS AFTER TREATED WITH ETHANOL EXTRACT OF PURPLE SWEET POTATO (*IPOMEA BATATA L.)*

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ABSTRACT

Vaginal atrophy is a common complain in menopausal women as the sexual organ dysfunction. The features of vaginal atrophy including thinning of epithelium and decreasing in collagen fiber in the stroma therewith reduced blood vessels and vascularization by reason of decreased angiogenesis factors stimulation. Vascular endothelial growth factor (VEGF), a crucial mediator regulated by estrogen in angiogenesis, is involved in this process. Hormone Replacement Therapy (HRT) has proven effective in alleviating vaginal atrophy symptoms and enhancing vaginal wall vascularization, but it poses potential serious side effects. Phytoestrogens serve as a safer alternative to estrogen, demonstrating the ability to increase VEGF expression in the vaginas of ovariectomized mice and improve vaginal atrophy symptoms. Anthocyanins, a group of flavonoids with known phytoestrogenic activity both in vitro and in vivo, are found in high concentrations in purple sweet potato tubers. Several studies confirmed the phytoestrogenic activity of ethanol extracts from purple sweet potato tubers. Previous research indicates that these extracts increased iepithelial thickness, epithelial maturation index, and the expression of estrogen receptors α and β, whilst maintaining vaginal pH. This pure experimental study followed a Post Test Only Control Group Design, aiming to investigate the effects of ethanol extract from purple sweet potato tubers on VEGF expression in the vaginal tissue of a menopausal animal model. Thirty-eight female Wistar rats, aged 16-18 weeks, subjected to bilateral ovariectomy, were divided into two groups: treatment group (P1) and control group (P0). After 30 days of treatment, the experimental animals were sacrificed, and vaginal tissues were collected for histological preparation and VEGF immunohistochemical staining. The research findings revealed that VEGF expression in the P1 group was 3.59±1.23, while in the P0 group, it was 0.8±0.57. Independent Sample T-test results indicated a significant difference ($p<0.05$) in VEGF expression between the two groups. In conclusion, the group treated with ethanol extract purple sweet potato tubers exhibited significantly higher VEGF expression in vaginal tissue compared to the control group.

Keywords: Anthocyanin.,Vaginal Atrophy., Phytoestrogen., Purple Sweet Potato., VEGF

INTRODUCTION

Menopause naturally occurs at the end of mestrual cycle that lasts for at least a year and is caused by a decrease in ovarian follicle activity, which lowers estrogen.[1] Since estrogen is necessary for the proliferation and development of vaginal cells, a drop in estrogen levels causes vaginal atrophy. Vaginal atrophy affects about 30% of women at the outset of menopause and rises to 47% by the end of the menopausal transition. According to Liu et al., symptoms of vaginal atrophy that are frequently described include dryness, pain during sexual activity, irritation, vaginal discharge, and dyspareunia.[2]

The thinning and impaired maturation of the vaginal epithelial mucosa are the alterations related to vaginal atrophy.[3] When the amount of collagen and elastic in the vaginal connective tissue decrease, it reduces the ability of the vaginal wall to retain water, thus attenuate the vaginal size and elasticity.[4] Moreover, the low vaginal blood flow in postmenopausal women decrease the glandular production compared to premenopausal women. Dryness and decreased vaginal lubrication are the results of this decrease in cervical gland production. Furthermore, under atrophic situations, decreased vascularization impedes the process of tissue repair.[2]

Menopausal vaginal atrophy can be effectively treated with hormone replacement therapy (HRT), which shown to improve vaginal wall vascularization. HRT has disadvantages despite its efficacy, including a higher risk of ovarian, endometrial, and breast malignancies. Because of the hazards to consider, scientists have been exploring alternate treatments for menopausal symptoms, which has led to the emergence of phytoestrogens.[5]

As defined by Francisco et al., phytoestrogens are the estrogenic substances derived from plants that bind to estrogen receptors thus enforcing target cells to respond identically as estrogen.[6] Based on several studies, phytoestrogens may benefit women suffering menopausal vaginal atrophy symptoms by improving the vaginal structure that has been affected by vaginal atrophy[7]. Studies carried out on menopausal models in animals have also shown that phytoestrogens can effectively lessen the symptoms of vaginal atrophy.[8]

