Original Research Report

ESTROUS CYCLE AND UTERINE WEIGHT OF OVARIECTOMIZED MENOPAUSE FEMALE RAT MODELS AFTER TREATMENT WITH *Leucaena leucocephala* (Lam.) de Wit LEAF EXTRACT

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<table>
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<th>ABSTRACT</th>
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Hormone replacement therapy has adverse effects that may cause additional health problems, such as hot flashes, cancer, ischemic stroke, and death. Phytoestrogen offers a safer alternative for hormone replacement therapy. The *Leucaena leucocephala* (Lam.) de Wit plant is widely used as a medicinal ingredient and animal feed. This study aims to determine the effect of *Leucaena leucocephala* leaf extract on the uterine weight and estrous cycle of ovariectomized rats. This study used a completely randomized control design with two treatment groups (P1 and P2) and two control groups (negative (K-) and positive (K+)) with six replications in each group (n = 24). The negative control (K-) received 2 mL of corn oil, while K+ received 0.1 mg/kg bw of 17β-estradiol. P1 and P2 received 250 and 300 mg/kg bw of *Leucaena leucocephala* leaf extract, respectively. The extract was administered daily by gavage for 30 days. Estrous cycle data were obtained on day 31 by previously collecting vaginal swabs twice a day for 15 days. After the rats were sacrificed under anesthesia using chloroform, their uterine organs were removed for weight measurement. The data were analyzed for normality and homogeneity using the Shapiro-Wilk and Levene tests. The data distribution was not normal, so the Kruskal-Wallis test was used to test the hypothesis. If there was a difference between controls and treatments, the analysis continued with the Mann-Whitney test (p < 0.05). Significant differences were found between the controls and treatments in the length of each phase and overall estrous cycle. The post-hoc Duncan’s test revealed that the highest uterine weight was found in the 17β-estradiol-treated group, which was significantly different from the extract-treated rats. However, no significant difference was found between the two extract-treated groups in terms of the uterine weight. In conclusion, administering *Leucaena leucocephala* leaf extract increased uterine weight and normalized estrous cycle in ovariectomized rats.

| Keywords: | Healthy lifestyle; *Leucaena leucocephala*; estrous cycle; uterus |

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Highlights:
1. This study found that *Leucaena leucocephala* leaf ethanol extract has the ability to stimulate the growth of vaginal epithelial cells in ovariectomised rats.
2. *Leucaena leucocephala* leaf extract can be developed as a safe and cost-effective natural alternative for hormone replacement therapy in premenopausal women and livestock.

INTRODUCTION

Infertility can affect both humans and animals. The problem becomes even more serious when infertility occurs in married couples or livestock. One of the reproductive disorders in women or females is related to the production of the hormone estrogen. A decrease in the amount of estrogen in the body will impact the menstrual cycle in humans and the estrous cycle in non-primate mammals. Reproductive work is regulated by reproductive physiology involving the endocrine system. Reproductive hormones regulate gametogenesis,
morphology, and biochemical changes in the ovaries and uterus. In non-primate mammalian animals, the estrous cycle is characterized by morphological modifications in the ovaries, uterus, and vagina (Aritonang et al. 2017, Rajan et al. 2017).

The estrous cycle is influenced by steroid hormones, such as estrogen and progesterone. Estrogen exerts its effects by binding to the estrogen receptor (ER) and initiating gene expression (Pacheco et al. 2019). Infertility due to hormonal disorders can be treated with hormone replacement therapy (HRT) using synthetic estrogen. In addition to its therapeutic usage for hormonal disorders, hormone replacement therapy is also used to reduce menopausal symptoms, including hot flashes, genitourinary changes, sexual dysfunction, mood disorders, bone loss, and metabolic changes. However, prolonged use of synthetic estrogen in hormone replacement therapy can potentially cause cancer, ischemic stroke, and death (Potter et al. 2018, Mehta et al. 2021).

