The Anti-Inflammatory Effect of Octyl Gallate Through Inhibition of Nuclear Factor-κB (NF-κB) Pathway in Rat Endometriosis Model

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Abstract

Background: Endometriosis is a chronic inflammatory condition associated with an increased risk of epithelial ovarian cancer. Our previous studies found that the anti-inflammatory effect of octyl gallate in endometriosis cell culture was more effective than gallic acid and heptyl gallate. This study aimed to analyze the anti-inflammatory effect of octyl gallate in rat endometriosis model.

Methods: Thirty female Wistar rats were randomly divided into three groups. Group I was the sham-operated group, group II was the surgically-induced endometriosis group, whereas group III was the surgically-induced endometriosis group and each rat was administered with 20 mg of octyl gallate dissolved in 1 ml Na-CMC via oral gavage once a day for 30 days. When all rats were euthanized, the endometrial tissue from group I and last two groups were collected for further analysis. TNF-α levels were measured using Luminex, while non-phosphorylated NF-κB and COX-2 levels were analyzed using ELISA.

Results: The average of non-phosphorylated NF-κB levels in group III (4.970±0.971 pg/mgP) was significantly higher than group II (3.97±0.656 pg/mgP). Moreover, the proportion of rats with the high level of non-phosphorylated NF-κB in group III was 45.6% higher than group II (p<0.05). The proportion of rats with the high level of COX-2 in group III was 22.3% lower than group II (p<0.05). However, there was no significant difference in TNF-α levels between all groups.

Conclusion: The anti-inflammatory effect of octyl gallate may has effects in NF-κB activation and reduction of COX-2 levels in rat endometriosis model.

Keywords: Chronic inflammation, COX-2, Endometriosis, NF-κB, Octyl gallate.

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Introduction

Endometriosis is a gynecologic condition characterized by the formation of endometrial like tissue outside the uterine cavity. Endometriosis affects 5-10% of all women of reproductive age and causes several symptoms including chronic pelvic pain, dysmenorrhea, and dyspareunia. Endometriosis is associated with infertility and an increased risk of epithelial ovarian cancer (EOC) (1, 2). Chronic inflammation in the peritoneal cavity has an important role in endometriosis progression. Inflammatory mediators, such as IL-1β and TNF-α, will activate transcription factor NF-κB that creates a positive feedback loop to increase
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Various inflammatory mediators, including TNF-α, IL-1, IL-6, and IL-12 (3, 4). Inflammatory condition can cause the formation of endometrial cell adhesion sites due to tissue damage process. Moreover, the increase of NF-kB activation is associated with the upregulation of COX-2 levels, which plays an important role in Prostaglandin E2 (PGE2) synthesis (1, 5, 6). The upregulation of PGE2 levels can enhance estrogen hormone and Vascular endothelial growth factor (VEGF) induced-angiogenesis, along with apoptosis and lymphocyte proliferative inhibition (5). This NF-κB induced-chronic inflammation then promotes the development of endometrial cysts and causes more profound clinical signs.

The current treatment of endometriosis, including hypoestrogenic induction and analgesic administration, is known to be ineffective because the treatment is unable to remove existing endometriosis tissue and provides adverse effects on long-term administration. Meanwhile, the removal of endometriosis tissue through surgery is also not optimal because the recurrence rate after therapy still reaches 40-80% (1). This underlies the need to develop the other potential alternative treatment.

Octyl gallate is one of the gallic acid derivatives, widely contained in natural sources, such as green tea, grapes, pineapple, and strawberries. Gallic acid is known to have an antioxidant, anti-inflammatory, and proapoptotic effect in cancer cells (7). Our previous studies on endometriosis primary cells culture have proven octyl gallate was more effective compared to gallic acid and heptyl gallate in suppressing inflammatory regulation, particularly in the expression of NF-κB, IL-6, COX-2, mRNA and a noticeable decrease in endometriosis cell viability was observed as tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (8,10). In addition, octyl gallate was also shown to have a more stable affinity and stronger bond to inhibit NF-κB compared to the other compounds (9). However, in vivo experiments on the anti-inflammatory effect of octyl gallate in endometriosis have never been studied before.