It has been demonstrated that phytoestrogens can enhance angiogenic factors in vaginal tissue. Reduced expression of estrogen receptors, VEGF, bFGF, angiopoietin, and vascular endothelial growth factor receptor-1 (VEGFR-1) is observed in rodents following ovariectomies. In ovariectomized rodent vaginas, the phytoestrogen YGW is administered to restore estrogen receptor expression and increase the expression of angiogenic factors, such as VEGF, VEGFR-1, bFGF, Angiopoietin-1, and Angiopoietin-2. According to Yin et al., the effects of phytoestrogen YGW are similar to those of premarin estrogen.[9]

Plants contain reddish-purple pigments called anthocyanins, which are members of the flavonoid group. Nanashima et al. have shown that anthocyanins possess estrogenic activity in both in vitro and in vivo studies, and their molecular structure is similar to that of estrogenic hormone[10]. Purple sweet potato tubers that contain high anthocyanins related to various health benefits, including improved lipid profiles together with antioxidant, antidiabetic, and antihypertensive effects. [11].

Purple sweet potato tubers have also been shown to be phytoestrogens; they have been shown to preserve vaginal pH,[12] increase estrogen receptor expression,[13] and improve vaginal epithelial thickness in menopausal animal model vaginas.[14] By assessing VEGF expression, this study attempts to examine the effect of ethanol extract from purple sweet potato tubers on the vascularization of the vaginal wall in an animal model of menopause.

MATERIALS AND METHODS

This is an experimental research with a randomized post-test only control group design method. This research is in vivo research using rats as experimental animals and has received information on ethical feasibility by the Research Ethics Commission Unit, Faculty of Medicine, Udayana University No. B.2169/UN14.2.2.V.32/PT.01.04/2022. The experimental animals were then randomly divided into two experimental groups with the following characteristics: adult female Wistar strain rats that had been ovariectomized and weighed between 180-220 grams. The subjects of this research were obtained from the Animal Laboratory Unit, Department of Pharmacology, Faculty of Medicine, Udayana University.

Animal Preparation

Ovariectomy was performed using the modified Ingle DJ and Grith JQ methods. The procedures were conducted as follows: First, ketamine at a dose of 40 mg/kg BW was injected intramuscularly then the abdominal fur was removed and the surgery area was sterilized using betadine, then covered with sterile mask. Next, a transabdominal incision was made above the uterus 1.5 – 2 cm long layer by layer until it penetrates the peritoneum. The oviducts and ovaries are cleaned from the surrounding fat and connective tissue, and the distal oviducts and ovaries are then tied and removed. This process was carried out on two sides, for both ovaries. The incision wound will then be closed with layer-by-layer sutures. Gentamicin injection was given at a dose of 60-80 mg/kg BW/day for three days, as post-operative therapy. The ovariectomized rats were then rested for 7 days while being given standard BR II food 20 grams per mouse every day and 2 ml distilled water per 200 grams of mouse body weight for drinking. These rats were then kept in cages and monitored in the Histology section of the Faculty of Medicine, Udayana University.

Preparation of Ethanol Extract of Purple Sweet Potato

A particular method was employed to produce the purple sweet potato tubers' ethanol extract. Purple sweet potato tubers were first picked, then washed and peeled. Following their cutting into cube shapes (widths of 2 to 2.5 cm), the tubers were ground into a powder. This powder was mixed with 70% ethanol (1 L of ethanol for 1 kg of tubers). Three layers of fine fabric were used to filter the mixture. In order to produce a concentrated extract, the filtrate was evaporated. Following that, this extract was once more dissolved in one liter of water and brought to a boil. The final product's anthocyanin concentration was determined to be 119 mg/mL.[15]

Distribution of Sample Groups

This study used two groups of animals. Based on Federer's formula and considering the mortality of experimental animals, it was determined that the number of samples needed for each group was 18 animals. The groups were divided as follows:

a. Control (P0): ovariectomy group given normal saline 1 ml/day.
b. Treatment (P1): ovariectomy group given ethanol extract of purple sweet potato at a dose of 400 mg/Kg BW/day for 30 days.

