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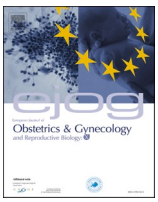

Sulawesi propolis induces higher apoptotic activity and lower inflammatory activity in a rat endometriosis model

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ABSTRACT

Background: Endometriosis has a major impact on women's quality of life. The two primary pathologies are chronic inflammation and altered apoptotic activity. Sulawesi propolis has been shown to have known anti-inflammatory and pro-apoptotic properties in other diseases.
Objective: To investigate the effects of Sulawesi propolis in the rat endometriosis model.
Methods: An autologous endometriosis model was created in 60 female Wistar rats by laparotomy. Rats were divided into four groups ($n = 15$ in each group): control group (CG), dienogest group (DG), propolis 50 mg/kg body weight (BW)/day (P50) group, and propolis 100 mg/kg BW/day (P100) group. Each treatment group was divided into three different treatment durations ($n = 5$ in each treatment group): 2, 4 and 6 weeks. After treatment, laparotomy was performed to determine endometriotic tissue growth, apoptosis [caspase-3 and Bcl-2-associated X/Bcl-2 (Bax/Bcl)] and inflammation [prostaglandin-E2 (PGE2) and interleukin-1B (IL-1B)].
Results: A significant difference was seen in endometriotic tissue growth between the P50 group and the CG, with the greatest reduction in the P50 6-week (P50–6) group, reaching 70.66% of the initial area. Highest Bax/Bcl-2 mRNA expression was shown in the P50–4 and P100–4 groups, highest caspase-3 expression was shown in the P50–2 and P50–4 groups, and lowest IL-1B expression was shown in the P50–4 group; all differed significantly from the CG. No significant difference in PGE2S mRNA was found between the groups.
Conclusion: Sulawesi propolis extract suppressed endometriotic tissue growth in the rat model by increasing apoptotic activity. The effects were time-dependent, with 50 mg/kg BW as the optimal dose.

1. Introduction

Endometriosis is a chronic inflammatory disease characterized by the presence of endometrium-like tissue outside the uterine cavity. It causes substantial morbidity, including chronic pelvic pain, multiple surgeries and infertility, leading to physical, mental and social impairment. This long-standing disease gives rise to a substantial economic burden and a decline in women's quality of life [1]. Sampson's retrograde menstrual theory supports the theory that endometriosis may originate from eutopic endometrium [2]. These ectopic endometrial-like tissues display similar characteristics, with a reduction in apoptotic activity, and increased cell proliferation and adhesion, enabling them to grow and survive [3]. Endometriotic tissue also shows changes in steroid hormone production, expressing more oestrogen receptor β than

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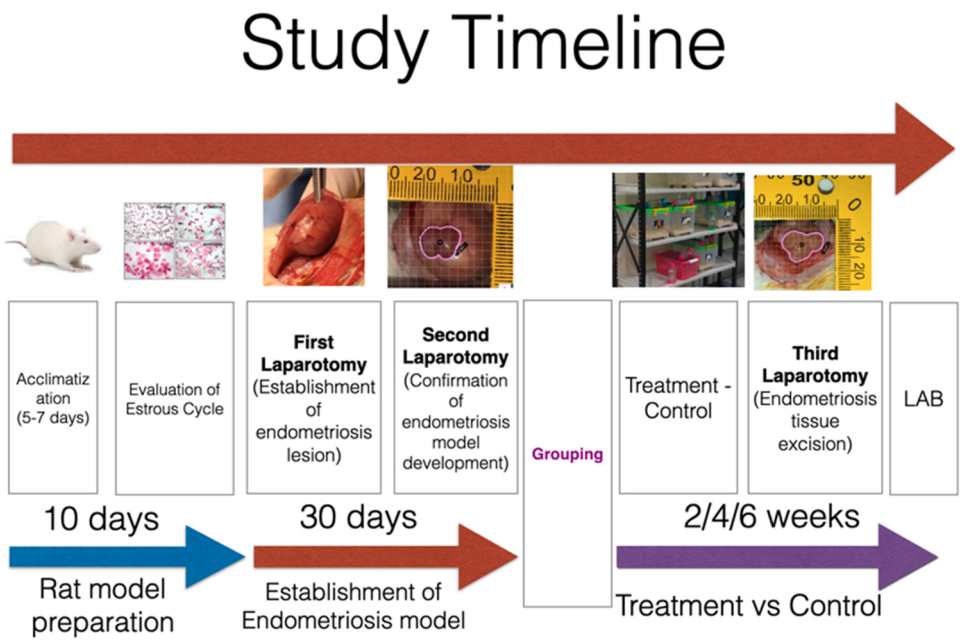

Fig. 1. Study timeline.

oestrogen receptor α, with its aromatase enzyme expressing fewer progesterone B receptors, which leads to oestradiol accumulation and progesterone resistance [4].

Endometriosis needs long-term management to alleviate pain and enhance fertility. Medical therapy mainly consists of hormone suppression therapy, in which the medication causes downregulation of the hypothalamo-pituitary-ovarian pathway. This long-term medication has several drawbacks, including high recurrence after treatment; side effects due to the hypo-oestrogenic state and analgesic-induced gastrointestinal problems; and the low chance of conceiving during medical treatment. Surgical therapy for endometriosis carries several risks for the patient, such as surgical complications, decreased ovarian and tubal function, and a high rate of recurrence.

Propolis – a mixture of various plants, beeswax and an enzyme in bee saliva – has been studied in various diseases and exerts many biological activities. It is an antimicrobial, anti-oxidant, anti-inflammatory, immunomodulatory and anti-apoptotic agent [5,6]. These biological properties are closely related to the pathogenesis of endometriosis, so propolis is a potential candidate for the treatment of endometriosis [7]. This study aimed to investigate the effect of Sulawesi propolis in a rat endometriosis model.

2. Materials and methods

2.1. Preparation of the rat endometriosis model

Six-week-old Wistar female rats obtained from the National Drug Agency and Food Control, weighing 150–300 g, were used to create an autologous endometriosis model. All rats were housed in a standard cage, five per cage, and were accommodated based on their innate physiological and behavioural needs. The endometriosis implant was established in accordance with do Amaral et al. [8], with modifications as follows: laparotomy was performed in the pro-oestrous or oestrous cycle; the endometrial side of the uterine segment was in contact with the peritoneum; and antibiotics were used to prevent infection. Rats were anaesthetized using ketamine 90 mg/kg body weight (BW) (PT. Dexa Medica, Tangerang, Indonesia) and xylazine 10 mg/kg BW (PT. Tekad Mandiri Citra, Bandung, Indonesia).

