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Changes in the M1/M2 ratio due to andrographolide therapy for deep endometriosis: An experimental study using a BALB/C endometriosis model

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ABSTRACT

Background: Andrographolide is a compound that serves as an anti-inflammatory agent. M2 macrophages promote the disease, while M1 macrophages inhibit endometriosis progression.

Aim: Andrographolide can modify the M1/M2 ratio, aligning with the pattern observed with standard treatment for deep endometriosis.

Methods: This experimental study used a post-test-only research design with a control group of 42 female Balb/C divided into 6 groups. Mice in group N (normal), normal control (KN) endometriosis (END) without therapy, while the positive control (KP) group received endometriosis + dienogest, group (P1) END and 0.05 mg/kg BW andrographolide (AND), group (P2) END + 0.1 mg/kg BW AND, group (P3) (END + 0.2 mg/kg BW AND. CD86 (M1) macrophages, while CD163 (M2) macrophages examine the number of M1 and M2 cells via flow cytometry in a specific color.

Results: There was a significant difference in M1, which was higher than M2 in the AND group, compared with KP. The number of M2 macrophages in the P2 group was significantly lower than that in the KN group. Similarly, the number of M2 macrophages in the P3 group was significantly lower than that in the KN group.

Conclusion: M1 was superior to M2 in the andrographolide group and exhibited a similar trend in the visanne group. This study serves as a preliminary investigation using experimental animals and can be further developed as a foundation for research on andrographolide as an alternative therapy for endometriosis.

Keywords: Andrographolide, Endometriosis, Peritoneal Fluid, M1/M2 ratio.

Introduction

Endometriosis, a chronic estrogen-dependent inflammatory disease characterized by the growth of endometrial-like tissue on the outer surface of the uterine cavity, affects 10% of women of reproductive age (Namazi *et al.*, 2021). In healthy conditions, increased macrophage infiltration in the endometrium primarily occurs during the late secretory phase and menstruation, suggesting that macrophages play a role in initiating endometrial detachment and regenerating the endometrial lining during menstruation. Previous studies have demonstrated that the innate immune system plays a significant role in the pathogenesis of ectopic lesions in endometriosis, including immune cells, chemokines, macrophages, natural killer (NK) cells, dendritic cells (DC), and neutrophils within the peritoneal immune system microenvironment (Akoum *et al.*, 2006; Giudice *et al.*, 2023; Reis *et al.*, 2024; Yao *et al.*, 2024) Macrophage grouping depends on the generating pathway; macrophages are divided into "classically or conventionally activated" (M1) or "alternatively activated" (M2) macrophages. M2 macrophages play a part in promoting the growth of endometriosis but not M1 macrophages. M2 macrophages encourage vascularization and the development of ectopic endometrial lesions in mice models, whereas M1 macrophages discourage the development of these lesions (Li *et al.*, 2021; Ochoa *et al.*, 2024)

An Indonesian medicinal plant known as Sambiloto can act as an immunomodulator. Sambiloto (*Andrographis paniculata* Nees) is widely recognized for its pharmacological effects, including anti-inflammatory properties. It contains deoxy andrographolide,

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andrographolide, 14-deoxy-11, neo-andrographolide, 12-didehydroandrografolide, homo-andrographolide, diterpenoid, and flavonoids. Andrographolide is a compound that serves as an immunomodulator, particularly as an immunostimulant, enhancing the immune system's function in humans. The role of andrographolide as an immunomodulator is to enhance the body's defense system, such as by boosting the activity of white blood cells to combat bacteria and other antigens, while flavonoids contribute to the anti-inflammatory response (Guan *et al.*, 2022; Li *et al.*, 2023).

In this study, we aimed to demonstrate that andrographolide can alter the M1/M2 ratio, matching the pattern of the M1/M2 ratio seen with visanne as a standard therapy for deep endometriosis.

Material and Methods

Study design and participants

This experimental study used a post-test with the control group research design. A total of 42 female mice (Balb/C) weighing 20–30 g and 2–3 months old were obtained from the Animal Engineering Laboratory, Faculty of Veterinary Medicine, Airlangga University Surabaya, Indonesia.

