

Research Article

Effect of Exercise, Metformin, and Their Combination on Ki-67 Expression and Endometriotic Implants in a Mouse Model of Endometriosis

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Abstract

Objective: To determine the effects of exercise, metformin, and their combination on Ki-67 expression and endometriotic implants in a mouse model of endometriosis.

Methods: This experimental study used 28 three-month-old BALB/c mice with induced endometriosis, which were randomly and equally divided into four groups (K, P1, P2, and P3). On day 15, group P1 received a swimming protocol (10 minutes/day) for 14 days, group P2 received metformin (4 mg/day) for 14 days, and group P3 received a combination of both treatments, while group K served as the control and received no treatment. Immunohistochemical analysis of Ki-67 expression was performed on peritoneal tissues from the abdominal and pelvic cavities and measured using the Remmele Scale Index. Endometriotic implant areas were analyzed using a computer-based tracing method. Statistical analysis was performed using SPSS version 32, with significance set at $p < 0.05$.

Results: There were significant differences in Ki-67 expression ($p = 0.002$) and endometriotic implant area ($p < 0.001$) among the groups. Ki-67 expression was significantly lower in the P1 group compared to the other groups, while the implant area was significantly lower in the P2 group compared to the other groups.

Conclusion: Exercise and metformin may be effective potential treatments for endometriosis by reducing Ki-67 expression and endometriotic implant size.

Keywords: Endometriosis; exercise; Ki-67; metformin

Pengaruh Olahraga, Metformin dan Kombinasi terhadap Ekspresi Ki-67 dan Implantasi Endometriosis pada Mencit Model Endometriosis

Abstrak

Tujuan: Penelitian ini bertujuan untuk menentukan pengaruh olahraga, metformin, dan kombinasinya terhadap ekspresi Ki-67 dan implan endometriotik pada model mencit endometriosis.

Metode: Penelitian ini menggunakan metode penelitian eksperimental dengan menggunakan 28 ekor mencit BALB/c berumur tiga bulan dengan endometriosis yang diinduksi, yang dibagi secara acak dan merata menjadi empat kelompok (K, P1, P2, dan P3). Pada hari ke-15, kelompok P1 diberikan protokol renang (10 menit/hari) selama 14 hari, kelompok P2 diberikan metformin (4 mg/hari) selama 14 hari, dan kelompok P3 diberikan kombinasi kedua perlakuan, sedangkan kelompok K sebagai kontrol tidak diberikan perlakuan. Analisis imunohistokimia ekspresi Ki-67 dilakukan pada jaringan peritoneum rongga abdomen dan pelvis, serta diukur menggunakan Indeks Skala Remmele. Luas implan endometriotik dianalisis menggunakan metode penelusuran berbasis komputer. Analisis statistik dilakukan menggunakan SPSS versi 32 dengan tingkat signifikansi $p < 0,05$.

Hasil: Hasil penelitian ini menunjukkan bahwa terdapat perbedaan bermakna pada ekspresi Ki-67 ($p = 0,002$) dan luas implan endometriotik ($p < 0,001$) antar kelompok. Ekspresi Ki-67 secara signifikan lebih rendah pada kelompok P1 dibandingkan kelompok lainnya, sedangkan luas implan secara signifikan lebih rendah pada kelompok P2 dibandingkan kelompok lainnya.

Kesimpulan: Olahraga dan metformin berpotensi menjadi terapi yang efektif untuk endometriosis dengan menurunkan ekspresi Ki-67 dan ukuran implan endometriotik.

Kata kunci: Endometriosis; Ki-67; metformin; olahraga

Introduction

Endometriosis is an inflammatory disease characterized by the growth of abnormal tissue resembling the endometrium, which triggers an inflammatory reaction.¹ It is an estrogen-dependent disease, as estrogen stimulates endometrial proliferation.² Endometriosis has significant clinical impacts, including increased infertility, chronic pelvic pain, and a higher risk of malignancy. The gold standard for diagnosing endometriosis is laparoscopy, with or without biopsy for histological confirmation.^{1,3}

The prevalence of endometriosis in women of reproductive age is approximately 10%, increasing to 35-50% in women with pain and/or infertility. Endometriosis reduces the quality of life in women and adolescents.¹ Management of endometriosis generally includes medical therapy and surgery. Medical therapy aims to suppress estrogen levels, leading to atrophy of endometriotic implants and regression of lesions without causing significant hypoestrogenic side effects. Various pharmacological agents have been used to treat endometriosis, including GnRH agonists, GnRH antagonists, progestagens, aromatase inhibitors, danazol, gestrinone, selective estrogen receptor modulators (SERMs), progesterone inhibitors, and selective progesterone receptor modulators.^{1,4}

