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Effect of DLBS3233, Metformin, and Their Combination on the Expressions of VEGF and Endometriosis Implants in Endometriosis Mice (A Mouse Model in Endometriosis Study)

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ABSTRACT

Background: Endometriosis is a gynaecological disorder characterized by the presence of endometrial tissue outside the uterine cavity. The process of angiogenesis is regulated by VEGF, which plays an essential role in the development of endometriosis implants. Metformin is an insulin sensitizer that is known to have a beneficial effect in the treatment of endometriosis, and DLBS3233 is a PPARγ agonist; it is hoped that it can reduce VEGF and reduce endometrial implants.
Objective: To explore the effect of DLBS3233, metformin, and combination on VEGF expression and endometrial implant area of endometriosis-induced mice.
Methods: This experimental study used 3-month-old 28 BALB/c mice of endometriosis that were randomly and equally divided into four groups (K, P1, P2, and P3). On the 15th day, the K group was given a placebo, the P1 group was given DLBS3233 0.25 mg/day for 14 days, the P2 group was given metformin 4 mg/day for 14 days, and the P3 group was given a combination. The immunohistochemistry of VEGF expression was performed from the mice's abdominal cavity and pelvic peritoneal tissues and measured by the Remmele Scale Index. In contrast, the extracted mice's endometrial implants were analyzed using computer tracing. All data normality tests were calculated with the Shapiro-Wilk test. The mean difference test of all groups was analyzed using the one-way ANOVA test and the Kruskal-Wallis test.
Results: There were significant differences in the expressions of VEGF (p=0.005) and endometrial implants (p=0.001). Expression of VEGF in the P3 group was significantly lower compared to others, and the endometrial implant area in the P2 group was significantly lower compared to others.
Conclusion: DLBS3233 and Metformin may be a potentially effective drug treatment for endometriosis by decreasing VEGF expression and endometrial implants.

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1. Introduction

Endometriosis is a gynaecological disorder characterized by the growth of endometrial glands and stroma outside the uterine cavity. Endometriosis has severe clinical implications; there are increased infertility, chronic pelvic pain, and the risk of malignancy. The primary method for the diagnosis of endometriosis is using laparoscopy, with or without biopsy, for histological diagnosis.[1,2]

The overall prevalence of endometriosis in women of reproductive age is 3-10%. The cause of endometriosis is still unknown. Several theories of the cause of endometriosis that have been known are the theory of retrograde menstruation accompanied by endometrial cell transport, coelomic epithelial metaplasia, and vascular or lymphatic spread of endometrial cells. The combination of several theories is thought to be the cause of endometriosis. Increased oxidative stress will increase the production of free radicals or reduce antioxidant defences in the body, which is said to be related to the pathogenesis of this disease. Oxidative stress can increase angiogenesis and the growth and proliferation of endometriosis implant tissue in the peritoneal cavity. The most important angiogenic agent, vascular endothelial growth factor (VEGF), is known to be involved in the progression of endometriosis ectopic lesions. This is evidenced by much vascularization and VEGF receptor expression in endometriosis. More than 80% of blood vessels in endometriosis tissue are susceptible to the influence of this VEGF. [2,3,4]

Endometriosis is a progressive disease with high numbers of recurrences and requires long-term treatment. The management of endometriosis is broadly based on medical therapy and surgery. Medical therapy is designed to suppress estrogen and cause tissue atrophy of endometriosis implants so that endometriosis lesions are

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expected to regress. However, the administration of hormonal drugs can suppress ovulation and make it difficult to use together with infertility treatment. Surgical therapy is carried out to take the tumour mass directly and improve the anatomy of the reproductive organs to improve the function of the organs. However, surgery often results in a decrease in ovarian reserves, adhesions, and endometriosis recurrences.[5]

Metformin is a synthetic form of insulin. Metformin, as a biguanide insulin sensitizer, works to inhibit Metalloproteinase-2, repair tissue Superoxide dismutase (SOD), and decrease VEGF. It is known that metformin also has antioxidant, anti-inflammatory, and antiproliferative effects and works by activating Adenosine monophosphate-activated protein kinase (AMPK) and inhibiting Nuclear Factor Kappa-Beta (NF-KB).[6,7] The drug DLBS3233 is an extract of *Cinnamomum burmannii* and *Lagerstroemia speciosa*. DLBS3233 is an agonist of Peroxisome Proliferator-Activated Receptor γ (PPARγ) and Peroxisome Proliferator-Activated Receptor δ (PPARδ).[8] PPARγ works to reduce VEGF, inhibit NF-KB, and Akt.[9]

