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Combination of vitamin C and E modulated monosodium glutamate–induced endometrial toxicity in female Wistar rats

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ABSTRACT

Objective: To investigate whether combination of vitamin C and E able to inhibit decreasing angiogenesis, endometrial thickness, and α -estrogen receptor level in female rats receiving orally MSG–treatment. **Methods:** Twenty five female Wistar rats were divided into five group, control group, MSG [140 mg/200 gram body weight (bw)] group non treated and treated with combined vitamin C (0.2; 0.4; or 0.8 mg/g bw) and E (0.04 IU/g bw). Analysis of vascular endothelial growth factor (VEGF) level were done by immunohistochemistry technique. Analysis of the number of arteriole and thickness of endometrium was done histopathologically with hematoxylin eosin staining. Analysis of uterus α -estrogen receptor was done using flowcytometer. **Results:** The expression of VEGF, number of arteriole, thickness of endometrium, and α -estrogen receptor were significantly lower in MSG–treatment group compared to control group ($P < 0.05$). Second and third dose of combined vitamin C and E significantly increased VEGF level and number of arteriole compared to MSG–treatment group ($P < 0.05$), to reach similar level in control group ($P > 0.05$). Administration of vitamin C and E significantly increased the thickness of endometrium, and expression of α -estrogen receptor compared to MSG–treatment group ($P < 0.05$), reaching the number in the control group ($P > 0.05$). **Conclusions:** The present data suggesting that combined vitamin C and E able to inhibit endometrial toxicity caused by orally MSG treatment via modulating angiogenesis, increase endometrial thickness and expression of α -estrogen receptor.

1. Introduction

Monosodium l–glutamate (MSG), chemically known as 2–amino pentane dioic or 2–amino glutaric acid, is normally used as a flavor–enhancing ingredient more commonly used in traditional Asian cuisine[1]. MSG contains 78% of glutamic acid, 22% of sodium and water[2]. MSG–treatment is able to produce metabolic changes, which can further

result in severe bodily disturbances, even at a relatively lower concentration[3–4]. Some reports indicated that MSG was toxic to human and experimental animals[5]. MSG could produce symptoms such as numbness, weakness, flushing, sweating, dizziness and headaches. In addition, ingestion of MSG has been alleged to cause or exacerbate numerous conditions, including asthma, urticaria, atopic dermatitis, ventricular arrhythmia, neuropathy and abdominal discomfort[6].

During the reproductive period, endometrium is dynamic and will experience cycles of proliferation, differentiation, and decay. In premenopausal women, endometrium is proliferative or secretory in nature, depending on the phase of the menstrual cycle[7]. Capability of the endometrium to

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provide proper environment for conception, implantation, early gestation and placentation is an important point for pregnancy and fertility[8]. As far we known there is no study to evaluate the toxicity of MSG on endometrium tissue.

Vitamin E is a powerful fat-soluble antioxidant and therefore can protect cell membranes against oxidative damage that can prevent further damage to deoxyribonucleic acid (DNA) and tissues. Vitamin E can prevent lipid peroxidation because this antioxidant can break the chains of lipid peroxyl rapidly through the provision of a hydrogen atom in the hydroxyl group[9]. Activity of vitamin E as an antioxidant can improve blood supply to the granulosa during ovarian follicles development thereby supporting the production of the hormone estrogen which plays a role in the thickening of the endometrium[10]. Vitamin C (ascorbic acid) has a considerable antioxidant activity: it scavenges reactive oxygen species and may, thereby, prevent oxidative damage to the important biological macromolecules, such as DNA, proteins, and lipids[11]. This study aimed to investigate whether combined of vitamin C and E able to inhibit decreasing angiogenesis, endometrial thickness, and α -estrogen receptor level in female rats receiving orally MSG-treatment.

2. Material and methods

2.1. Animal

Twenty five adult female Wistar rats weighing 150–200 g obtained from the Laboratory Pharmacology Brawijaya University were randomly divided into the following five group: control group; MSG group non treated(140/200 gram body weight); MSG group treated by combined vitamin C (0.2 mg/g BW) and vitamin E (0.04 IU/g bw); MSG group treated by combined vitamin C (0.4 mg/g BW) and vitamin E (0.04 IU/g bw); and MSG group treated by combined vitamin C (0.8 mg/g BW) and vitamin E (0.04 IU/g bw). The rats were kept in a room with a 12-h light/dark cycle at 22 °C and were provided access to food and water ad libitum. This study was done in the Pharmacological, Biomedical, Pathological Anatomical Laboratories, Medical Faculty of Brawijaya University, Malang, East Java, Indonesia.