This study aimed to analyze the anti-inflammatory effect of octyl gallate through the inhibitions of the NF-κB pathway in rat endometriosis model.

**Methods**

**Materials:** Octyl gallate 99% and Sodium carboxy methyl cellulose (Na-CMC) were purchased from Sigma-Aldrich (Singapore); Nuclear factor-kappa B (NF-kB) ELISA kit was purchased from My Biosource (MBS722386, USA) with range of assay: 0-1000 ng/ml; COX-2 ELISA kit was purchased from My BioSource (MBS725633, USA) with range of assay: 0-100 ng/ml; TNF-α Magnetic LumineX Multiplex Assay kit was purchased from R&D Systems (LXSARM-03, USA).

**Experimental design:** Thirty female Wistar rats, aged 7-8 weeks with an average weight of 150-200 g, were obtained from the Institute for Health Research and Development, Indonesia. All rats were housed with free access to food and water. The temperature was maintained at room temperature (23-25°C) in a simulated daylight condition (12 hr light: 12 hr dark). All protocols in this study were approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia, with ethical code 0661/UN2.F1/ETIK/2018. A week after the acclimatization, all rats were randomly divided into three groups, defined as the followings:

- **Group I:** Underwent laparotomy (Sham-operated group)
- **Group II:** Surgically-induced endometriosis administered with Na-CMC
- **Group III:** Surgically-induced endometriosis administered with octyl gallate dissolved in Na-CMC

**Induction of endometriosis:** Group II and III underwent autotransplantation technique to develop endometriosis tissue (Figure 1). The autotransplantation process was performed in rats during their estrous cycle, proven by a vaginal pap smear. Surgically-induced endometriosis method by autotransplantation was adopted from Fischer et al. (10) with minor modification. Rats were anesthetized with ketamine at dose 73 mg/Kg BW and xylazine at dose 8.8 mg/Kg BW. The left uterine horn was excised±0.5 mm length, placed in a petri dish containing 0.9% NaCl, then cut open. The cut-opened horn was attached on the right peritoneal wall near a vein using a 3-0 size chromic catgut thread with the endometrial tissue facing the peritoneal wall. On the other hand, group I underwent laparotomy without the autotransplantation of uterine tissue. Injection of antibiotics 100 mg/Kg BW was given intraperitoneally (IP) before the abdominal wall was closed. The abdominal wall was then sutured using 3-0 size chromic catgut thread and the outer skin was sutured using 3-0 size silk thread. For the next two days, rats were injected with antibiotics and evaluated for any sign of
illness. Two months after the endometriosis induction, the second laparotomy was performed in groups II and III to evaluate the formation of the endometrial cyst. The endometriosis lesions were previously confirmed through HE tissue staining (Figure 2).

Octyl gallate induction: Group II was administered with 1 ml of Na-CMC solution, while group III was administered with 20 mg of octyl gallate dissolved in 1 ml Na-CMC via oral gavage once a day for 30 days.

Tissue collection: All rats were sacrificed using high-dose ketamine. The endometrial tissues of the group I, along with endometriosis tissue of the group II and III were collected. Then, tissues were washed in 0.9% NaCl and immediately stored in PBS with a ratio of 100 mg/ml. Sample tissues were homogenized using a high-pressure sonication method, then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was separated and analyzed.

Inflammatory mediator measurement: Inflammatory mediators NF-κB and COX-2 were analyzed using ELISA kits. Pro-inflammatory mediators, TNF-α, were analyzed using Magnetic Luminex Multiplex Assay kit. Inflammatory mediator levels were divided by total protein concentration of each sample. Total protein levels were measured with spectrophotometry at 280 nm wavelength using the Warburg-Christian method.

Figure 1. Surgically-induced endometriosis (Autotransplantation). The autotransplantation was performed in rats during their estrous cycle, proven by a vaginal pap smear. Rats were anesthetized with ketamine at dose 73 mg/Kg BW and xylazine at dose 8.8 mg/Kg BW, then underwent a laparotomy. The left uterine horn was excised±0.5 mm long, placed in a petri dish containing 0.9% NaCl, then cut open. The cut-opened horn was attached on the right peritoneal wall near a vein using a 3-0 size silk thread with the endometrial tissue facing the peritoneal wall. The formation of the endometrial cyst was evaluated with second laparotomy 60 days after autotransplantation.