Metformin and DLBS3233 are currently used as treatment options for Polycystic Ovary Syndrome (PCOS). Metformin has now been used in cases of endometriosis that have contraindications to surgery and hormonal therapy. Research on DLBS3233 on endometriosis has yet to be done. Research conducted by Lebovic in 2007 using a thiazolidinediones (TZD) class, namely rosiglitazone, which is a PPARγ agonist, showed that the surface area of endometriosis lesions was significantly reduced in the experimental group compared to the control group. Treatment of the thiazolidinediones class has side effects, namely worsening or causing congestive heart failure, oedema, weight gain, anaemia, liver toxicity, decreased bone density, and increased risk of fractures.10 These things support the therapeutic potential of DLBS3233 in endometriosis, especially the natural drug content and minimal drug side effects. Treatment of metformin and DLBS3233 in endometriosis is expected to help with the regression of endometriosis lesions and to increase pregnancy rates in patients with endometriosis-related infertility.

2. Methods

This research is an experimental study on mice in Obstetrics and Gynecology. The research was conducted at the Faculty of Veterinary Medicine, Airlangga University, Surabaya. The research was carried out from December 2022 to March 2023. This research is a randomized actual experimental type with post-test only with a control group design. The treatment given was DLBS3233, metformin, and combination, while the outcome was VEGF expression and endometrial implant tissue area in endometriosis model mice. The experimental animals, namely female BALB/c mice aged approximately three months weighing 20–30 grams, were selected based on inclusion criteria. The study began with the acclimatization of BALB/c mice for one week. It was kept individually in a room with a temperature of ±260C and light-dark cycles. Mice were randomly divided into four groups, each consisting of 7 mice. The treatment was given together, and there were no dead or "drop-out" mice from the four groups.

3. Result

Effect of treatment on VEGF expression

Histopathological picture of VEGF expression from endometriosis peritoneal tissue of mice using immunohistochemical staining. Comparison of the expression of VEGF in the vascular endothelium of endometriosis tissue marked by the presence of brown chromogen

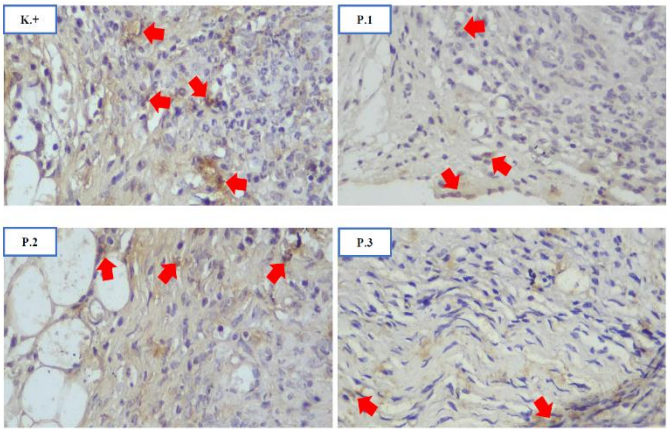

Figure 1 Comparison of peritoneal VEGF expression in groups (K, P1, P2, P3)

The data normality test used the Shapiro-Wilk test and obtained VEGF expression data in all groups usually distributed (p>0.05)

Table 1. Test the different expressions of VEGF in all groups

Group	Mean±SD	Median	(Min-Max)
K	3.686±0.452	3.50	3.20-4.40
P1	2.386±0.915	2.40	1.00-3.50
P2	2.857±1.047	2.70	1.40-4.80
P3	2.000±0.746	2.00	1.20-2.90

Based on the different tests of VEGF expression in all groups using the Oneway Annova test, p = 0.005 was obtained, indicating a significant difference in the mean of VEGF expression in all groups.

Subsequent analysis to determine differences in the mean between groups using the Post Hoc LSD test showed that the mean VEGF expression in group K differed from group P1 (p=0.007) and group P3 (p=0.001). There was no difference in the mean VEGF expression in the K group compared to the mean VEGF expression in the P2 group (p=0.071). There was no difference in VEGF expression between the P1 and P2 groups (p=0.293) and the P3 group (p=0.388). There was no difference in VEGF expression between the P2 and P3 groups (p=0.063).

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Effect of treatment on endometriosis implant area.

Comparison of the macroscopic appearance of implant lesions and hypervascularization of blood vessels in the peritoneal tissue of endometriosis model mice in various groups. The area of the implant lesion was measured using an image roster.