Development of an endometriosis model was confirmed 1 month after the first surgery. Successfully developed models were divided at random into four treatment groups ($n = 15$ in each group): control group (CG) – received water only; dienogest group (DG) – received dienogest 0.25 mg/day; P50 group – received propolis 50 mg/kg BW/day; and P100 group – received propolis 100 mg/kg BW/day. Each treatment group was divided into three different treatment durations ($n = 5$ in each treatment group): 2, 4 and 6 weeks. After completing treatment, a third laparotomy was performed to measure lesion size; mRNA expression of Bax/Bcl-2 and caspase-3 as apoptotic markers; and mRNA expression of prostaglandin E2 synthase (PGE2S) – a rate-limiting enzyme in PGE2 production – and interleukin 1B (IL-1B) as inflammatory markers. During treatment, all rats were examined for any alterations in the oestrous cycle. The study timeline is shown in Fig. 1.

2.2. Treatment groups

2.2.1. Propolis group

Propolis from *Tetragonula* spp. was obtained from Masamba, North Luwu district of South Sulawesi Province, Indonesia. The dosages of 50 and 100 mg/kg BW were chosen based on the study by Sahlan et al. [7]. The wax-free propolis preparation was extracted using the method of Sahlan et al. [7], provided by PT. RIN Biotek (Taman Tekno Bumi Serpong Damai, Banten, Indonesia; product batch number 19I19E13004). Liquid propolis 0.2–0.4 mL/day was administered orally with metal sound.

2.2.2. Dienogest group

Dienogest was selected as progestins have become the first-line treatment in Indonesian guidelines for the treatment of endometriosis [9]. Of the progestins, only dienogest and dydrogesterone have been studied in randomized controlled trials with placebo controls, and only dienogest has been proven to be safe for long-term use [10].

The DG received dienogest 1 mg/kg BW/day based on the study by Katayama et al. [11]. Dienogest 0.2–0.4 mL/day in 0.5% carboxymethylcellulose suspension was given orally with metal sound.

2.3. Evaluation of the oestrous cycle

Vaginal discharge of rats was obtained through vaginal lavage, fixed using 90% alcohol solution and stained using Giemsa. Specimens were examined microscopically to determine the oestrous cycle.

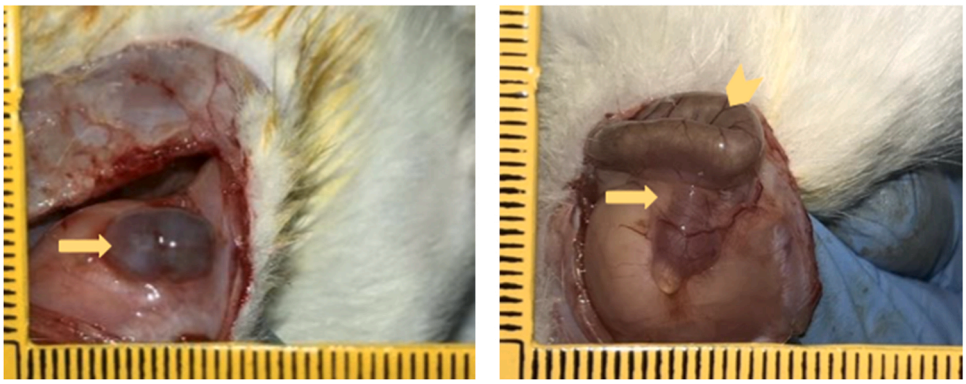

Fig. 2. Endometriotic lesion in rat models. The yellow arrow points to a smooth-surfaced, hyperaemic cystic mass surrounded by blood vessels. Bowel adhesion can also be seen (yellow arrowhead).

2.4. Measurement of lesion size

Following confirmation of development of the endometriosis model, the lesion formed was photographed to evaluate its macroscopic appearance. The lesion area was measured using SketchAndCalc software (iCalc Inc., Palm Coast, FL, USA).

2.5. Measurement of mRNA expression in endometriotic tissue

Expression of Bax/Bcl-2, caspase 3, IL-1B and PGE2S mRNA was assessed by isolating the RNA samples of endometriotic tissue based on the modified QIAamp RNA Blod Mini Handbook protocol. RNA spectrophotometric measurements and cDNA synthesis were performed based on the Instruction Manual ReverTra Ace qPCR RT Master Mix with gDNA Remover protocol (Toyobo, Osaka, Japan). On real-time polymerase chain reaction (PCR) examination, the melting temperature was determined to perform quantitative real-time PCR based on the QuantiTech SYBR Green PCR handbook protocol (Qiagen, Hilden, Germany) using a three-step cycling cycle. The results were compared with the standard curve and analysed.

2.6. Ethical consideration

All procedures were approved by the Ethical Committee of the Faculty of Medicine, University of Indonesia on 24 February 2020 (Ref. No. 227/UN2. F1/ETIK/PPM.00.02/2020).

2.7. Statistical analysis

Statistical analysis was undertaken using SPSS Version 25 (IBM Corp., Armonk, NY, USA). Data distribution was tested using Kolmogorov–Smirnov test. Analysis of variance was used to compare normally distributed data, and Kruskal–Wallis test and Mann–Whitney test were used to test non-normally distributed data. Comparison of changes in the