Figure 2. Hematoxylin-eosin (HE) staining of rat surgically-induced endometriosis (40x magnification). HE staining from our previous study proved that endometrial cyst induced with autotransplantation was truly an endometriosis tissue. The red arrow sign indicates the presence of the endometrial gland.

Statistical analysis: Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software version 23. The mean difference of non-phosphorylated NF-κB, COX-2, and TNF-α levels among three groups was analyzed using Analysis of Variance (ANOVA), while the different proportions of the low and high level of non-phosphorylated NF-κB levels (Cut off point=5.002 pg/mgP), COX-2 levels (Cut off point=16.151 ng/mgP), and TNF-α levels (Cut off point=75.888 pg/mgP) were analyzed using Fisher’s Exact Test. A value of p<0.05 was considered statistically significant.
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Results

Rat in group I of this study underwent laparotomy as a sham group, while group II and III underwent surgically-induced endometriosis using autotransplantation technique (Figure 1). This surgically-induced endometriosis method was proved to be able to develop endometriosis lesions, confirmed through HE tissue staining from our previous study (Figure 2).

The average of non-phosphorylated NF-κB levels in group I (5.827±0.909 pg/mgP) was significantly higher compared to group II (3.908±0.664 pg/mgP) (p<0.05), indicated the increase of NF-κB activation in rat endometriosis model. Oral octyl gallate induction significantly upregulated the non-phosphorylated NF-κB levels in group III (4.970±0.971 pg/mgP) compared to group II (p<0.05), indicated the decrease of NF-κB activation (Figure 3).

The decrease of non-phosphorylated NF-κB levels in group II was followed by a significant increase of COX-2 levels (19.285±7.575 ng/mgP) compared to group I (13.897±2.621 ng/mgP). Oral octyl gallate induction then reduced the average of COX-2 level in group III (14.721±4.940 ng/mgP) in comparison to group II (Figure 4).

Furthermore, the average of TNF-α levels was elevated in group II (73.564±23.936 pg/mgP) comparing to group I (58.909±14.188 pg/mgP), but the average levels among three groups were not significantly different (Figure 5).

The different proportions of non-phosphorylated NF-κB, COX-2, and TNF-α levels between the three groups were used for further analysis. Figure 6 shows that the proportion of rats with high level...
of non-phosphorylated NF-κB in group II was 80.9% lower than those in group I. On the other hand, the proportion of rats with high level of COX-2 was 56.7% higher. Oral octyl gallate intake caused the proportion of rats with the high level of NF-κB in group III to be 45.6% higher than group II, which led to the reduction of the proportion of rats with the high level of COX-2 in group III (44.4%) compared to group II (66.7%). However, there were no significant differences in TNF-α high category levels between the three groups.

**Discussion**

The NF-κB signaling pathway is the main transcription factor that plays a major role in many chronic inflammatory diseases, such as endometriosis (13). The enhancement of NF-κB activation in endometriosis leads to the maintenance and progression of endometriosis lesions, making NF-κB a potential drug target in endometriosis condition. The NF-κB proteins are normally sequestered in the cytoplasm with its inhibitory proteins called IκB family members. Pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 could induce the degradation of IκB, resulting in the nuclear translocation of NF-κB members (9, 13). Furthermore, binding of the members of NF-κB to DNA promoter would induce the expression of pro-inflammatory cytokines, intercellular adhesion molecules, and angiogenesis factors (11-13). Moreover, high activation of NF-κB in endometriosis then increases the synthesis of COX-2 (1, 2, 5, 14, 15). These theories are consistent with our result. In this study, the proportion of rats with the high level of TNF-α and COX-2 was increased in the surgically-induced endometriosis rats compared to the sham-operated rats. In addition, the proportion of rats with the high level of non-phosphorylated NF-κB was decreased, indicating that activation of NF-κB was elevated in endometriosis rat model.