2.2. Ethics

All experimental procedures were compliant with the Medical Faculty Brawijaya University Committee Guidelines on the Use of Live Animals in Research, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. MSG treatment

The powder of MSG was dissolved with aquades 1 mL then treatment by gavage into rats at 10 a.m every day for 42 days according previous study[12].

2.4. Vitamin C and E treatment

Vitamin C was dissolved with aquades 0.5 mL, but vitamin E was dissolved with sesame oil 0.5 mL. All these substances were orally treatment by gavage into rats at 10 a.m every day for 42 days.

2.5. Determination estrous cycle

The estrous cycle was determined to know the execution time of experimental animals. Cotton buds, cover glass, glass objects, Giemsa and the microscope were prepared for vaginal swap. Put cotton buds soaked with 0.9% physiological saline into the vaginal opening and rotate 360° to obtain vaginal discharge, and then put the vaginal discharge on glass objects, dried and then soaked in methanol 9% for 10 minutes. It was then stained with Giemsa for 30 minutes. After stained with Giemsa, it was then washed in running water and dried, then observe using a microscope with a magnification 40 times. Results of a vaginal swap for phase determination of white rats were based on the presence of and quantity of vaginal epithelial cells[13].

2.6. Hematoxylin–eosin (HE) staining

Histopathological profile of uterine tissues was analyzed using HE staining. Sample was deparaffinized using xylene and dehydrated using alcohol. Slides were then washed with running water for 15 minutes and soaked in a hematoxylin solution for 10–15 minutes. Slides were then washed with running water for 15 minutes and dipped in acid alcohol 1%. The slides were then dipped in liquid ammonia and stained with eosin 1% for 10–15 minutes. Slides were dehydrated again with alcohol series (80%, 90%, and absolute alcohol) each for 3 minutes. Slides were then soaked with xylene for \pm 2 hours and then dried. The results of staining were observed with a microscope XC 10 (Olympus); endometrial tissues were observed to calculate the amount of branches of endometrial spiral arteries and thickness. The thickness of the endometrium was determined by calculating the average thickness of the endometrium with the highest and lowest sizes at each incision (totally 10 locations) for sample using Dot Slide camera.

2.7. Analysis of uterus α -estrogen receptor

Analysis of uterus α -estrogen receptor was done with Rabbit Anti Phospoestrogen Receptor Alpha (SER 104+SER 106) Polyclonal Antibody PE Conjugated (bs-3131R-PE) (Bioss, USA) using flowcytometer BD FACSCalibur and software cell quest pro software.

2.8. Immunohistochemical (IHC) staining

IHC staining was carried out to see the expression of VEGF protein in endometrial tissues. Slides were deparaffinized using xylene and dehydrated using alcohol series. The slides were immersed in citrate buffer of pH 6 and heated in a temperature of 95 °C for 20 minutes. After slides were

blocked using H₂O₂ 3% in methanol for 15 min (endogenous blocking), they were then washed with PBS and blocked back with sniper and incubated for 60 min. Furthermore, the primary antibody (VEGF) was added in BSA 0.2 % and incubated overnight in 4 °C. Once the slides were washed with PBS, they were then incubated with biotinylated universal secondary antibody for 60 minutes at room temperature. Incubation of the enzyme SA–HRP (Streptavidin Horseradish Peroxidase) was performed for 40 minutes at room temperature, and then DAB (Diaminobenzidine) was added with ratio of DAB chromagen: DAB buffer = 1:50 for 10–20 minutes. After the slides were washed with PBS and distilled water, they were counterstained with Mayer's Hematoxin for 5–10 minutes at room temperature. The slides were mounted and observed with a microscope at 400x magnification to count the number of cells expressing VEGF.

2.9. Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using 1–way ANOVA with SPSS 15.0 statistical package. Post Hoc test was used if the ANOVA was significant. $P < 0.05$ was considered statistically significant.

3. Results

Table 1 shows the VEGF expression in all groups. The

expression of VEGF were significantly lower in MSG–treatment group compared to control group ($P < 0.05$). Second and third dose of combined vitamin C and E significantly increased VEGF expression compared to MSG–treatment group ($P < 0.05$), to reach similar expression in the control group ($P > 0.05$).

The exposure of MSG to rat endometrium affected the arteriole number, as shown in Table 1. There were significantly ($P < 0.05$) decreased the arteriole number in groups exposed to MSG compared to non–treatment group. The administration of combined vitamin C and E (second and third dose) significantly ($P < 0.05$) increased the the arteriole number compared to the MSG–exposed groups, to achieve the level in control group ($P > 0.05$).