Phytoestrogens can function as an alternative medicine to reduce severe menopausal symptoms. These compounds are considered safe in the long term and can be used until the body adjusts to the changes in hormonal physiology (Canivenc-Lavier & Bennetau-Pelisséro 2023). Phytoestrogens are commonly found in the diets of humans and animals. This hytoestrogens are polyphenolic compounds abundantly present in plants. Thus, edible plants are considered a nutritional source rich in phytoestrogens. Non-steroidal polyphenolic compounds derived from plant metabolism have a conformational structure similar to 17β-estradiol (E2), the major pre-menopausal bioactive estrogen. Phytoestrogens can act as selective modulators of estrogen receptors and function as endocrine disruptors in an agonist or antagonist manner, depending on the dose consumed (Di Gioia & Petropoulos 2019). Prior research by Peña-Corona et al. (2019) showed that phyto-estrogen intake causes reproductive changes in animals of both sexes as well as temporary infertility syndrome. The use of phytoestrogens has a lower risk of side effects compared to synthetic estrogens or hormonal replacement drugs. Furthermore, the benefits of phytoestrogens are not limited to their function as an alternative ingredient for alleviating menopausal symptoms (Canivenc-Lavier & Bennetau-Pelisséro 2023). Pourhoseini et al. (2022) conducted a study to evaluate the impact of phytoestrogens derived from the combination of the Cimicifuga racemosa plant with clomiphene on the endometrial thickness and follicle number among women with polycystic ovaries. The study determined that phytoestrogens can serve as a substitute for clomiphene in inducing ovulation in patients with polycystic ovaries. Fernandez et al. (2020) performed a phytochemical test of Leucaena leucocephala leaf extract and demonstrated the presence of flavonoid compounds, such as quercetin. The flavonol content in Leucaena leucocephala has both estrogenic and antiestrogenic effects on reproduction. Specifically, it contains quercetin, which reduces apoptosis of ovarian cells and increases ovarian weight, oocyte quality, and the number of litters in young female mice (Beazley & Nurmikoska 2016). According to Deivasiagamani (2018), Leucaena leucocephala contains secondary metabolites, including flavonoids, saponins, phenols, tannins, cardiac glycosides, phlorotannins, and terpenoids. Leucaena leucocephala leaf extract has a total phenolic content of 51.04±0.91 mg GAE/g and a total flavonoid content of 0.13±0.01 mg catechin/g. A study conducted by Chatchanayuenyong et al. (2017) proved that Leucaena leucocephala leaf extract has antioxidant activity as determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The results showed that at a concentration of 369.6 μg/mL, the scavenging activity of Leucaena leucocephala leaf extract was found to be 50%. This is equivalent to the activity of 576.79±16.08 μg of vitamin C. It is necessary to investigate the effects of Leucaena leucocephala leaf extract on the uterine weight and estrous cycle of ovariectomized rats. Ovariectomized rats was used as a model of impaired fertility and menopause.

**MATERIALS AND METHODS**

Leucaena leucocephala leaves were obtained from the Serangan Island area, Denpasar, Indonesia (8°42′53.8″S 115°13′21.9″E). The leaves were separated from the stems and washed with running water. The next stage was drying the leaves by direct exposure to sunlight for approximately two weeks. Once the leaves had dried, they were ground using a blender and sifted until a fine powder. The leaf powder was macerated using 95% ethanol for 48 hours. The ratio between the amounts of Leucaena leucocephala leaf powder and the solvent used was 1:10. The macerated product was strained using filter paper. The resulting filtrate was then evaporated using a vacuum rotary evaporator until a coarse paste-like extract was formed (Abubakar & Haque 2020).