The previous studies suggested that gallic acid and its derivative were able to inhibit the activation of NF-κB (7, 16, 17). This is consistent with our result indicating that octyl gallate was able to inhibit NF-κB translocation to the nucleus, illustrated through the significant increase of non-phosphorylated NF-κB levels in the rats which were administrated with octyl gallate compared to the surgically-induced endometriosis rats. Moreover, our previous study proved that octyl gallate had strong binding power and stable affinity with NF-κB, analyzed using in silico docking method. This binding inhibited the nuclear translocation of NF-κB and suppressed the transcription of pro-inflammatory genes.

Several studies by Wei et al. (18), Kim et al. (19), and Jiang et al. (20) proved that gallic acid induction was able to inhibit inflammatory reaction in rat model that exists in suppressing the inflammatory cytokines, including TNF-α levels. In addition, research by Mori et al. (21) also showed the effectiveness of octyl gallate combined with ferulic acid in reducing TNF-α levels in Alzheimer's mice model. Nevertheless, octyl gallate in this study did not appear to reduce TNF-α levels as it was evident by the average of TNF-α levels in the rats administered with octyl gallate which increased compared to the surgically-induced endometriosis rats. However, the increase was not statistically significant, so octyl gallate likely had no effect on TNF-α levels. TNF-α can act both as one of the activators and the result of the nuclear translocation of NF-κB. Therefore, the ineffectiveness of octyl gallate in suppressing TNF-α levels might happen due to the ability of octyl gallate in inhibiting the activation of NF-κB directly without affecting TNF-α level as its activator.

Despite its ineffectiveness in suppressing TNF-α levels, oral octyl gallate induction was able to reduce COX-2 levels, another inflammation mediator that plays an important role in the progression...
of endometriosis. This result was consistent with our previous study that showed the effectiveness of octyl gallate in reducing COX-2 levels in endometriosis primary cell cultures (9). In addition, a study by Das et al. (7) also demonstrated that octyl gallate was capable in reducing PGE\textsubscript{2} levels through the suppression of COX-2 synthesis. COX-2 is one of the enzymes that plays an important role in PGE\textsubscript{2} synthesis, so the decrease of COX-2 levels in endometriosis tissue can suppress PGE\textsubscript{2} synthesis. PGE\textsubscript{2} levels in patients with endometriosis are known to be quite high and trigger immune dysfunction. Consequently, immune cells are not able to eliminate endometrial cysts. Furthermore, the increase of PGE\textsubscript{2} can promote the development of endometrial cysts through apoptosis inhibition by increasing the anti-apoptotic protein Bcl2 and reducing the pro-apoptotic protein Bax (1, 5, 6). Based on this description, the decreased COX-2 levels in endometriosis induced by octyl gallate can ultimately play an important role in inhibiting the development of endometrial cysts.

The anti-inflammatory effect of gallic acid and its derivatives in rat model was able to decrease the expression of pro-inflammatory cytokines, including TNF-\textalpha, which led to the inhibition of phosphorylated NF-kB (18). However, our finding suggested that the anti-inflammatory mechanism of octyl gallate in rat endometriosis model involved the direct inhibition of NF-kB activation which led to suppression of COX-2 levels.

**Conclusion**

Oral administration of octyl gallate was able to reduce COX-2 levels of rat endometriosis tissues through the inhibition of NF-kB activation. However, this study showed that octyl gallate was ineffective in suppressing TNF-\textalpha levels. It might be related to the ability of octyl gallate that directly inhibits NF-kB without affecting TNF-\textalpha level. However, further studies are needed to prove this mechanism.

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**Conflict of Interest**

We declare there are no conflicts of interest in this research.

**References**

1. Sacco K, Portelli M, Pullaco J, Schembri-Wismayer P, Calleja-Agius J. The role of prostaglandin E2 in endometriosis. Gynecol Endocrinol. 2012;28(2):134-8.

2. Ahn SH, Monsanto SP, Miller C, Singh SS, Thomas R, Tayade C. Pathophysiology and immune dysfunction in endometriosis. Biomed Res Int. 2015:1-12.

3. De Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. Lancet. 2010;376(9742):730-8.

4. Stilley JA, Birt JA, Sharpe-Timms KL. Cellular and molecular basis for endometriosis-associated infertility. Cell Tissue Res. 2012;349(3):849-62.