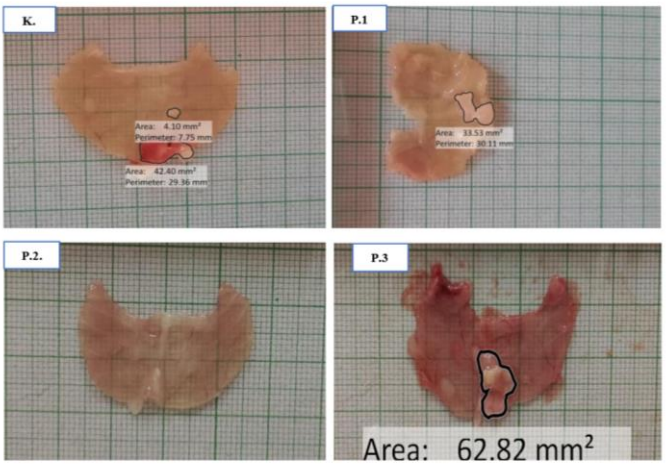

Figure 2 Comparison of group endometriosis implant areas (K, P1, P2, P3).

Data normality test using the Shapiro-Wilk test obtained endometriosis implant area data in groups K, P1, and P3 were not normally distributed ($p < 0.05$), for group P2 had the same value. Hence, the normality test results were absent. Hence, the following analysis used the Kruskal-Wallis test.

Based on the different tests of the average endometriosis implant area ratio in all groups using the Kruskal-Wallis test, the value of $p < 0.001$ ($p < 0.05$) was obtained, indicating that there was a significant difference in the average endometriosis implant area ratio in all groups.

Table 2. Difference test of endometriosis implant area across all groups

Group	Mean±SD	Median	(Min-Max)
K	38.492±38.343	40,79	4.27-116.68
P1	4.790±12.673	0	0-33.53
P2	0	0	0
P3	13.688±24.912	0	0-62.82

Subsequent analysis to find out the mean differences between groups using the Mann-Whitney test showed that the average endometriosis implant area ratio in group K was significantly different compared to the mean endometriosis implant area ratio in group P1 ($p = 0.004$), group P2 ($p = 0.001$) and the P3 group ($p = 0.043$). There was no difference between the implant area between the P1 group and the P2 group ($p=0.317$), and the P3 group ($p=0.534$). There was no difference in the implant area between the P2 and P3 groups ($p=0.143$).

Calculation of the treatment effect on regression of total endometriosis implants

Table 3. Treatment effect on implant regression across groups

Treatment	Implant regression	
	No Total	Total
DLBS3233	1	6
Metformin	0	7
Combination	2	5
Control	7	0

The value calculation number needed to treat (NNT) is sequentially at P1 (1.167), P2 (1.000), and P3 (1.400). Calculation of Absolute Risk Reduction (ARR) values sequentially at P1 (0.857), P2 (1.000), and P3 (0.714). Calculation of Relative Risk (RR) and Confidential Interval (CI) for each group, respectively P1 = 0.143 (0.023 – 0.877); P2 cannot be calculated; and P3 = 0.286 (0.089 – 0.922).

4. Discussion

In this pilot study, all 20 volunteers participated in this 2-month-long intervention without dropping out.

The mean expression of VEGF in endometriosis implant tissue in the peritoneum of endometriosis model mice that received a combination of metformin with DLBS3233 was lower compared to the other groups. Based on the different expression tests between groups, a significant comparison of VEGF expression was obtained only between the control group and the group given DLBS3233 and the combination. The results of this study are in line with previous research by Foda, which showed metformin administration caused a decrease in VEGF with an average of 179.96 (63.54 to 196.38) compared to before metformin administration, namely 292.6 (263.53 to 321.66).[6] Research conducted by Mulyantoro found that combining stem cells and metformin resulted in a significant decrease in implant area ($p = 0.001$), and a decrease in VEGF was insignificant.

VEGF is the most widely known angiogenic growth factor expressed in endometriotic lesions and released into the peritoneal fluid of patients with endometriosis. That is a potent, selective endothelial mitogen and survival factor.[12] Peroxisome proliferator-activated receptors (PPARs), namely PPARα and PPARγ, significantly inhibit cell migration VEGF-induced vascular endothelium.[13]

The result of implant area in this study showed a difference in the average ratio of endometriosis implant area in the group of mice given DLBS3233, metformin, and the combination compared to the control group. The administration of DLBS3233, metformin, and the combination can reduce the area of endometriosis implants in endometriosis model mice. This study's results align with previous studies conducted by Indrapraja on endometriosis model rats that were given metformin, and it was found that metformin significantly reduced endometriosis implants after 28 days of administration.[14] Another similar study was conducted by Lebovic using the drug pioglitazone (a PPAR γ agonist).[10]