The preparation for the ovariectomized rats started by surgically removing the ovaries a week after feed adaptation. The rats were intramuscularly anesthetized using a combination of 50 mg/kg bw of ketamine (Ketamil, 3526004001-TPO-136521835, WAM.BDOH.62, CV Wahana Agro Mandiri, Denpasar, Indonesia) and 10 mg/kg bw of xylazine (Xyla, 3526004001-OHD-176355624, WAM.T.OH.27, PT Tekad Mandiri Citra, Bandung).
Indonesia). The hair in the surgical area was shaved, and the incision area was cleaned using 70% alcohol. A small transverse peritoneal incision (1 cm) was made slightly to the right in the middle of the abdomen, allowing for the opening of the transverse abdominal muscles. After dissecting the muscles, the peritoneal cavity and adipose tissue covering the ovaries were exposed. The right and left ovaries were removed at once with one incision in the ventral abdomen. The incision, spanning the peritoneum, muscle, and skin, was sutured aseptically once the ovary removal procedure was completed. The surgical wound was treated post-operation by applying gentamicin sulfate antibiotic ointment (GKL9912700630A1, PT Kimia Farma Tbk, Indonesia) until it dried (Sankar et al. 2014).

This study used a completely randomized design and involved 24 female rats with a weight range of 195–200 g. The ovariectomized rats were randomly divided into four treatment groups: a negative control group (K-), a positive control group (K+), and two treatment groups (P1 and P2). K- was given 2 mL of corn oil, whereas K+ was administered 0.1 mg/kg bw of 17β-estradiol (Progynova 2 mg, DK10915900716B1, Bayer, Indonesia). P1 and P2 received treatments using *Leucaena leucocephala* leaf extract at doses of 250 mg/kg bw and 300 mg/kg bw, respectively (Festing 2020). Each treatment consisted of six replications. The independent variables in this research were the doses of *Leucaena leucocephala* leaf extract, while the dependent variables were estrous cycle length and uterine weight.

The rats were kept in cages made of plastic boxes measuring 33x25x14 cm at the animal laboratory. These cages were equipped with wire covers. The rearing cages were lined with husks to absorb urine and feces. During the experimental period, the room’s environment was maintained on a 12-hour cycle of light and darkness at a temperature of 25±2 °C. The rats underwent a seven-day acclimatization period in the laboratory. The food provided was CP 551 concentrate, which was produced by PT Charoen Pokphand in Indonesia. Additionally, the rats were provided with ad libitum water (Parhizkar et al. 2016). After the acclimatization, the rats’ estrous cycles were regularly monitored for a duration of two complete cycles. One month after the rats were ovariectomized, they received treatment with *Leucaena leucocephala* leaf extract. The treatment was administered for 30 days by force-feeding the rats 2 mL of the extract per day using a feeding syringe with a gavage needle tube.

Data on the estrous cycle of the rats were collected on the 31st day following the administration of *Leucaena leucocephala* leaf extract. The data were obtained by collecting vaginal swab specimens twice a day for 15 days at 09.00 and 17.00 local time (Central Indonesia Time), following the procedure described by Ajayi & Akhigbe (2020). The vaginal smears were prepared by using sterile cotton buds that were moistened with 0.9% sodium chloride (NaCl). A cotton bud was rubbed into the vaginal opening and rotated slowly. The results of the swabs were smeared on microscope slides that had been dripped with 0.9% NaCl. The preparations were fixed with 70% alcohol for five minutes. Afterwards, the preparations were stained with 3% Giemsa (Indo Reagen, 0340544B, PT Segara Husada Mandiri, Jakarta, Indonesia) and left for two minutes. Finally, the preparations were washed with distilled water and left until they formed a ring. The epithelial cells in the vaginal smear preparations were observed using a light microscope at 100X magnification.

Following data collection on the estrous cycle, the rats were sacrificed using chloroform anesthesia. The euthanized rats underwent surgery to extract their uterine organs (Ajayi & Akhigbe 2020). The uterus was placed in a petri dish filled with a 0.9% NaCl solution, and then connective tissue and fat were removed from the uterus. Subsequently, the uterus was drained using paper suction, followed by the measurement of the uterine weight (Setyawati et al. 2021).

The data were analyzed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, N.Y., USA). The normality test was carried out using the Shapiro-Wilk test, while the homogeneity test was conducted using the Lavene test. A one-way analysis of variance (ANOVA) was used if the data were normally distributed and homogeneous. It was continued with the Duncan’s test if there was a significant difference between groups (p<0.05). Due to the non-normal distribution of the data, the Kruskal-Wallis test was employed. If there was a difference between the control and treatment groups, the test continued with the Mann-Whitney test (p<0.05) (Childs et al. 2021).