Endometriosis involves an inflammatory response mediated by cytokines. Interleukin-6 (IL-6) and interleukin-8 (IL-8) in peritoneal fluid may play important roles in its pathogenesis. The secretion of IL-6 and IL-8 from endometriotic cells contributes to disease progression. Increased local estrogen production within endometriotic tissue also plays a crucial role in lesion growth, with aromatase enzyme activity detected in these lesions. An *in vitro* study demonstrated that metformin, an insulin sensitizer, suppresses inflammatory responses, aromatase activity, and proliferation of endometriotic stromal

cells. The mechanism of action of metformin in endometriosis includes reducing aromatase activity in endometriotic implants and suppressing ovarian steroidogenesis.⁵

Metformin also exhibits antioxidant, anti-inflammatory, and anti-proliferative effects by activating adenosine monophosphate-activated protein kinase (AMPK) and inhibiting nuclear factor kappa B (NF- κ B). These properties support its therapeutic potential in endometriosis by promoting regression of lesions and potentially improving pregnancy rates in endometriosis-related infertility.⁵

Symptoms associated with endometriosis arise from a local peritoneal inflammatory response caused by ectopic endometrial implants.⁶ Adhesion of ectopic tissue to the peritoneum is believed to occur via integrins and is associated with an imbalance between extracellular matrix-degrading enzymes, such as matrix metalloproteinases (MMPs), and their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). Following peritoneal invasion, the survival of endometriotic lesions is supported by local angiogenesis.⁷

Increased tumor mass, recurrence, and invasive phenotype of endometriotic lesions are associated with enhanced proliferative capacity of endometrial stem cells, both eutopic and ectopic, in women with endometriosis. This is characterized by increased expression of the Ki-67 antigen, a nuclear marker present only during active cell proliferation.⁸

These environmental alterations contribute to the development and persistence of inflammatory processes in endometriosis.⁹ Conversely, regular physical exercise has a protective effect against inflammatory diseases.^{6,10} Physical activity increases levels of hormone-binding globulins, thereby reducing estrogen bioavailability and benefiting estrogen-dependent conditions such as endometriosis.⁹ Exercise is also

associated with reduced insulin resistance, decreased menstrual flow, increased anti-inflammatory cytokines, and modulation of oxidative stress. Oxidative stress plays a key role in the pathophysiology of endometriosis, as reactive oxygen species (ROS) are elevated in the peritoneal fluid of affected women.^{9,11,12}

Exercise guidelines from major public health organizations recommend a combination of aerobic exercise (AE) and resistance exercise (RE). For example, the CDC and the American College of Sports Medicine (ACSM) recommend moderate-intensity aerobic exercise for 150 minutes per week or vigorous-intensity exercise for 75 minutes per week, combined with resistance training at least 2 days per week.¹³

Methods

This study was a randomized true experimental study with a post-test-only control group design. The interventions included exercise, metformin, and their combination, while the outcomes measured were Ki-67 expression and the area of endometriotic implant tissue in a mouse model of endometriosis. Ethical approval was obtained from the Health Research Ethics Committee, Faculty of Medicine, Diponegoro University (Number 122/EC/H/FK-UNDIP/X/2022).

Sampling was performed using simple random sampling. The study was conducted at the Faculty of Veterinary Medicine, Airlangga University, Surabaya, from November 2022 to February 2023. The exercise intervention consisted of swimming in a pool with a diameter of 60 cm and a height of 75 cm, with a water depth of 15 cm, for 10 minutes per day over 14 days. Metformin was administered in powder form dissolved in distilled water at a dose of 4 mg/day for 14 days.

The experimental animals were female BALB/c mice aged approximately 3 months and weighing 20-30 grams, selected based on

inclusion criteria. Prior to the intervention, the mice underwent a one-week acclimatization period and were housed individually in a controlled environment at a temperature of 26 with a light-dark cycle.

The mice were randomly divided into four groups (K, P1, P2, and P3), with six mice in each group. Group P1 received the swimming protocol (10 minutes/day) for 14 days, group P2 received metformin (4 mg/day) for 14 days, and group P3 received a combination of both treatments. Group K served as the control and received no treatment. No mortality or dropouts occurred during the study period.

Data normality was assessed using the Shapiro-Wilk test. Differences between groups were analyzed using one-way ANOVA for normally distributed data and the Kruskal-Wallis test for non-normally distributed data. Statistical analysis was performed using SPSS version 32, with significance set at $p < 0.05$.