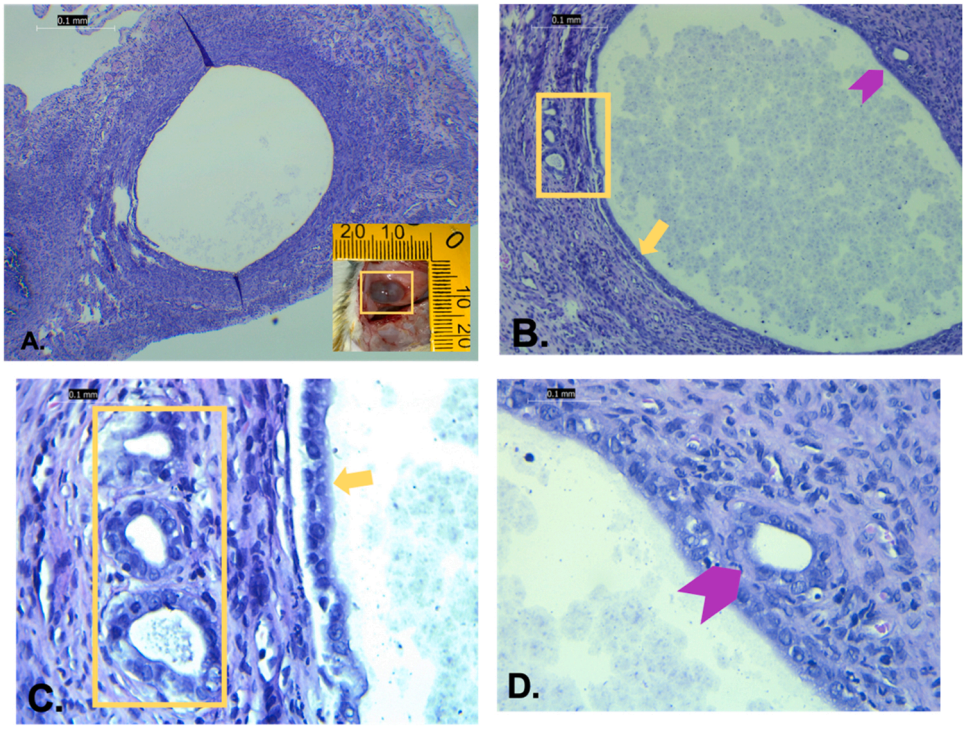

Fig. 3. Microscopic view of endometriotic tissue in rat model, haematoxylin and eosin staining. (A) 4 × 10 enlargement of endometriosis model. (B) A cystic mass filled with leukocytes and endometrial cells, with the cyst wall consisting of a layer of cuboidal epithelium (yellow arrow), 4 × 10 enlargement. (C, D) Cystic mass surrounded by endometrial stromal (yellow arrow) and epithelial-like structure (purple arrowhead), 40 × 10 enlargement.

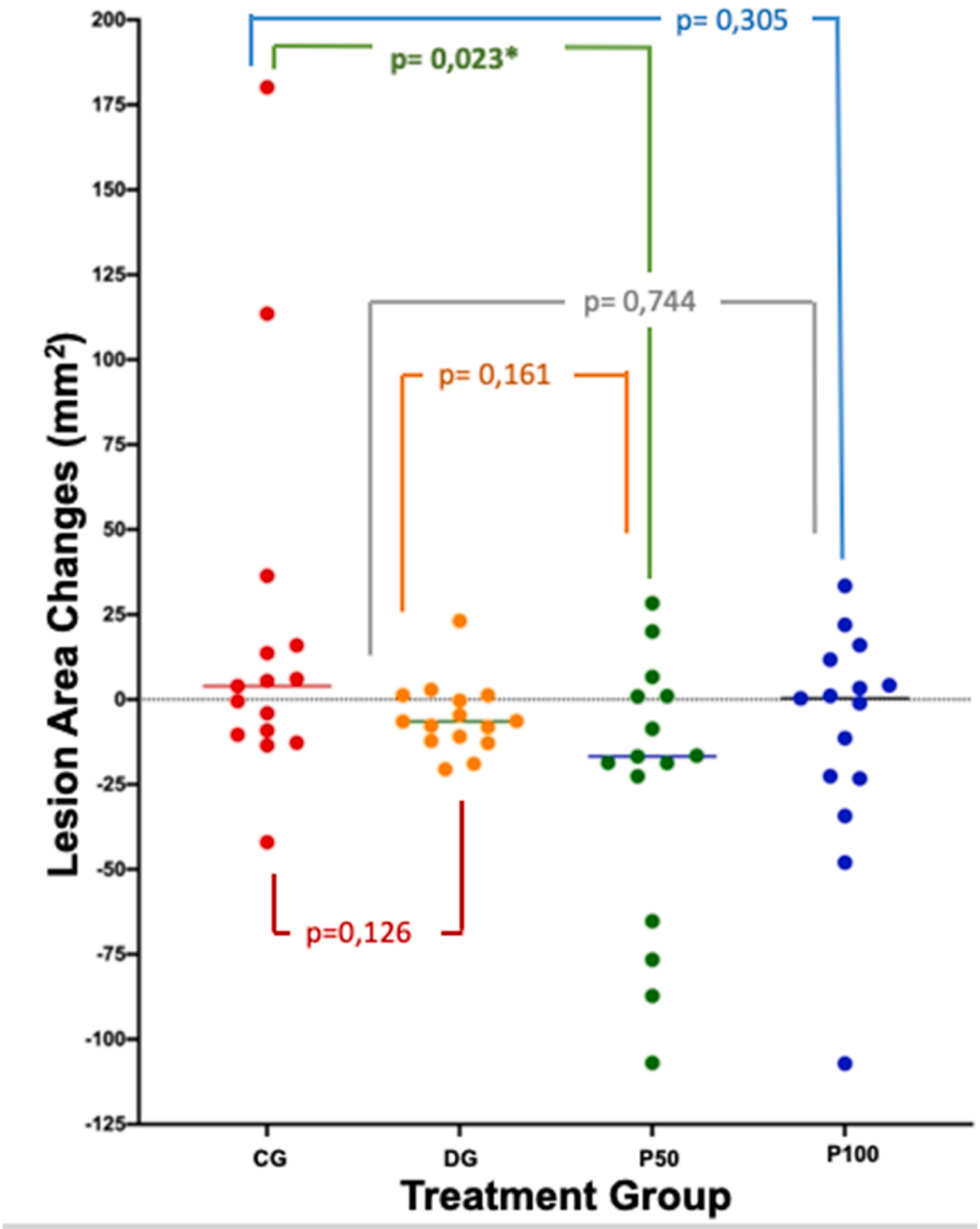

Fig. 4. Changes in lesion area for all treatment groups. CG, control group; DG, dienogest group; P50, propolis 50 mg/kg body weight (BW)/day; P100, propolis 100 mg/kg BW/day (Mann–Whitney test).

oestrous cycle between groups was tested using Chi-squared test, and $p \leq 0.05$ was considered to indicate significance. Change in endometriotic lesion area was assessed as comparison of the lesion area between the third laparotomy and the second laparotomy in the treatment groups and the CG. Inflammatory and apoptotic activity were assessed in comparison with Bax/Bcl-2, caspase-3, IL-1B and PGE2S expression in the CG.