After the mice underwent a process of adaptation when they were kept in the cage by receiving the same food for 1 week, cyclosporine A was injected into the mice at concentrations of as much as 0.2 cc/mouse. Cyclosporine injection is used to repress the immune status of mice to ease the growth of endometriosis implants in the peritoneal cavity. Then, the experimental animals were categorized into 6 groups, each group consisting of 7 mice according to Federer's formula with these experimental treatments: mice in group N (normal) without treatment, mice in the normal control group (KN) that were injected with endometrial cells, and mice in the positive control group (KP) that were injected with endometrial cells and received dienogest therapy at a dose of 0.005 mg per kg body weight; treatment group 1 (P1) included mice injected with endometrial cells and given andrographolide therapy at a dose of 0.05 mg per kg body weight; treatment group 2 (P2) consisted of mice injected with endometrial cells and given andrographolide therapy at a dose of 0.1 mg per kg body weight; and treatment group 3 (P3) involved mice injected with endometrial cells and treated with andrographolide therapy at a dose of 0.2 mg per kg body weight. Additionally, mice were intramuscularly injected with ethinyl estradiol at a dose of 0.2 µg per mouse in the thigh using a 1 ml disposable syringe. On the 15th day, the mice were dissected to determine the extent of endometriosis implantation within the peritoneum (Fig. 1).

Prior to euthanization, peritoneal fluid was collected in phosphate-buffered saline and centrifuged twice (2,500 rpm). The abdominal wall and peritoneum were then detached, after which the peritoneum was cut and stretched on millimeter paper and documented using photographs to fully observe the amount of endometriosis. The results were recorded using a data collection sheet and analyzed statistically. An anatomical pathology examination was next performed using the reddest peritoneal tissue, which was collected during preparation, and then preserved with 10% formalin. The peritoneum was examined using a Nikon H600L microscope equipped with a 300-megapixel DS Fi2 digital camera and Nikon image processing software (Nikon Corporation). The area of endometriosis implants was macroscopically evaluated for areas of hyperemia, which was confirmed by sampling the most hyperemic area for endometriosis lesions. The peritoneal fluid collection was performed after 14 days after the intraperitoneal injection of syngeneic endometrial tissue. After centrifuging the peritoneal fluid, CD 86 (105008, PE anti-mouse CD86 Antibody, 200 µg 1 Biolegend) was used as a marker for M1 macrophages, whereas CD 163 (156704, PE anti-mouse CD163 Antibody, 100 µg 1, Biolegend) was used as a marker for M2 macrophages.

After that, the macrophage fluid was measured by flow cytometry and processed with the PE anti-mouse CD86 Antibody kit from BioLegend® for M1 and PE anti-mouse CD163 Antibody from BioLegend® for M2. The percentage (ratio) of M1/M2 will appear in the form of a graph using flow cytometry and calculating specific color for M1 or M2. The data were tested for normality and showed a normal distribution; thus, a one-way ANOVA test followed by a post hoc test was conducted to analyze the differences. All calculations were performed using SPSS software, version 19.

Ethical approval

The medical ethics committee of the Medical Faculty of Diponegoro University approved this research (permission no. 108/EC/KEPK/FK-UNDIP/XI/2024) November 6, 2024.

Results

The study revealed the formation of endometriosis foci after day 14. Macroscopically, endometriosis appears on the peritoneum, which is pink in color and features reddish patches, thin-walled cysts infiltrated by blood vessels, and attached to the peritoneum. Andrographolide is a natural compound with anti-inflammatory properties that can modulate M1 and M2 macrophages, which are part of the innate immune system and can change their phenotype in response to stimuli. There are two types of macrophages: M1 macrophages, which are pro-inflammatory and release cytokines to help fight pathogens and inhibit tumor growth, and M2 macrophages, which are anti-inflammatory and produce cytokines that support tissue repair and tumor growth (Table 1).