Procedures for Euthanasia and Burial of Experimental Animals

The euthanasia procedure uses ketamine at a dose three times the anesthetic dose, namely 120 mg/kg BW intramuscularly. After that, the mouse spleen will be taken to make spleen histology preparations. Rats whose spleens have been removed will be wrapped in medical waste bags and buried in the ground.

VEGF Staining

Organs were prepared in paraffin blocks, trimmed 6 μm thickness then embedded on a clean poly-L-lysine-coated glass slide to obtain a spleen tissue section. Prior to this, PBS pH 7.4, 1% FBS, and sodium citrate buffer pH 6 were prepared. The tissue on the poly-L-lysine slides underwent deparaffinization with xylene for 3 cycles of 5 minutes each. Subsequent rehydration involved immersion in 100% ethanol for 2 cycles of 5 minutes, 95% ethanol for 2 cycles of 2 minutes, 70% ethanol for 2 cycles of 2 minutes, and rinsing with PBS for 2 cycles of 5 minutes. Antigen retrieval was performed using citrate buffer in a 500 cc Duran bottle in the microwave (800 watts; 6.5 minutes; 500 cc). After boiling, slides were inserted and left for 20 minutes. Following microwaving, slides were taken out and allowed to reach room temperature for approximately 60 minutes. PBS washing was performed for 3 cycles of 5 minutes, excess buffer cleaned with tissue. Slides were treated with dual endogenous enzyme block for 15 minutes, washed with PBS for 3 cycles of 5 minutes, and excess buffer removed with tissue. Application of the primary antibody (VEGF = 1:200) followed, with overnight incubation at room temperature. Subsequent washing with PBS for 3 cycles of 5 minutes and cleaning excess buffer with tissue were carried out. Slides were then treated with a sufficient amount of labeled polymer-HRP to cover the specimen, followed by a 30-minute incubation at room temperature. Further washing with PBS for 3 cycles of 5 minutes and cleaning excess buffer with tissue were performed. Substrate-Chromogen was applied, and the slides were incubated for 10 minutes. Washing with distilled water from a wash bottle was followed by staining with hematoxylin Meyer for 60

seconds. After rinsing with distilled water and dipping the slides 10 times in tap water, dehydration ensued with 2 cycles of 2 minutes in distilled water, 2 minutes in 70% ethanol, 2 cycles of 2 minutes in 95% ethanol, 2 cycles of 5 minutes in 100% ethanol, and 3 cycles of 5 minutes in xylene. Mounting was done with DPX, covered with a cover slip.

Histological Observations

Three fields of view and 400x magnification were used for the immunohistochemistry analysis using the Optilab light microscope. The presence of several brown patches or brown staining with granular uniformity indicated positive VEGF expression. At first, the entire field was examined at a 100x magnification to find "hot spots," or locations where there was a significant concentration of positively labeled cells. The number of cells displaying brown staining was then counted within the designated hot spot region at a 400x magnification using raster image analysis software.

Data Analysis

The amount of Vascular Endothelial Growth Factor (VEGF) expressed in vaginal tissues was determined by taking the average of the results obtained from three separate counting points for each sample. Using SPSS 15.0 software, statistical analysis was done to evaluate the variations in VEGF expression levels between the groups. Since the VEGF expression data is numerical, group differences should be ascertained using an Independent T-test after a normal distribution of the data has been confirmed. The p-value was considered statistically significant if it was less than 0.05.

RESULTS

Figures 1 and 2 display the immunohistochemistry staining results for the control and treatment groups, respectively. Positive VEGF expression, identified by brown staining, is indicated by yellow arrows in the vaginal tissue of the menopausal animal model in both figures. After being observed at 400x magnification in three different fields of view and quantified, the VEGF expression of the control group (P0) was 0.8±0.57 and the treatment group was 3.59±1.23 (Figure 3). The result of the Shapiro-Wilk test showed that the data had a normal distribution, with values of 0.184 for the treatment group and 0.173 for the control group. The Independent Sample T-Test yielded a 0.000 p-value. Therefore, it can be said that as compared to the control group, the treatment group's vaginal tissue showed noticeably higher levels of VEGF expression.