3. Results

3.1. Development of endometriotic tissue in rat models

Endometriotic tissue was developed successfully in all 60 rat models, appearing as a smooth-surfaced, hyperaemic cystic mass surrounded by blood vessels. Intestinal and omental adhesion were common (Fig. 2).

Microscopically, endometriotic lesions consisted of a layer of cuboidal epithelium forming the cyst wall, surrounded by endometrial stromal and epithelial-like structures (Fig. 3).

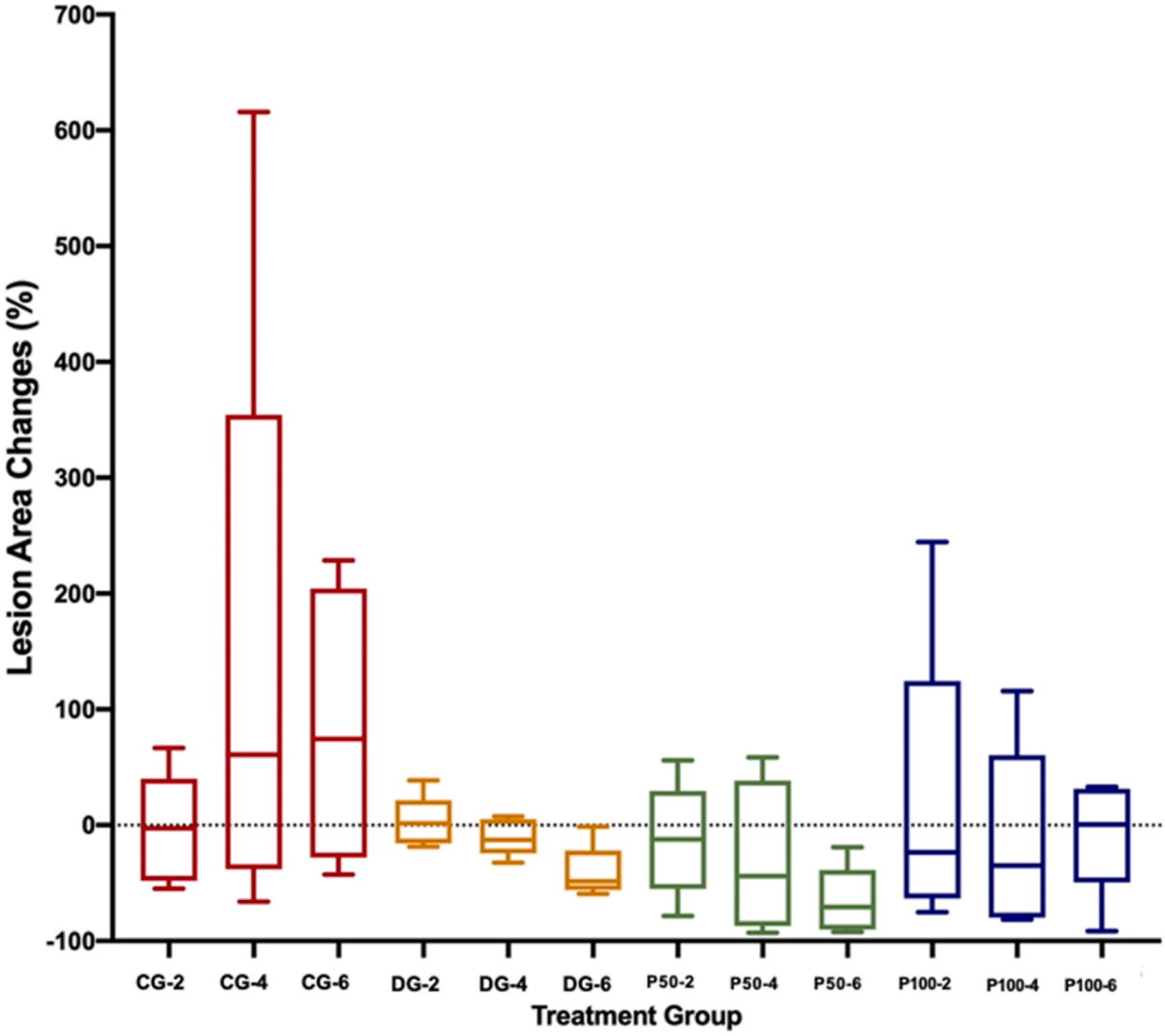

Fig. 5. Relative changes in lesion area for each treatment duration compared with the initial lesion area. CG, control group; DG, dienogest group; P50, propolis 50 mg/kg body weight (BW)/day; P100, propolis 100 mg/kg BW/day (Mann–Whitney test). The bars and tails represent the minimum, quartile (Q) 1, Q2 (median), Q3 and maximum value of each measurement.

3.2. Growth of endometriotic lesions

The absolute change in endometriotic lesion area for each rat can be seen in Fig. 4. Compared with the CG, lesion reduction was significantly greater in the P50 group ($p = 0.023$, Mann–Whitney). However, no significant difference was found between the DG and the P50 group, or the DG and the P100 group.

The relative change in endometriotic lesion area compared with the initial lesion area for each group (treatment and duration) is shown in Fig. 5. Lesion area decreased with longer treatment duration in the P50 group and the DG. However, in the CG, lesion area continued to increase with longer treatment duration. In the P100 group, there was a decrease in lesion area up to week 4, but the reduction was less in week 6. The greatest reduction in lesion area was seen in the P50 6-week (P50–6) group (−70.66% of the initial lesion area), while the greatest increase in lesion area was seen in the CG at 6 weeks (+74.32% of the initial lesion area). When comparing treatment durations, the P50–6 and DG-6 groups showed a significant reduction in lesion area compared with the CG group at 6 weeks (Fig. 5). However, no significant difference was found between the propolis groups and the DG.

3.3. Changes in apoptotic activity

mRNA expression of Bax/Bcl-2 and caspase-3 compared with the CG is portrayed in Figs. 6 and 7. For Bax/Bcl-2 expression, all groups revealed a similar pattern, with highest expression in the 4-week group and this reduced in the 6-week group. Highest Bax/Bcl-2 mRNA expression was shown in the P50–4 group, followed by the P100–4 group. Expression of Bax/Bcl-2 mRNA differed significantly in both the P50 and P100 groups compared with the CG.