The flow cytometry showed that in the peritoneal fluid from all research samples, the number of M1 CD86 macrophages in the peritoneal fluid of mice

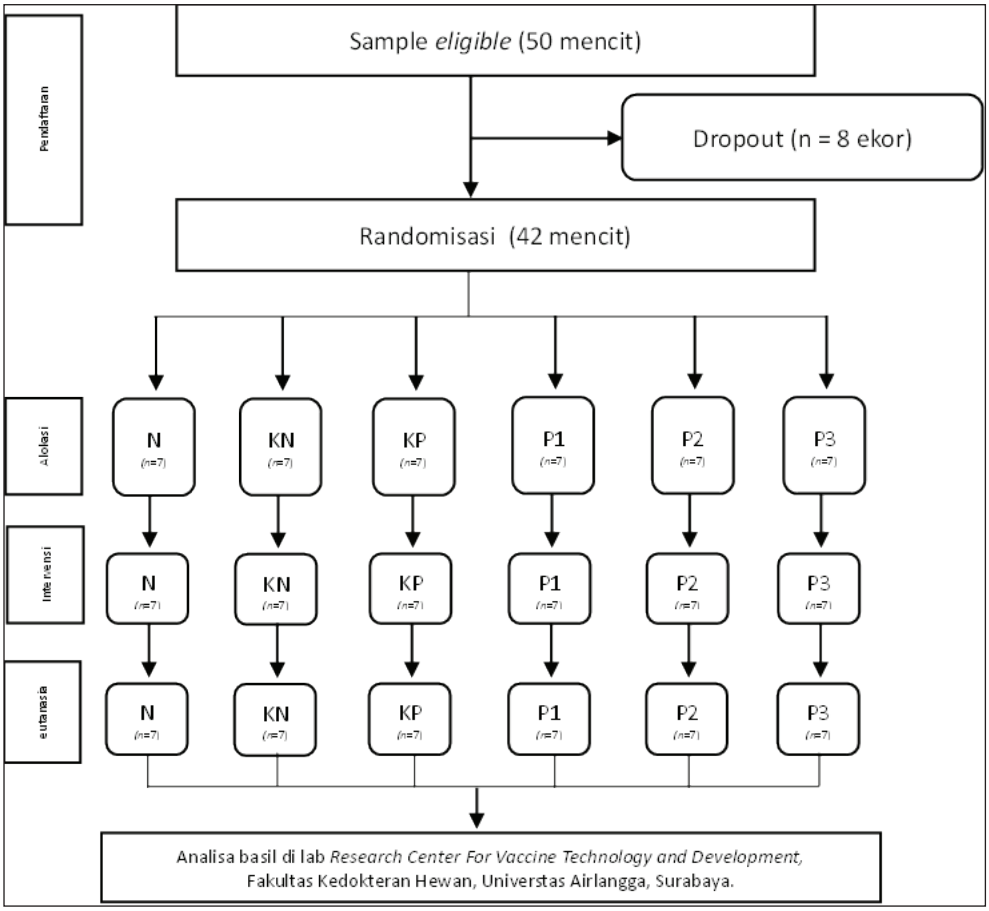

Fig. 1. Schematic illustration for allocation and research flow.

Table 1. Mean Number of M1 CD86 and M2 CD 163 macrophages in mouse peritoneal fluid in the research group ($n = 42$).

Group	M1 CD 86 (%)		M2 CD163 (%)	
	Mean	SD	Mean	SD
K Normal	61.2	5.11	35.5	4.31
K Negative	54.9	10.86	67.7	3.26
K Positive	61.5	14.41	58.6	2.31
P1	42.4	4.35	42.5	5.54
P2	80.0	4.42	60.5	2.24
P3	20.4	1.86	15.5	4.17

was highest in group P2, at 80.02% ± 4.423%, while the lowest was in group P3, at 20.42% ± 1.864%. The number of M2 CD163 macrophages in the peritoneal fluid of mice was highest in the negative control group (67.67%), while the lowest was observed in the P3 group (15.48%). The statistical test results indicated significant differences in the number of M2 CD163 macrophages in the peritoneal fluid between the study groups ($p < 0.001$; Kruskal–Wallis test). The number of M2 CD163 macrophages in the peritoneal fluid of the P1 group was significantly lower than that of the negative control group ($p = 0.002$). The statistical test results indicated a significant (S) difference in the number of M1 CD86 macrophages in the peritoneal fluid among the study groups ($p < 0.001$; Kruskall–Wallis test). The significance value of the intergroup comparison of M1 CD86 macrophages in the peritoneal fluid of mice was noted between the research groups. Endometriosis lesions in the P3 group exhibited a significantly higher M1/M2 ratio than those in the KN group and were almost equivalent to the KP group (Fig. 3).