5. Kalinski P. Regulation of immune response by prostaglandin E2. J Immunol. 2012;188(1):21-8.

6. Fujikawa M, Ibuki T, Matsumura K, Sawa T. Inflammatory hyperalgesia: the role of prostaglandin system in the spinal cord. Adv Neuroimmune Biol. 2012;3(2):197-207.

7. Das ND, Das A, Chai YG. The anti-oxidative and anti-inflammatory roles of gallic acid on transcriptional regulation. In: Thompson MA, Collins PB, editors. Handbook on gallic acid. New York: Nova Science Publishers, Inc.; 2013.

8. Bustami A, Dachniar H, Adyasa ZM, Nareswari SS, Sopiah P, Arsianti A, et al. The apoptotic effect of gallic acid and its derivatives on primary cultured endometriosis cells. Adv Sci Lett. 2017;23(7):6697-700.

9. Bustami A, Utami FS, Budiarti R, Wibowo H. Interleukin-1\beta and cyclooxygenase-2 proinflammation analysis and in silico docking nuclear factor kappa B on endometriosis cell culture given heptyl gallate and octyl gallate treatment. Asian J Pharm Clin Res. 2019;12(2):503-6.

10. Bustami A, Nareswari SS, Sopiah PP, Zoya MA, Ade A, Muharram R. Cell viability inhibition effect of gallic acid and its synthetic derivative forms on primary cultured endometriosis cells. Adv Sci Lett. 2017;23(7):6681-4.

11. Fischer OM, Kaufmann-Reiche U, Moeller C, Fuhrmann U. Effects of dienogest on surgically induced endometriosis in rats after repeated oral administration. Gynecol Obstet Invest. 2011;72(3):145-51.

12. Ockinghaus A, Ghosh S. The NF-kB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol. 2009;1(4):a00034.

13. Liu T, Zhang L, Joo D, Sun SC. NF-kB signalling in inflammation. Signal Transduct Target Ther. 2017;2, pii: 17023.
14. Kaponis A, Iwabe T, Taniguchi F, Ito M, Deura I, Decavalas G, et al. The role of NF-kappaB in endometriosis. Front Biosci (Schol Ed). 2012;4:1213-234.
15. McKinnon BD, Kocbek V, Nirgianakis K, Bersinger NA, Mueller MD. Kinase signalling pathways in endometriosis: potential targets for non-hormonal therapeutic. Hum Reprod Update. 2016;22(3):382-403.
16. Cho YJ, Lee SH, Park JW, Han M, Park MJ, Han SJ. Dysfunctional signaling underlying endometriosis: current state of knowledge. J Mol Endocrinol. 2018;60(3):R97-R113.
17. Yahfoufi N, Alsadi N, Jambi M, Matar C. The immunomodulatory and anti-inflammatory role of polyphenols. Nutrients. 2018;10(11). pii: E1618.
18. Wei G, Wu Y, Gao Q, Shen C, Chen Z, Wang K, et al. Gallic acid attenuates postoperative intra-abdominal adhesion by inhibiting inflammatory reaction in a rat model. Med Sci Monit. 2018;24:827-38.
19. Karimi-Khouzani O, Heidarian E, Amini SA. Anti-inflammatory and ameliorative effect of gallic acid on fluoxetine-induced oxidative stress and liver damage in rats. Pharmacol Reports. 2017;69(4):830-5.
20. Kim SH, Jun CD, Suk K, Choi BJ, Lim H, Park S, et al. Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. Toxicol Sci. 2006;91(1):123-31.
21. Jiang DX, Zhang MH, Zhang Q, Chen YS, Ma WJ, Wu WP, et al. Influence of gallic acid on porcine neutrophils phosphodiesterase 4, IL-6, TNF-α and rat arthritis model. J Integr Agr. 2015;14(4):758-64.
22. Mori T, Koyama N, Tan J, Segawa T, Maeda M, Town T. Combination therapy with octyl gallate and ferulic acid improves cognition and neurodegeneration in a transgenic mouse model of Alzheimer’s disease. J Biol Chem. 2017;292(27):11310-25.