The expression of caspase-3 mRNA in various treatment groups is shown in Fig. 7. Both the P50 and P100 groups showed a similar pattern: highest expression was seen in the 2-week groups and decreased gradually with time. The highest expression of caspase-3 was found in the P50–2 group (18.95 times CG), followed by P50–4 and P100–2 (9.93 and 4.97 times CG, respectively). Over 6 weeks, no increase in caspase-3 mRNA expression was seen in any of the groups. Compared with the CGs with the same duration, the P50–2 and P50–4 groups showed a significant difference. However, no significant difference was found between the propolis groups in comparison with the DG.

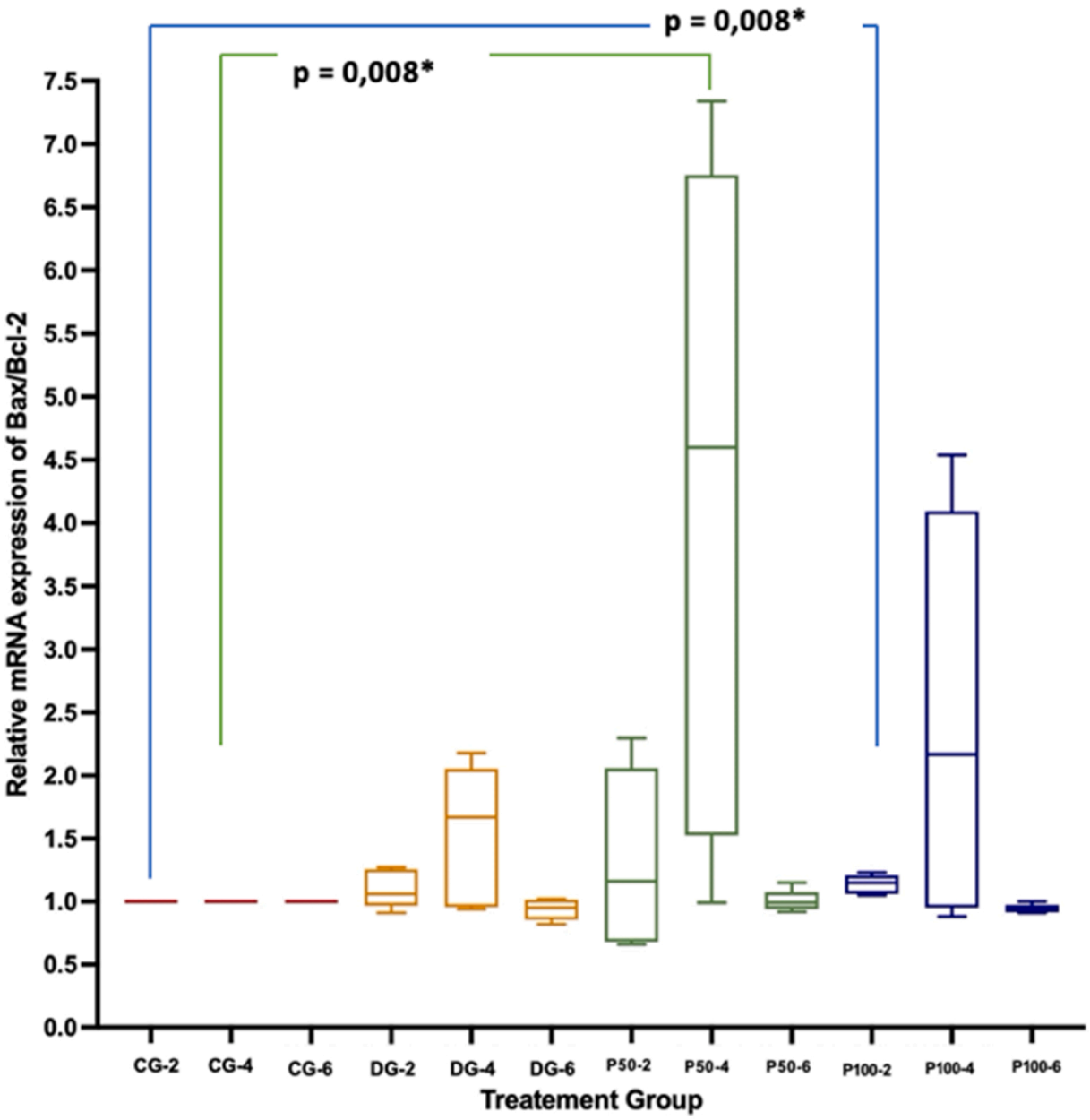

Fig. 6. Bax/Bcl-2 mRNA expression in endometriotic tissue in various treatment groups compared with the control group (CG). DG, dienogest group; P50, propolis 50 mg/kg body weight (BW)/day; P100, propolis 100 mg/kg BW/day (Mann–Whitney test). The bars and tails represent the minimum, quartile (Q) 1, Q2 (median), Q3 and maximum value of each measurement.

3.4. Changes in inflammatory activity

In this study, inflammatory activity was assessed using mRNA expression of PGE2S and IL-1B relative to the CG (Figs. 8 and 9). The DG-2 group showed the lowest expression of PGE2S mRNA (0.18 times CG), but this increased gradually in the DG-4 and DG-6 groups.

Both the P50 and P100 groups showed a similar pattern: highest inflammatory activity in the 2-week groups, with gradual decrease in the 4- and 6-week groups. Lowest expression was seen in the 6-week groups. However, no significant difference was observed between the propolis groups and the CG (Fig. 8).

Regarding the expression of IL-1B, the greatest reduction occurred in the P50–4 group (0.07 times CG), followed by the P100–4 group (0.19 times CG) and DG-2 (0.40 times CG). A similar pattern was seen in both propolis groups, with a decrease in the 4-week groups, followed by an increase in the 6-week groups. In the DG, decreased expression was seen in the 2- and 4-week groups, but increased expression was seen unexpectedly in the 6-week group. Compared with the CG for the same treatment duration, the P50–4 group showed a significant reduction in IL-1B mRNA expression (Fig. 9).

3.5. Changes in the oestrous cycle

Changes in the oestrous cycle are shown in Table 1. Rats in the propolis groups showed no changes in the oestrous cycle; however, all rats in the DG showed abnormal oestrous cycles (>7 days) post-treatment.