**RESULTS**

The average length of each phase (i.e., proestrus, estrous, metestrus, and diestrus) in one estrous cycle was analyzed using the Kruskal-Wallis test because the data were not normally distributed. The results of the analysis showed that there was a significant difference (p<0.05) between the control and treatment groups (Table 1).
The average length of each phase in the estrous cycle would typically influence the overall length of the estrous cycle. The average length of the estrous cycle in this study showed significant differences between K- and K+ as well as between P1 and P2. The Mann-Whitney test revealed that the estrous cycle length of K- was 109.3 hours, with only the diestrous phase being observed. The estrous cycle length of P1 was 110 hours, which was not significantly different from K-. The longest estrous cycle was observed in P2, lasting 125.33 hours (around five days). This duration was not significantly different from the estrous cycle of K+, which lasted 122.66 hours.

Table 2. The results of the Mann-Whitney test for the rats' estrous cycle (hours) and the length of each phase.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Phases</th>
<th>Preovul</th>
<th>Proestr</th>
<th>Estrous</th>
<th>Metestr</th>
<th>Diestru</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-</td>
<td>3.50</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.026</td>
</tr>
<tr>
<td>K+</td>
<td>15.08</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.394</td>
</tr>
<tr>
<td>P1</td>
<td>12.67</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.015</td>
</tr>
<tr>
<td>P2</td>
<td>18.75</td>
<td>0.485</td>
<td>0.002</td>
<td>0.026</td>
<td>0.002</td>
<td>0.015</td>
</tr>
</tbody>
</table>

The uterine weight data were analyzed using a one-way ANOVA because they were normally distributed. The results of the analysis showed significant differences between the control and treatment groups. The post-hoc Duncan's test revealed that the K+ rats had the highest uterine weights, which were significantly different from those of P1 and P2. However, there was no significant difference observed between P1 and P2, as indicated in Table 3. Figure 1 displays the results of the vaginal smear observation conducted using a light microscope at 100X magnification. The results indicated that leukocytes and nucleated epithelial cells were the distinctive characteristics of the diestrous phase.

Table 3. The rats' uterine weight after treatment with Leucaena leucocephala leaf extract.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uterine weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-</td>
<td>0.18±0.00</td>
</tr>
<tr>
<td>K+</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>P1</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>P2</td>
<td>0.24±0.02</td>
</tr>
</tbody>
</table>

Legends: A value of p<0.05 indicates a significant difference. K- = negative control group (corn oil); K+ = positive control group (0.1 mg/kg bw of estradiol); P1 = treatment group 1 (250 mg/kg bw of Leucaena leucocephala leaf extract); P2 = treatment group 2 (300 mg/kg bw of Leucaena leucocephala leaf extract).
DISCUSSION

Phytoestrogens have a structure that closely resembles that of estrogen, specifically estradiol. Phytoestrogens are able to bind to estrogen receptors in the nuclear membrane due to this similarity (Sergio et al. 2019). This binding will induce biologically detectable effects on female activity, such as the proliferation of vaginal epithelial cells, the thickening of the uterine wall, and the development of mammary glands. The analysis of the estrous cycle length, initiation period, and other phases is beneficial in education and research, as well as for examining the effects of drugs or chemicals on reproductive function (Ajayi & Akhigbe 2020). The estrous cycle consists of four phases: proestrous, estrous, metestrous, and diestrous. This study found that the female rats in the negative control group had the shortest average length of the estrous cycle. The estrous cycle was short because it only consisted of the diestrous phase. The vaginal smear results revealed the presence of leukocytes and nucleated epithelial cells, which are indicative of the diestrous phase. This could happen because the negative control rats were only given corn oil. A previous study discovered that ovariectionmized rats only went through a diestrous phase due to a low rate of internal estrogen blood levels (Yousefzadeh et al. 2020).