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The effect of metformin on the endometriosis implant area is thought to be due to metformin's action on AMPK, inhibiting prostaglandins and inflammatory reactions. In rat models implanted with endometriosis, there was a regression of the implant with the administration of metformin. Metformin reduces the activity of the aromatase enzyme. It improves the hyperandrogenic environment by increasing levels of sex hormone-binding globulin (SHBG), including the epithelium and stroma, thereby reducing estradiol levels in the circulation and endometrium. Metformin reduces StAR expression stimulated by PGE2 by preventing the translocation of cAMP response element binding protein (CREB)-regulated transcription coactivator 2 (CRTC2) which is generally induced by PGE2 where metformin does this by increasing AMPK phosphorylation so that CREB-CRTC2 complexes do not form. This StAR, in the process of steroidogenesis, plays a very important role by providing a continuous supply of cholesterol for the production of estradiol. Star facilitates the entry of cholesterol into the mitochondria, where cholesterol is converted to pregnenolone, androstenedione is further converted to estrone and estradiol. As a result, the capacity of stromal cells to convert androstenedione to estrone, which depends on aromatase activity, decreases. DNA synthesis as a marker of proliferation also decreased, possibly due to the influence of metformin, which regulates and activates AMPK, which then inhibits the mTOR pathway. Then, androstenedione is further converted to estrone and then to estradiol. As a result, the capacity of stromal cells to convert androstenedione to estrone, which depends on aromatase activity, decreases. DNA synthesis as a marker of proliferation also decreased, possibly due to the influence of metformin, which regulates and activates AMPK, which then inhibits the mTOR pathway. Then, androstenedione is further converted to estrone and then to estradiol. As a result, the capacity of stromal cells to convert androstenedione to estrone, which depends on aromatase activity, decreases. DNA synthesis as a marker of proliferation also decreased, possibly due to the influence of metformin, which regulates and activates AMPK, which then inhibits the mTOR pathway.[7,11]

There is currently no research on the use of DLBS3233 in endometriosis. Another drug that has the same way of working as a PPAR-γ agonist is the drug Thiazolidinediones (TZD). The main goal of these two drugs is to treat DM disease by increasing insulin sensitivity, namely increasing the disposal of insulin-dependent glucose in muscle and adipose tissue and reducing hepatic glucose output. TZD drugs have side effects related to secondary weight gain and fluid retention, so their use in patients with cardiovascular problems is limited. PPAR-γ agonists initiate transcription, causing increased adiponectin levels to decrease the expression of leptin, resistin, visfatin, TNF-α, IL-6, and inhibit proliferation, suppress angiogenesis and promote apoptosis.[15,16,17]

The limitations of this study were not using multiple dose tests for each therapy and the shorter exposure time. This study also did not compare with therapies that are often used in the treatment of endometriosis, such as GnRH agonists as the drug of first choice for the treatment of endometriosis, so it is not known whether the administration of DLBS3233 and metformin is as effective for the treatment of endometriosis. Nonetheless, the overall picture of this study shows that the administration of DLBS3233 and metformin has a good effect on VEGF and the size of endometriosis implants so that it can be studied further to be used as an alternative therapy for endometriosis. Measuring estradiol concentrations is also very important in the management of endometriosis because some treatments for endometriosis result in hypoestrogenic conditions that cause anovulatory conditions that affect fertility. In studies using mice, it is challenging to measure adverse events. This research is a preclinical study, so it still requires several phases of clinical trials so that it can provide benefits to the community. Before proceeding to the clinical trial phase, new drugs must undergo a toxicity test. However, the drugs metformin and DLBS3233 have already passed that stage, so all that remains is to proceed to the clinical phase for use in the treatment of endometriosis.

5. Conclusion

VEGF expression in endometriosis implant tissue in the peritoneum of endometriosis model mice that received combination therapy was lower than those that received DLBS3233 and metformin. The tissue area of endometriosis implants in the peritoneum of endometriosis model mice that received metformin therapy was lower than those that received DLBS3233 and the combination.

Ethical Approval

The research was approved by the Health Research Ethics Committee, Faculty of Medicine, Diponegoro University – Dr. Kariadi and carried out by the principles of the Declaration of Helsinki.

Conflicts of Interest

The authors certify that they have no competing financial interests or personal relationships that could influence the work reported in this paper.

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Author Contributions

Conceptualization, SGW, YL, and SH; methodology, SGW, and YL; formal analysis, SGW; investigation, SGW; data curation, SGW; writing—original draft preparation, SGW, and YL; writing—review and editing, SGL, YL, IM, RDC and JD; visualization, SGW; supervision, IM, RDC and JD; funding acquisition, SGW.

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