4. Discussion

Propolis has been reported to have beneficial effects for various diseases [12–22]. However, the study of propolis in endometriosis is

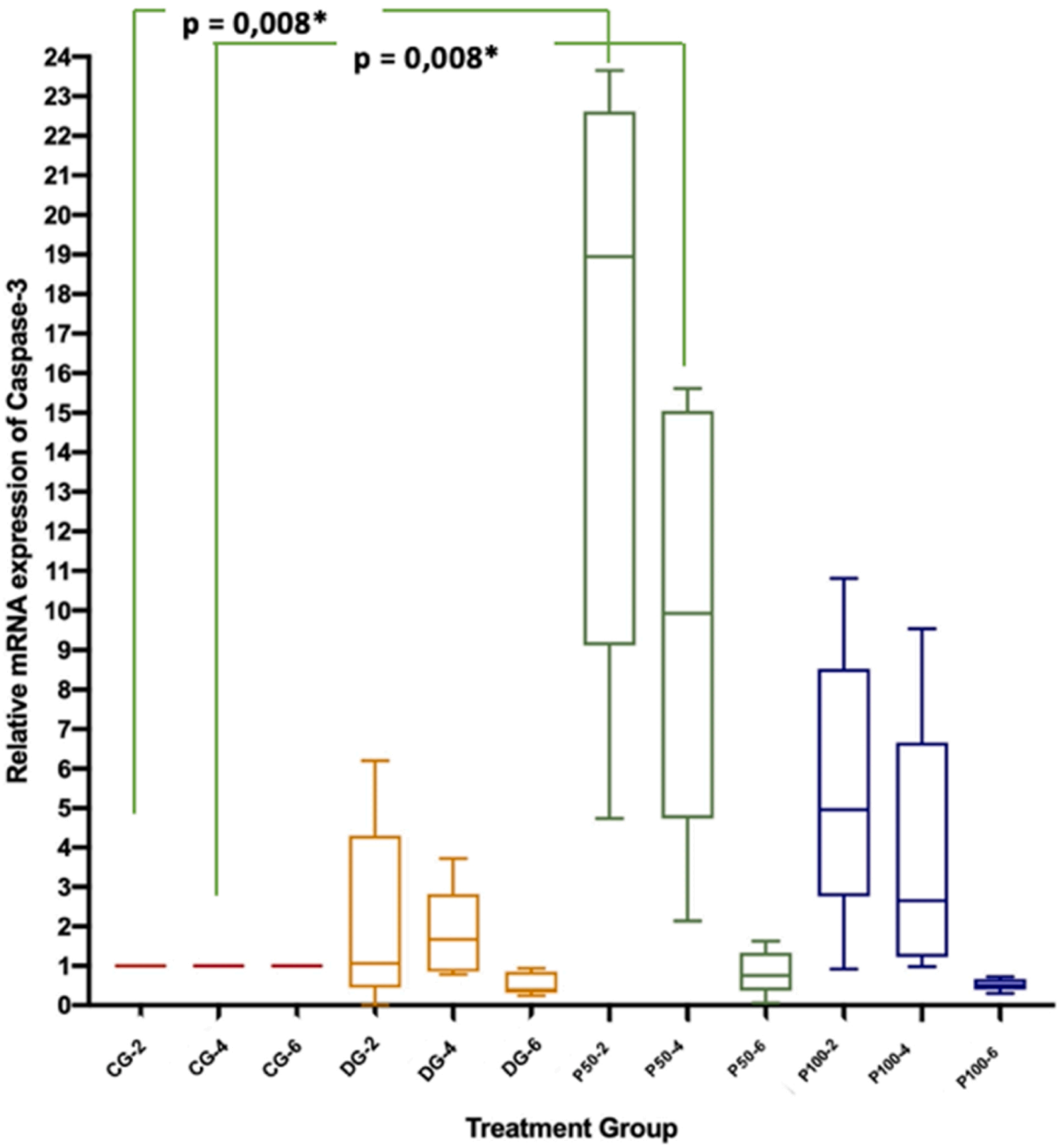

Fig. 7. Caspase-3 mRNA expression in endometriotic tissue in various treatment groups compared with the control group (CG). DG, dienogest group; P50, propolis 50 mg/kg body weight (BW)/day; P100, propolis 100 mg/kg BW/day (Mann–Whitney test). The bars and tails represent the minimum, quartile (Q) 1, Q2 (median), Q3 and maximum value of each measurement.

scanty. In a recent in-silico study, the present authors showed that Sulawesi propolis binds strongly to endometriosis-related receptors [23]. While propolis can be found worldwide, different active components and compositions may exert different properties [24]. This study specifically investigated the effect of *Tetragonula sapiens* propolis from Luwu District, South Sulawesi, and its active components have been identified [6].

The rat endometriosis model used in this study has similar characteristics to peritoneal lesions (superficial endometriosis) in humans. Its macroscopic appearance showsblood vessels around the superficial lesion, indicating angiogenic activity, and the presence of intestinal adhesion, implicating active inflammatory activity. Microscopically, the endometrial stromal and glandular-like cells, as seen all over the lesion, mimic endometriosis cells in humans (Fig. 3).

Changes in the endometriotic lesion area are an objective indicator for determining the progression of endometriosis. In this study, all groups, except the CG, showed a reduction in lesion area with treatment time. The P50 group (all treatment durations) showed a significant reduction in growth compared with the CG. The lesion area in the P50 group decreased over time, reaching a 70.66% reduction in the P50–6 group. However, in the P100 group, the maximum reduction occurred in the P100–4 group (up to 34.93% of the initial area). This finding showed that the effect of propolis on lesion growth was time- and dose-dependent. Total dosage above the maximum therapeutic dosage will not result in a better outcome.