Following the removal of the ovaries through a procedure known as ovariectiony, the levels of estrogen are reduced to such an extent that the hormone can no longer stimulate vaginal epithelial cell proliferation. This is because, in addition to producing egg cells, the ovaries also contribute to the production of estrogen. According to Li et al. (2018), a lack of estrogen hormone prevents vaginal epithelial cell growth. The development of vaginal epithelial cells causes the estrous cycle to be shortened. The regulation of vaginal epithelial cell development in the lower female reproductive tract (LFRRT) is dependent on the estrogen receptor 1 (ESR1) gene, which encodes the receptor in the vaginal epithelium.

The administration of synthetic estrogen (Progynova 2 mg) at a dose of 0.1 mg/kg bw causes the proestrous, estrous, metestrous, and diestrous phases of the estrous cycle to last five days. The analysis of the vaginal smears revealed that the epithelial cells had become cornified, indicating the occurrence of the estrous phase. This happened due to the synthetic hormone causing a rise in estrogen levels in the blood. High estrogen levels induce the process of mitosis and the proliferation of vaginal epithelial cells. Furthermore, the epithelial cells will become cornified and detach from the vaginal wall. A prior study conducted by Sato et al. (2016) reported a positive correlation between estrogen levels and the length of the estrous cycle. This means that the higher the estrogen levels, the longer the estrous cycle.

Phytoestrogen compounds have the ability to bind to estrogen receptors, either estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ), in the granulosa cells of ovarian follicles. Afterwards, both the genomic mechanism involving deoxyribonucleic acid (DNA) and the non-genomic mechanism involving signaling molecules occur to stimulate active proliferation of granulosa cells, causing the diameter of the follicle to increase (Gogos et al. 2015). A shorter duration of the follicular phase leads to a shorter ovulation and an increased corpus luteum. A shorter estrous cycle leads to accelerated ovulation, resulting in a quicker availability of egg cells in the female reproductive tract, specifically the fallopian tubes, where fertilization occurs (Mardika et al. 2018).

Cornified epithelial cells were found in the vaginal smears of P1 and P2 following treatment with Leucaena leucocephala leaf extract. The presence of cornified epithelial cells indicated that Leucaena leucocephala leaf extract could increase estrogen levels in the blood. This was in line with the finding of a study conducted by Fernandez et al. (2020), who revealed that the administration of Leucaena leucocephala leaf extract caused an increase in the hormone estrogen in ovariectionomized rats. In this study, the length of the estrous cycle in P1, which received a dose of 250 mg/kg bw, was shorter compared to P2, which was administered a dose of 300 mg/kg bw. Despite the fact that P1 had a shorter estrous cycle than P2, the uterine weights of the two treatment groups were not statistically different. Meanwhile, the overall estrous cycle length in P2 was five days, which was the same as the rats in K+.
that were given synthetic estrogen. This suggests that both groups had a normal estrous cycle length of five days. Female rats generally have a short estrous cycle length that ranges from four to five days, including the phases of proestrous, estrous, metestrous, and diestrous (Sato et al. 2016).

In this study, the rats in the positive control group (K+) that were given synthetic estrogen had the highest uterus weight in comparison to the other groups. In contrast, the rats in the negative control group (K-) had the lowest uterine weight due to their estrous cycle being limited to the diestrous phase. The diestrous phase marked the initial stage of the cycle, during which the uterine wall was still very thin. According to Narulita et al. (2017), mice induced by synthetic estrogen experienced an increase in the concentration of estradiol in the blood, leading to a significant thickening of the endometrium during the estrous phase. The thickening of the endometrium, due to high levels of estrogen in the blood, stimulates the proliferation of glands in the uterine wall.