The number of M1 CD86 (red) macrophages was more dominant than that of M2 CD163 (blue) in the group receiving andrographolide therapy compared to the positive group (Fig. 2). Additionally, the number of M2 CD163 macrophages in the P2 group was significantly lower than that of the negative control group ($p = 0.002$). Similarly, in the P3 group, the number of M2 CD163 macrophages was also significantly lower than that of the negative control group ($p = 0.002$) (Fig. 4).

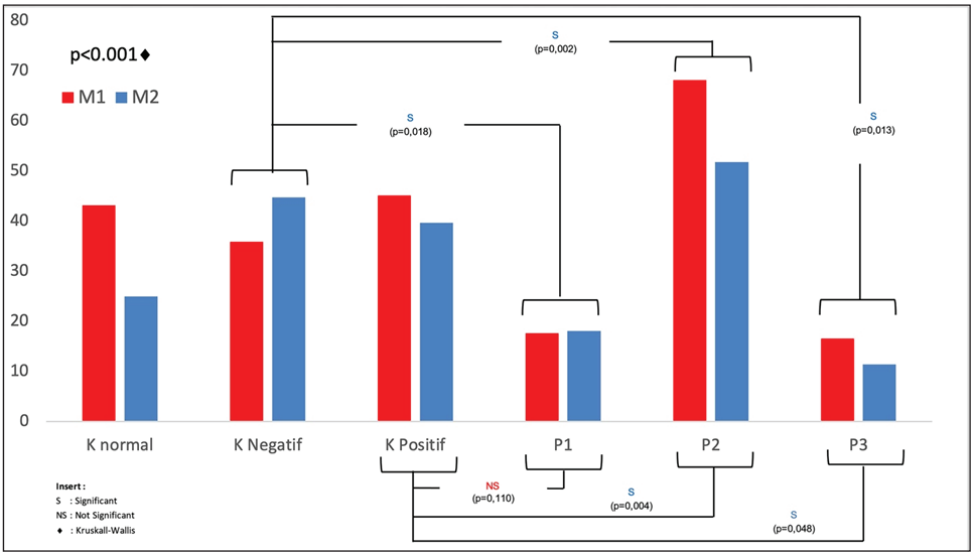

Fig. 2. The changing ratio of each group (N, KN, KP, P1, P2, and P3) shows that M1 (red) is superior to M2 (blue).