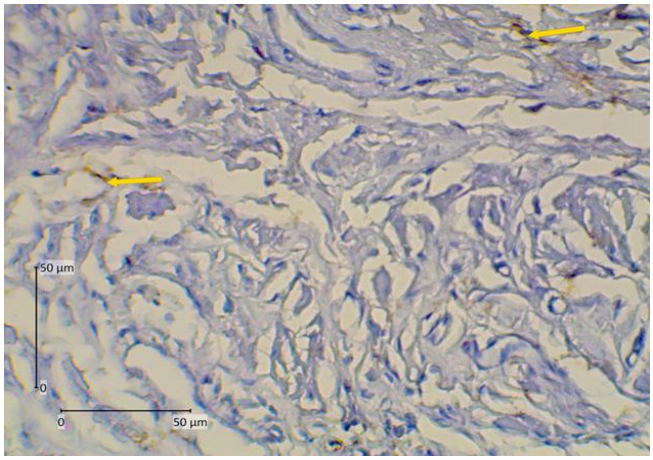

Figure 1. Shows the expression of Vascular Endothelial Growth Factor (VEGF) in the control group (P0) at 400x magnification. The arrow designates and highlights in brown the VEGF-positive cells.

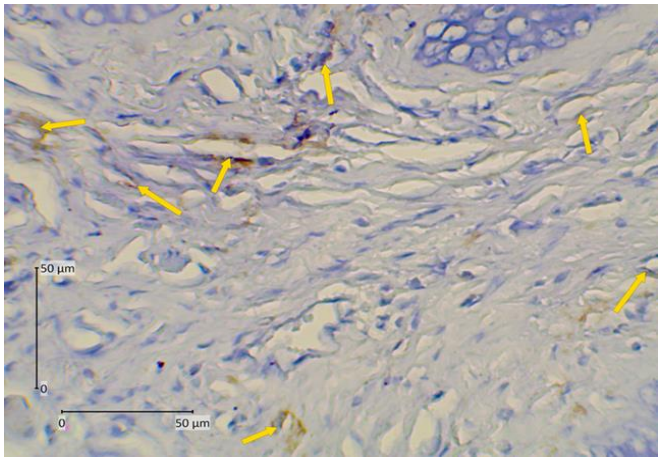

Figure 2. Shows the expression of Vascular Endothelial Growth Factor (VEGF) in the treatment group (P1) at 400x magnification. The arrow designates and highlights in brown the VEGF-positive cell

DISCUSSION

During menopause of women, some changes occur such as in the composition of estrogen hormones, marked by a significant decrease in the levels of estradiol and estrone, the primary estrogen hormones.[16] This condition leads to a state of hypoestrogenism, as estrone exhibits the weakest potential among other estrogen hormones. The substantial decline in estradiol levels results in vaginal atrophy characterized by alterations in mucosal layers, smooth muscles, and connective tissues.[17]

The thickness of the epithelium lining the mucosa decreases, and there is a reduction in the proportion of superficial epithelial cells.[18] In the lamina propria, there is a decrease in the synthesis of collagen and elastin fibers by fibroblast cells, accompanied by an increase in the breakdown of collagen fibers.[17] The smooth muscle layer and collagen fibers experience a decline, resulting in the shrinkage and reduced elasticity of the vagina.[4]

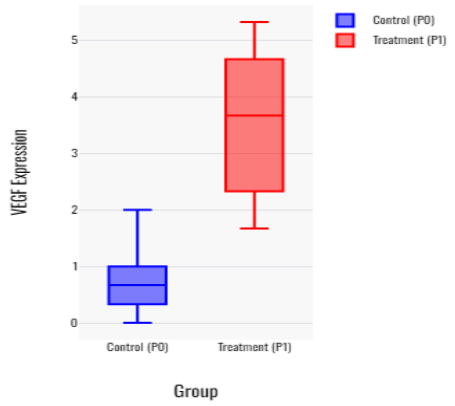

Figure 3. The average expression of VEGF (vascular endothelial growth factor) in the treatment and control groups.