Results

Histopathological images of Ki-67 expression in peritoneal endometriotic tissue of mice were obtained using immunohistochemical staining. The comparison of Ki-67 expression in the vascular endothelium of endometriotic tissue was indicated by the presence of brown chromogen (Figure 1).

The normality of the data was assessed using the Shapiro-Wilk test, which showed that Ki-67 expression data in all groups were normally distributed ($p > 0.05$).

Based on the analysis of differences in Ki-67 expression among groups using the one-way ANOVA test, a p-value of 0.002 was obtained, indicating a significant difference in mean Ki-67 expression across all groups (Table 1). Subsequent post hoc analysis using the LSD test showed that the mean Ki-67 expression in group K was significantly different compared to groups P1 ($p = 0.002$),

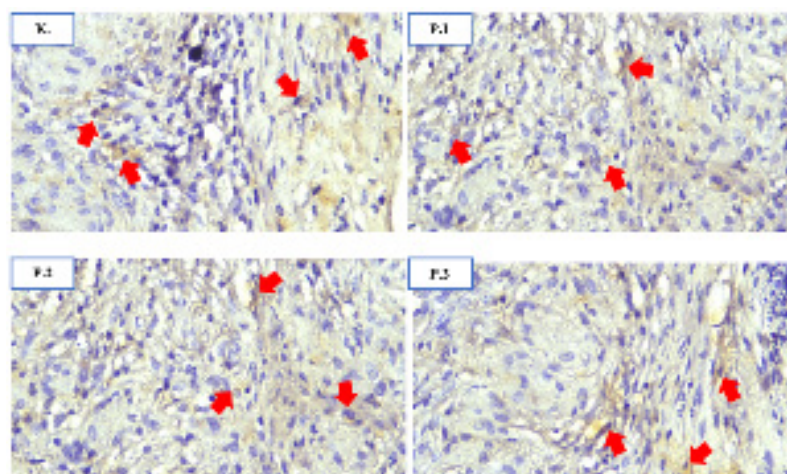


Figure 1 Comparison of Peritoneal Ki-67 Expression among Groups (K, P1, P2, P3)

Table 1 Comparison of Ki-67 Expression among All Groups

Group	N	Ki-67 Expression		P Value
		Mean	SD	
K	6	4.900	1.390	0.002
P1	6	2.067	0.816	
P2	6	2.300	1.064	
P3	6	2.967	1.329	

P2 ($p = 0.005$), and P3 ($p = 0.044$). There were no significant differences in Ki-67 expression between group P1 and group P2 ($p = 0.985$), or between group P1 and group P3 ($p = 0.556$). Additionally, no significant difference was observed between group P2 and group P3 ($p = 0.759$). Macroscopic comparison of implant lesions and hypervascularization in the peritoneal tissue of endometriosis model mice across groups was also performed. The area of the implant lesions was measured using an image analysis method (Figure 2).

Normality testing showed that the data on endometriotic implant area in group K were not normally distributed ($p < 0.05$), whereas those in groups P1, P2, and P3 were normally distributed ($p > 0.05$). Therefore, further analysis was performed using the Kruskal-Wallis test.

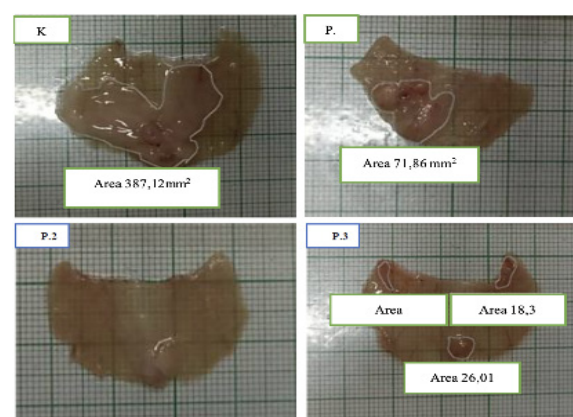


Figure 2 Comparison of Endometriotic Implant Areas among Groups (K, P1, P2, P3)

Based on the analysis of differences in endometriotic implant area among groups using the Kruskal-Wallis test, a p -value of < 0.001 was obtained, indicating a significant difference in implant area across all groups. Subsequent pairwise comparisons using the

Table 2 Comparison of Endometriotic Implant Area among Groups

Group	N	Mean	SD	Min-Max
K	6	175.42	108.76	112.3-387.1
P1	6	70.49	14.40	51.9-90.5
P2	6	13.03	15.49	0-36.3
P3	6	36.67	22.53	0-64.6

Mann-Whitney test showed that the mean implant area in group K was significantly different compared to groups P1 ($p = 0.004$), P2 ($p = 0.004$), and P3 ($p = 0.004$). The mean implant area in group P1 was also significantly different compared to groups P2 ($p = 0.004$) and P3 ($p = 0.016$). However, there was no significant difference between groups P2 and P3 ($p = 0.061$) (Table 2).