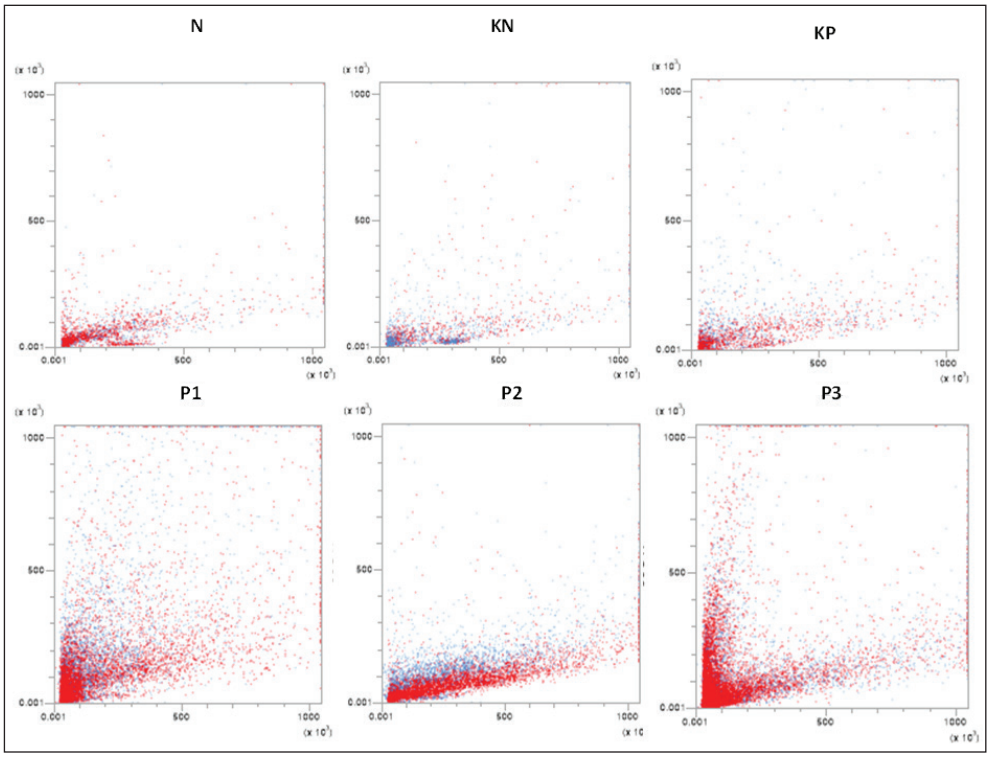

Fig. 3. Flow cytometry analysis of macrophages M1 CD86 (red) is higher than that of M2 CD 163 (blue) in the P1, P2, and P3 groups that received andrographolide compared with the negative control group.

Discussion

Ono *et al.* (2021) stated that fractalkine (FKN), which is secreted by eutopic endometrial stromal cells, increases IL-10 production and inhibits IL-12 production, inducing M2 polarization of macrophages, which is advantageous for endometriosis proliferation and invasion. (Ono *et al.*, 2021) In addition, Lu *et al.* (2021) found that Smad2/Smad3 upregulation occurred in macrophages exposed to eutopic and ectopic endometrial homogenates in women with endometriosis, supporting the hypothesis of M1 to M2 macrophage polarization via the Smad2/Smad3

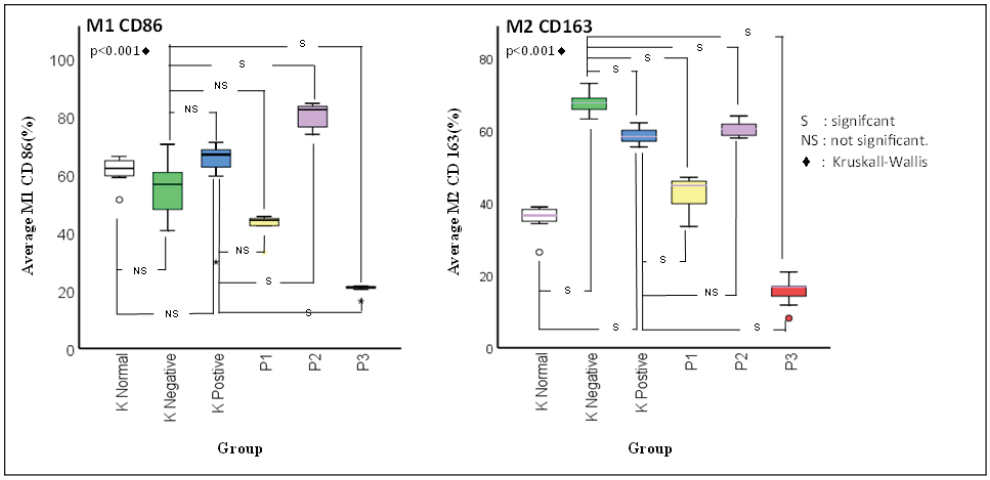

Fig. 4. Comparison of the number of M1 CD86 (left image) and M2 CD163 (right image) macrophages in the peritoneal fluid of mice in the study group (n = 42).

pathway. Li *et al.* (2021) argued that M1 macrophages were the most abundant macrophage population in the endometrium of endometriosis patients (Li *et al.*, 2021).