The decrease in systemic estrogen levels also results in a reduction in the blood supply to the vaginal wall. In the connective tissue layer of the vaginal wall, large blood vessels accompanied by bundles of nerve fibers, smaller blood vessels, and nerve fibers penetrating the muscle layer, lamina propria, and basal membrane are identified. The blood supply to the vaginal wall is regulated by the levels of nitric oxide produced by the endothelium. Research findings indicate that during menopause, there is a decrease in nitric oxide secretion leading to a reduced blood supply to the vagina. Estrogen deficiency also contributes to a decrease in the number of blood vessels in the vaginal lining, as estrogen plays a role in regulating angiogenesis in the vaginal wall,[19]

Estrogen works through estrogen receptors to regulate the vascularization of the vaginal wall. Estrogen induces an enhancing effect on the expression of vascular growth markers, specifically Vascular Endothelial Growth Factor (VEGF). Additionally, estrogen plays a role in the regulation of Angiopoietin-1 and Angiopoietin-2, which are associated with VEGF expression to signal angiogenic processes. Ovariectomy treatment in mice results in a decrease in the expression of estrogen receptors, VEGF, basic fibroblast growth factor (bFGF), angiopoietin, and vascular endothelial growth factor receptor-1 (VEGFR-1).[9]

Several research studies have demonstrated the effectiveness of phytoestrogens in alleviating symptoms of vaginal atrophy. Administering a soy-rich diet for six months to menopausal women showed improvements in vaginal epithelium, evidenced by an increased karyopyknotic index. Another study involving isoflavone supplementation over 16 weeks in menopausal women revealed a significant reduction in urogenital symptoms compared to the placebo group.[20] The combination of isoflavones and lignans given for 12 weeks was reported to improve vaginal atrophy symptoms in menopausal women, including enhanced sexual quality, improved vaginal pH, and an increase in the epithelial maturation index.[7]

This research demonstrated that daily administration of purple sweet potato ethanol extract at a dosage of 400 mg/kg body weight for 30 days significantly increased VEGF expression in the vaginal tissues of a menopausal animal model. These findings suggest that purple sweet potato, abundant in anthocyanins, may act as a phytoestrogen, thereby influencing VEGF expression. The estrogen-like effects of anthocyanins are attributed to their structural similarity to estrogen, enabling them to bind to estrogen receptors.[21, 22] The phytoestrogen activity of anthocyanins is mediated via their binding to estrogen receptors. Anthocyanins bind to estrogen receptors, albeit with lower affinity compared to endogenous estrogen. Polyphenols, derivatives of red wine rich in anthocyanins, act by binding to ERα on endothelial cells to stimulate nitric oxide (NO) production. Nanashima et al. concluded that anthocyanins exhibit estrogenic activity through binding to both ERα and ERβ.[23]

The present findings indicate that purple sweet potato ethanol extract, abundant in anthocyanins, acts as a phytoestrogen by promoting vascularization in the vaginal wall via the upregulation of VEGF expression. This aligns with previous research demonstrating the extract's efficacy in preventing vaginal epithelial thinning [14] and maintaining normal vaginal pH.[12] The mechanism underlying these effects is likely attributable to the phytoestrogenic activity of the anthocyanins present in the ethanol extract. The structural resemblance of anthocyanins to estrogen, coupled with the known estrogenic effects of numerous flavonoids, supports evidence from studies suggesting the binding of anthocyanins to both ERα and ERβ receptors, thereby highlighting the potential estrogenic activity of anthocyanin-rich purple sweet potato extract. Moreover, the capacity of purple sweet potato ethanol extract to modulate estrogen receptor expression has been confirmed in studies using menopausal animal models. [13]

SUMMARY

The findings of this study lead to the conclusion that the treatment group receiving purple sweet potato ethanol extract exhibits a significantly higher expression of Vascular Endothelial Growth Factor (VEGF) in the vaginal wall compared to the control group.

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