MSG–treatment lowered the thickness of endometrium significantly compared with no exposure ($P < 0.05$). The combined of vitamin C and E increased the thickness of endometrium, reaching the level in the control group ($P > 0.05$), as seen in Table 1.

Table 1 shows the expression of α –estrogen receptor in all groups. Treatment to MSG could significantly reduce the expression of α –estrogen receptor compared to the control ($P < 0.05$). Administration of vitamin C and E significantly increased the expression of α –estrogen receptor compared to MSG–treatment group ($P < 0.05$), reaching the number in the control group ($P > 0.05$).

Table 1

Expression of VEGF, number of arteriole, endometrial thickness, and alpha–ER expression in MSG–treatment groups and control female rats.

Parameters	Control	Treatment groups			
		MSG	MSG + CE1	MSG + CE2	MSG + CE3
VEGF (%)	64.64 ± 2.66	37.97 ± 5.20 ^a	38.30 ± 3.82 ^a	66.85 ± 4.50 ^{bc}	70.43 ± 2.45 ^{ab}
Number of arteriole	25.60 ± 2.88	18.80 ± 2.68 ^a	22.60 ± 2.70	28.40 ± 3.50 ^{bc}	29.60 ± 1.94 ^{bc}
Thickness (μm)	633.30 ± 70.50	555.54 ± 88.58 ^a	678.15 ± 105.22 ^b	696.55 ± 60.31 ^b	754.57 ± 105.07 ^{ab}
α –ER expression	33.51 ± 3.31	27.68 ± 2.89 ^a	33.35 ± 4.43 ^b	35.20 ± 3.53 ^b	35.46 ± 1.93 ^b

Note: values are presented as mean ± SD; ^a $P < 0.05$; in comparison with control (non exposure) group; ^b $P < 0.05$; in comparison with MSG–treatment groups; ^c $P < 0.05$; in comparison with first combined dose groups; ^d $P < 0.05$; in comparison with second combined dose groups; MSG: monosodium glutamate; VEGF: vascular endothelial growth factor. α –ER: α –estrogen receptor.

4. Discussion

In this study, the level of VEGF were significantly lower in MSG–treatment group compared to control group. VEGF is a potential growth factor required in the process of endometrial proliferation, as well as plays a role in inducing increased permeability and angiogenesis in endometrial spiral arterioles^[14]. In endometrial tissues, an increase in the number of spiral arterioles is associated with the increased expression of VEGF as a critical factor in the process of angiogenesis. Second and third dose of combined vitamin C and E significantly increased VEGF expression compared to MSG–treatment group, to reach similar expression in control group. Increased expression of VEGF as a result of the

administration of vitamin C and E in rats treatment to MSG showed that the antioxidant these vitamins able to modulate the angiogenic activity of VEGF. In the proliferative phase, the blood vessels will undergo angiogenic process through a formation of spiral arterioles. VEGF and all its receptors are known to be essential components in the angiogenic process and function of blood vessels in endometrium^[15, 16].

In addition, treatment to MSG could significantly reduce the expression of α –estrogen receptor than that the control group. Estrogen can act through both membrane associated α –estrogen receptor and a structurally unrelated, integral membrane, G–protein coupled estrogen receptor, GPR30, to stimulate one or more cytoplasmic signalling cascades in response to oestrogen. The effects of signalling via GPR30 in the endometrium are unclear, but there is a profound cyclic regulation of this receptor^[17]. α –estrogen receptor act at proliferative and secretion phase of both epithelial

and stroma endometrial[18]. This finding indicated that MSG inhibit α -estrogen receptor in endometrium that confirmed by reduced endometrial thickness. Combined of vitamin C and E significantly increased the expression of α -estrogen receptor compared to MSG-treatment group, reaching the number in the control group. This finding indicate that vitamin C and E able to modulate upregulation α -estrogen receptor to increased endometrial thickness.

In this study, MSG-treatment also lowered the thickness of endometrium significantly compared with no exposure. The combined of vitamin C and E increased the thickness of endometrium, reaching the level in the control group, as seen in Table 1. This finding indicated that MSG inhibit endometrial proliferation that consistent with previous studies found that the injection of monosodium glutamate (4 mg/g b.w.) to rats resulted in a decrease the thickness of endometrial[19]. Bone marrow derived stem cells can specifically give rise to endometrial stromal and epithelial cells in both humans and mice. These bone marrow-derived stem cells likely contribute to both normal tissue homeostasis and repair[20]. Although not characterized in this study, we speculate that MSG treatment may also affect the physiology of stem cells in endometrium and this may be a novel mechanism underlying pathogenesis of multiple diseases associated with MSG toxicity.