Furthermore, an understanding of the metabolic pathways linked to macrophage polarization, specifically glycolysis for M1 and oxidative phosphorylation for M2, opens new avenues for targeted therapies. By manipulating macrophage polarization or their metabolic pathways, researchers may develop novel strategies to manage endometriosis, potentially leading to improved patient outcomes. Thus, the implications of macrophage roles extend beyond mere observation; they challenge researchers to consider innovative therapeutic approaches that could alter the course of this debilitating condition (Moni and Uddin, 2018; Liang *et al.*, 2023; Jeljeli *et al.*, 2020).

M1 macrophages play a significant role in the pathogenesis of peritoneal endometriosis by increasing the levels of pro-inflammatory cytokines, which help suppress the development of chronic peritoneal inflammation. In contrast, M2 macrophages contribute to tumor growth and conditions characterized by tissue remodeling owing to their ability to regenerate endometriosis-related tissue (Li *et al.*, 2021; Lu *et al.*, 2021; Wong *et al.*, 2024). The macrophages present in the lesions represent a heterogeneous population of macrophages in the peritoneal fluid of endometriosis, causing an immune reaction. Some immune and inflammatory responses closely related to the occurrence of angiogenesis in endometriosis include the activities of TNF-a and COX-2 inducible hypoxia-inducible factor 1α, which cause extracellular matrix degradation and serve as key components of endometriosis adhesion angiogenesis (Lagana *et al.*, 2020; Calmon *et al.*, 2024; Wong *et al.*, 2024; Peng *et al.*, 2024; Quan *et al.*, 2024).

Andrographolide is the primary active component of the herb *A. paniculata* and is, known for its pharmacological activities, including immunosuppressant, antithrombotic, antiviral, antioxidant, and anti-inflammatory effects. As an anti-inflammatory and antioxidant, andrographolide promotes the expression of phosphoinositide 3-kinase, protein kinase B, and endothelial nitric oxide synthase polymorphism enzymes, which are inhibited by endothelial dysfunction. This process effectively suppresses the gene expression of interleukin 1β, interleukin 6, tumor necrosis factor-alpha, vascular endothelial growth factor, and tumor growth factor-α (Meresman *et al.*, 2021; Rajanna *et al.*, 2021; Jain and Sudandiradoss, 2023; Mudasir Ahmad *et al.*, 2024). Sambiloto (*A. paniculata* Nees) has been widely known to have pharmacological effects, one of which is as an anti-inflammatory (Jadhav and Karuppayil, 2021). One class of compounds in Sambiloto is flavonoids that inhibit the inflammatory process, a normal protective response to injury or tissue caused by physical trauma, damaging chemicals, or microbiological substances (Lotfizadeh *et al.*, 2020; Mishra *et al.*, 2021; Li *et al.*, 2021; Prasetyo *et al.*, 2021).

In conclusion, the findings of this study suggest that M1 macrophages in peritoneal fluid regulate environmental differentiation, indicating that the growth of endometriosis is more pronounced in the M2 phenotype due to the andrographolide group than in the normal group. These M1/M2 shifting ratios mirror those in the group using visanne as the standard medication. First, the activation of M1 macrophages indicates heterogeneity in the growth of endometriosis in the peritoneum, as deep endometriosis resulting from environmental stimuli shows variations in immune responses, which may arise from complex individual reactions to infectious agents and endogenous

alarm signals. Second, andrographolide may serve as a local inhibitor of the reparative action of M1 macrophage therapy in endometriosis. This study is a preliminary investigation using experimental animals and can provide a foundation for further research on andrographolide as an alternative therapy for endometriosis.

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Conflicts of interest

There are no conflicts of interest to declare.

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Author contributions

Indra Adi Susianto: conceptualization of data collection, data analysis, and writing. Neni Susilaningsih: data collection and analysis. Syarief Taufik Hidayat: conceptualization and writing. Banundari Rachmawati: conceptualization of data analysis and writing. All authors have read and approved the final manuscript.

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