Studies related to the dose of propolis in other diseases in rats are variable. However, 50–250 mg/kg BW/day is the dose commonly administered to the rat model. This study confirmed that propolis

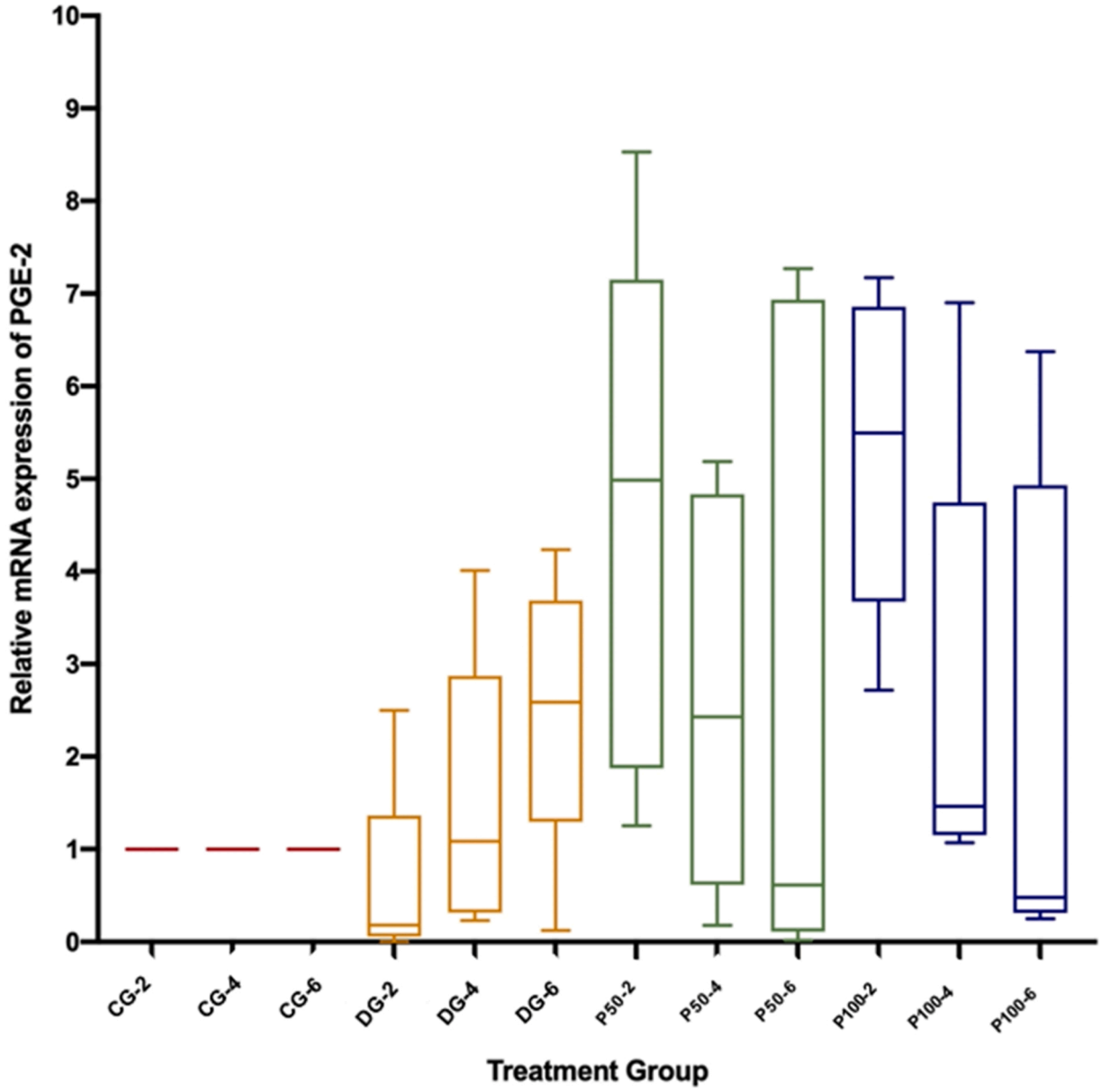

Fig. 8. Prostaglandin E2 (PGE2) mRNA expression in endometriotic tissue in various treatment groups compared with the control group (CG). DG, dienogest group; P50, propolis 50 mg/kg body weight (BW)/day; P100, propolis 100 mg/kg BW/day (Mann–Whitney test). The bars and tails represent the minimum,quartile (Q) 1, Q2 (median), Q3 and maximum value of each measurement.

50 mg/kg BW/day is a therapeutic dose for inhibiting the growth of endometriotic lesions, and propolis 100 mg/kg BW/day showed less efficacy. This finding is in line with research by Sahlan et al. [7] and Silva et al. [25] which demonstrated that administration of propolis at doses of 200 and 300 mg/kg BW/day begins to be toxic to animal models. When interpolated between the lifespan of rats and humans, 2 weeks of age in rats is equivalent to 1 year of age in humans. Hence, although speculative, it is probable that the optimum propolis dosage in humans would be either 50 mg/kg BW/day for 3 years or 100 mg/kg BW/day for 2 years [6,26].

Changes in apoptotic activity in the ectopic endometrium compared with the eutopic endometrium have been acknowledged as significant factors in the development of endometriotic lesions. Caspase-3 and Bax/Bcl2 depict apoptotic activity dynamically, and can be induced by several pathways. Activation of caspase-3 protein is a common apoptotic pathway through both the caspase-8 and caspase-9 pathways [27], while Bax is a pro-apoptosis protein, and its translocation to the mitochondria is an early sign of apoptosis.

This study showed that the pro-apoptotic effect of Sulawesi propolis was shown as early as 2 weeks after treatment initiation by upregulation of caspase-3, and later by upregulation of Bax/Bcl-2. This result may imply that propolis has biological activity by increasing apoptotic activity very early, especially in caspase-3 expression. This protein is a common execution phase of several apoptotic pathways. This may explain why caspase-3 mRNA expression can be seen earlier than Bax/Bcl mRNA expression. A previous study by Begnini et al. [28] found an apoptotic alteration in human bladder cancer cells following treatment with Brazilian propolis 25–50 μg/mL.

Chronic inflammation is another essential feature of endometriosis. The endometriotic implant has been known to secrete oestradiol; progesterone; monocyte chemo-attractant protein-1; transforming growth factor-β; vascular endothelial growth factor; and pro-inflammatory cytokines such as IL-1, IL-6, IL-8, tumour necrosis factor-alpha and PGE2 that lead to the development and progression of endometriosis [29].