Discussion

The mean Ki-67 expression in endometriotic implant tissue in the peritoneum of endometriosis model mice was lower in the exercise group compared to the other groups. Statistical analysis showed significant differences between the control group and all treatment groups. These findings are consistent with a study by Montenegro, et al.⁹, in which swimming at varying intensities (once, three times, and five times per week for five weeks) reduced MMP-9 expression and oxidative stress in mice, although the differences were not statistically significant compared to the control group. Similarly, previous research by Mulyantoro, et al.¹⁴ demonstrated that the combination of stem cells and metformin significantly reduced implant area and Ki-67 expression.

Ki-67 expression reflects the level of cellular proliferation in endometriotic tissue. Metformin affects endometriosis by suppressing the secretion of proinflammatory cytokines, including VEGF, IL-6, and IL-8, and by reducing Ki-67 expression. In addition to its anti-inflammatory effects, metformin also exhibits anti-estrogenic and

anti-proliferative properties on endometriotic stromal cells, supporting its potential as a therapeutic agent for endometriosis.¹⁵ Exercise also promotes cellular apoptosis and reduces MMP-9 levels, thereby decreasing endometriotic cell proliferation, migration, and differentiation, as well as reducing oxidative stress.^{16,17}

The results of this study also demonstrated significant differences in endometriotic implant area among groups receiving exercise, metformin, and combination therapy compared to the control group. These findings indicate that exercise, metformin, and their combination can reduce the size of endometriotic implants in a mouse model of endometriosis. This is consistent with previous findings by Indrapraja, et al.¹⁸, which showed that metformin significantly reduced endometriotic implants after 28 days of administration.

The effect of metformin on reducing endometriotic implant size is thought to be mediated through activation of AMPK and inhibition of prostaglandin synthesis and inflammatory pathways. In animal models, metformin has been shown to induce regression of endometriotic implants. It also reduces aromatase activity and improves the hormonal environment by increasing levels of sex hormone-binding globulin (SHBG) in epithelial and stromal tissues, thereby lowering estradiol levels in circulation and within the endometrium. Furthermore, metformin decreases PGE2-stimulated StAR expression, which plays a crucial role in steroidogenesis by regulating cholesterol transport for estradiol production. DNA

synthesis, as a marker of proliferation, is also reduced, likely due to AMPK activation and subsequent inhibition of the mTOR pathway.^{14,19}

Exercise contributes to increased activation of adenosine monophosphate-activated protein kinase (AMPK), a key metabolic regulator, leading to decreased anabolic processes (e.g., lipid synthesis) and increased catabolic processes (e.g., lipid oxidation). Exercise also enhances cellular apoptosis, reduces MMP-9 levels, and decreases endometriotic cell proliferation, migration, and differentiation, as well as oxidative stress.^{16,20}

This study has several limitations. It did not evaluate different therapeutic doses or longer durations of exposure. Additionally, it did not compare the interventions with standard therapies for endometriosis, such as GnRH agonists, which are considered first-line treatments. Therefore, the relative effectiveness of exercise and metformin compared to established therapies remains unclear. The study also evaluated only one type of exercise (swimming), limiting the ability to determine the most effective form or intensity of physical activity for reducing Ki-67 expression and endometriotic implant area.

Despite these limitations, the findings suggest that exercise and metformin have beneficial effects on Ki-67 expression and endometriotic implant size. As this is a preclinical study, further clinical trials are required to confirm these results and assess their applicability in human populations.

Conclusions

The untreated group was significantly different from all treatment groups. The exercise group did not differ significantly from the metformin and combination groups in reducing Ki-67 expression. However, in terms of reducing endometriotic implant area,

the exercise group showed a smaller effect compared to the metformin and combination groups. No significant difference was observed between the metformin and combination groups. Overall, treatment with exercise, metformin, and their combination reduced Ki-67 expression and endometriotic implant area in the peritoneal tissue of a mouse model of endometriosis.

Conflicts of Interest

The authors declare no conflicts of interest.

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