In conclusion, the present data suggesting that combined vitamin C and E able to inhibit endometrial toxicity caused by MSG treatment via increasing angiogenesis and endometrial thickness, and modulating α -estrogen receptor.

Conflict of interest statement

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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References

- [1] U. S. Department of Health and Human Services, U. S. Food and Drug Administration. *FDA and Monosodium Glutamate (MSG)*. Rockville: FDA; 1995.
- [2] Samuels S. The toxicity/safety of MSG: a study in suppression of information. *Account Res* 1999; **6**(4):259–310.
- [3] Egbuonu A, Obidoa O, Ezeokonkwo C, Ezeanyika L, Ejikeme P. Hepatotoxic effects of low dose oral administration of monosodium glutamate in male albino rats. *Afr J Biotechnol* 2009; **8**: 3031–5.
- [4] Diniz Y, Fernando A, Campos K, Mani F, Ribas B, Novelli E. Toxicity of hyper caloric diet and monosodium glutamate: oxidative stress and metabolic shifting in hepatic tissue. *Food Chem Toxicol* 2004; **42**: 319–325.
- [5] Biodun D, Biodun A. Spice or poison? Is monosodium glutamate safe for human consumption. *Natl Concord* 1993; **4**: 5.
- [6] Geha R, Beiser A, Ren C, Patterson R, Grammer L, Ditto A, et al. Review of allergic reaction to monosodium glutamate and outcome of a multicenter double blind placebo-controlled study. *J Nutr* 2001; **130**: 1032S–1028S.
- [7] Mingels MJJ, Geels YP, Pijnenborg JMA, van der Wurff AA, van Tilborg AAG, van Ham MAPC, et al. Histopathologic assesment of the entire endometrium in asymptomatic women. *Hum Pathol* 2013; **44**: 2293–2301.
- [8] Kathryn BH Clancy. Reproductive ecology and the endometrium: physiology, variation, and new directions. *Yearb Phys Anthropol* 2009; **52**: 137–154.
- [9] Palan PR, Woodall AL, Anderson PS, Mikhail MS. Alpha-tocopherol and alpha-tocopheryl quinone levels in cervical intraepithelial neoplasia and cervical cancer. *Am J Obstet Gynecol* 2004; **190**(5): 1407–1410.
- [10] Cicek N, Eryilmaz OG, Sarikaya E, Gulerman C, Yasemin G. Vitamin E effect on controled ovarian stimulation of unexplained infertile women. *J Assist Reprod Genet* 2012; **29**: 325–8.
- [11] Konopacka M. Role of vitamin C in oxidative DNA damage. *Postepy Higieny i Medycyny Doswiadczalnej* 2004; **58**: 343–348.
- [12] Seiva FRF, Chuffa LGA, Braga CP, Amorim JPA, Fernandes AAH. Quercetin ameliorates glucose and lipid metabolism and improves antioxidant status in postnatally monosodium glutamate-induced metabolic alterations. *Food Chem Toxicol* 2012; **50**: 3556–3561.
- [13] Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. *Toxicol Pathol* 2008; **36**: 375–84.
- [14] Fonseca-Moutinho JA. Smoking and cervical cancer. *ISRN Obstet Gynaecol* 2011; Epub Article ID 847684.
- [15] Kazi AA, Koos RD. Estrogen-induced activation of hypoxia-inducible factor-1 α , vascular endothelial growth factor expression, and edema in the uterus are mediated by the phosphatidylinositol 3-kinase/akt pathway. *Endocrinol* 2007; **148**(5): 2363–74.
- [16] Moller B, Lindblom B, Olvsson M. Expression of the vascular endothelial growth factor B and C and their receptors in human endometrium during the menstrual cycle. *Acta Obstet Gynecol Scand* 2002; **81**: 817–24.
- [17] Plante BJ, Lessey BA, Taylor RN, Wang W, Bagchi MK, Yuan L, et al. G protein-coupled estrogen receptor (GPER) expression in normal and abnormal endometrium. *Reprod Sci* 2012; **19**: 684–93.
- [18] Young SL. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. *Reprod Biomed Online* 2013; **27**: 497–505.
- [19] Miskowiak B, Kesa B, Limanowski A, Pantayka M, Filipiak B. Long-term effect of neonatal (MSG) treatment on reproductive system of the level rats. *Folia Morphol (Warsz)* 1999; **58**(2): 105–113.
- [20] Du HL, Taylor HS. Contribution of bone marrow-derived stem cells to endometrium and endometriosis. *Stem Cells* 2007; **25**(8): 2082–2086.