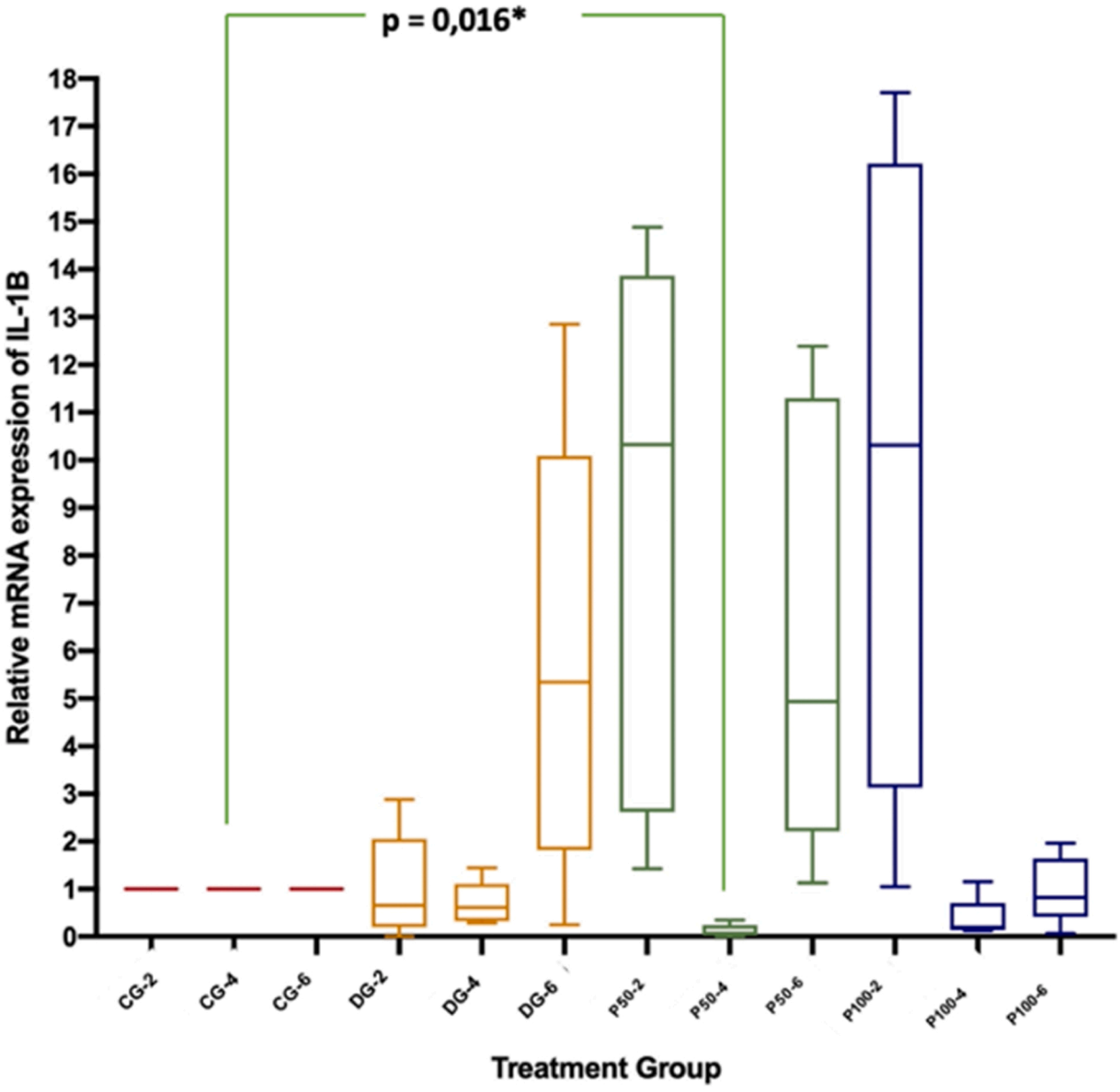

Fig. 9. Interleukin-1B (IL-1B) mRNA expression in endometriotic tissue in various treatment groups compared with the control group (CG). DG, dienogest group; P50, propolis 50 mg/kg body weight (BW)/day; P100, propolis 100 mg/kg BW/day (Mann–Whitney test). The bars and tails represent the minimum, quartile (Q) 1, Q2 (median), Q3 and maximum value of each measurement.

Table 1
Rats' oestrous cycles post-treatment.

Oestrous cycle	Group		*p*-value (Chi-squared)
	PC (2,4,6)	P50/P100 (2,4,6)	
Normal	0	30	< 0.001
Abnormal	15	0	

P50, propolis 50 mg/kg body weight/day; P100, propolis 100 mg/kg BW/day. Data presented as length of rat oestrous cycle post-treatment (days).

This study showed that IL-1B expression in both the P50 and P100 groups was downregulated in the 4-week treatment groups (0.07 and 0.19 times CG, respectively). In comparison with the CG with the same duration of treatment, the P50–4 group showed significantly lower expression of IL-1B mRNA. This result confirms the anti-inflammatory property of propolis demonstrated in the study by Sahlan et al. [7] which investigated the effect of propolis in reducing oedema in rat paws.

PGE2S expression in both the P50 and P100 groups was downregulated significantly over time. However, the lowest expression was observed in the PC-2 group (0.18 times CG). The administration of dienogest showed its efficacy in reducing inflammation, marked by the expression of IL-1B and PGE2, as shown in human studies. This reduction in inflammation contributes to the improvement of pain symptoms in human. However, although a reduction in lesion area and inflammatory activity were evident with propolis treatment in this study, the relationship with pain level was not assessed [30].

The current oestradiol-targeted hormonal therapy for endometriosis causes downregulation of the hypothalamo-pituitary-ovarian pathway. Several notable drawbacks have been reported due to this treatment, such as a hypo-oestrogenic state and a lower chance of conceiving in infertile patients with endometriosis. In this study, the DG showed abnormal oestrous cycles (>7 days) after 2–6 weeks of treatment, while rats in the propolis group showed normal oestrous cycles. Research in humans demonstrated that the administration of dienogest > 2 mg/day resulted in inhibition of ovulation [31]. In contrast, inhibition of ovulation did not occur in the propolis group, representing an advantage

of propolis compared with dienogest or other progestin therapy. Propolis appears to have a positive effect on female fertility, mainly based on animal studies, as it reduces inflammation and oxidative stresses [32].

5. Conclusion

Sulawesi propolis extract was shown to suppress the growth of endometriotic tissue in the rat model by increasing apoptotic activity and reducing inflammatory activity. The effects are time-dependent, with 50 mg/kg BW/day as the optimal dose. This effect is comparable with dienogest treatment without causing ovarian suppression.

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Declaration of Competing Interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. **H. Situmorang, A. Hestiantoro, S. Purbadi, P.E. Wuyung, R.A. Werdhani, A. Harahap, W. Permadi, M. Sahlan, W. Hadisaputra**.

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