This study presents the results of a phytochemical test conducted on Leucaena leucocephala leaf extract, which revealed the presence of flavonoid compounds. Flavonoids are a class of compounds that exhibit phytoestrogen activity, such as genistin, kaemferol, and daidzein (Ma’arif et al. 2019). Prior research reported by Hassan et al. (2013) stated that the flavonoid contents isolated from the chloroform, ethyl acetate, and n-butanol fractions of Leucaena leucocephala alcoholic extract were identified as caffeic acid, isorhamnetin, chrysoberylyl, isorhamnetin 3-O-galactoside, kaempferol-3-O-rubinoside, quercitin-3-O-rhamnoside, and luteolin-7-glucoside. Additional evidence was obtained from this study, supporting the presence of phytoestrogens in Leucaena leucocephala leaf extract. This study analyzed the mechanism of action of Leucaena leucocephala leaf extract and found that it acted as a phytoestrogen, producing estrogenic effects in ovariectomized rats.

The ESR1 gene, which encodes the estrogen receptor in the vaginal epithelium, is responsible for hypertrophy when estrogen levels increase during the estrous cycle. This is especially true for E2, the endogenous ligand estrogen, which is the major premenopausal bioactive estrogen. Without ESR1, the epithelial cells lose their shape and size or become atrophic, as seen in women after menopause (Li et al. 2018). Research conducted by Ybañez-Julca et al. (2022) reported that the administration of phytoestrogens derived from Lepidium meyenii leaf extract in ovariectomized rats reduced cornified endometrial walls and increased uterine weight. ESR1 in epithelial cells regulates genes that maintain cell integrity (keratins) as well as genes that are involved in producing mucus (mucins). Insufficient epithelial ESR1 leads to several problems, such as a loss of cornification, decreased cell integrity, and glycoprotein overproduction. Epithelial ESR1 is likewise critical to immunological suppression (Li et al. 2018).

The absence of ESR1 in epithelial cells prevents vaginal leukocyte suppression, which will result in excessive matrix metalloproteinase (MMP) activity. Matrix metalloproteinase breaks down proteins in the structure of the cell, causing changes in the extracellular matrix (ECM) and triggering cell detachment. When the extracellular matrix breaks down, it may result in the need for more neutrophils to clear out cellular debris. This new way of working shows that epithelial ESR1 is required to maintain vaginal homeostasis, and it can help create new treatments for symptoms experienced by women after menopause (Li et al. 2018).

Strength and limitations

This study provides additional evidence on the effects of quercetin, a flavonol compound found in Leucaena leucocephala (Lam.) de Wit leaf extract, on the uterine weight and estrous cycle of ovariectomized rats. The phytoestrogen potential of this plant was demonstrated through the reduction in apoptosis of ovarian cells and the increase in ovarian weight and oocyte quality. These effects could potentially contribute to the reproductive capacity of young female rats. This study used ovariectomized rats as a livestock model for impaired fertility and menopause. However, further research is necessary as this study did not examine animal behavioral parameters, uterine histological structure, or conduct assays for estrogen and progesterone hormone levels. It is important to analyze the potential phytoestrogen of Leucaena leucocephala and its effectiveness in treating hormonal disorders and reducing menopausal symptoms.

CONCLUSION

The administration of Leucaena leucocephala leaf extract increased uterine weight and maintained a normal estrous cycle in ovariectomized white rats.

Acknowledgment

The authors would like to thank Universitas Udayana, Badung, Indonesia, as well as the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for their support in this study.
Conflict of interest

None.

Ethical consideration

The ethical clearance for this study was obtained from the Animal Ethics Committee of the Faculty of Veterinary Medicine, Universitas Udayana, Badung, Indonesia, under protocol No. B/170/UN14.2.9/PT.01.04/2021 dated 19/8/2021.

Funding disclosure

This study was funded by the Institute for Research and Community Services and the Faculty of Mathematics and Natural Sciences of Universitas Udayana, Badung, Indonesia, under contract No. B/781/UNI4.2.8.II/PT.01.03/2021 dated 19/08/2021. The funding was provided through the Study Program Excellent Research (Penelitian Unggulan Program Studi) grant from the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia.

Author contribution

NIW conceptualized and designed the study as well as collected, analyzed, and interpreted the data, provided statistical expertise, and drafted the article. AASAS provided statistical expertise and validation for the study. IS contributed by drafting the article and revising the article for important intellectual content. All authors have read and approved the final version of the article for publication.

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