Int. J. Morphol.,
43(4):1458-1469, 2025.

Therapeutic Effects of Biochanin A on Endometriosis: A Comprehensive Biochemical, Molecular and Histopathological Study in Rat

Efectos Terapéuticos de la Biocanina A en la Endometriosis: Un Estudio Bioquímico, Molecular e Histopatológico Exhaustivo en Ratas

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ZHANG, L. & HE, Q. Therapeutic effects of biochanin A on endometriosis: A comprehensive biochemical, molecular, and histopathological study in rat. *Int. J. Morphol., 43(4)*:1458-1469, 2025.

SUMMARY: Endometriosis, characterized by the growth of endometrial tissue outside the uterus, poses significant challenges in female reproductive health. This study investigates the therapeutic potential of Biochanin A (BCA) in a rat model of endometriosis, where fifty female rats were divided into five groups: a sham group, an endometriosis (EM) group, and three treatment groups receiving danazol (DZ), BCA, or a combination of both. Over 28 days, various biochemical and molecular parameters were assessed, including serum levels of inflammatory markers such as CRP, IL-6, IL-1β, TNF-α, and VEGF-A, alongside hormonal profiles. Results indicated that the EM group exhibited elevated inflammatory cytokines and reduced hormone levels compared to the sham group, with significant alterations in gene expression related to apoptosis and endometriosis. Notably, BCA treatment, both alone and in combination with DZ, effectively modulated these inflammatory responses and restored hormone levels, demonstrating its potential to enhance the expression of protective genes against endometriosis. Histopathological evaluations revealed that BCA significantly reduced cystic follicles while promoting the development of antral follicles and corpora lutea. These findings underscore the efficacy of BCA in mitigating the adverse effects of endometriosis, suggesting its role as a promising therapeutic agent in managing this complex condition.

KEY WORDS: Experimental endometriosis model; Biochanin A; Danazol; Rat; Endometriosis.

INTRODUCTION

Endometriosis is a complex and multifaceted condition characterized by the presence of endometrial-like tissue outside the uterus. Understanding its etiology is crucial for effective treatment. The etiology encompasses various clinical factors, such as hormonal influences—where high levels of estrogen promote the proliferation of ectopic endometrial tissue while progesterone resistance hampers menstrual regulation—as well as immune system dysfunction, which can hinder the elimination of these cells (Saunders & Horne, 2021). Genetic predispositions and environmental factors, including exposure to endocrine disruptors and lifestyle choices, further contribute to the risk of developing endometriosis. Additionally, theories such as retrograde menstruation and metaplasia suggest alternative origins for the disease (Chiorean *et al*., 2023).

From a molecular perspective, endometriosis is associated with altered gene expression related to inflammation and cell proliferation, increased levels of inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α), and epigenetic modifications that affect hormonal responses (Kozlowski *et al*., 2024). Treatment for endometriosis often involves a combination of pharmaceutical interventions and complementary therapies, including medicinal plants. Pharmaceutical treatments primarily consist of hormonal therapies, which are the first line of management due to their ability to target hormonal factors involved in the disease. Combined oral contraceptives (COCs) containing estrogen and progestin inhibit ovulation, stabilize the endometrial lining, and reduce menstrual flow by decreasing the production of gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] from the pituitary gland, thereby stabilizing endometrial tissue (Koninckx *et al*., 2021). Their action involves modulating estrogen (ERα and ERβ) and progesterone receptors (PR) signaling pathways. Progestins such as

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Received: 2025-04-30 Accepted: 2025-06-06

medroxyprogesterone acetate and norethisterone promote decidualization and apoptosis in endometrial cells while inhibiting growth through reduced estrogen stimulation, primarily activating the PR signaling pathway to suppress genes associated with proliferation and inflammation. GnRH agonists like leuprolide and goserelin target GnRH receptors, initially stimulating LH and FSH release but leading to downregulation with prolonged use, inducing a "functional menopause" by suppressing ovarian estrogen production, thus reducing activation of estrogen signaling pathways (Allen *et al*., 2025). Aromatase inhibitors, such as anastrozole, danazol, and letrozole, block the aromatase enzyme to lower estrogen levels, consequently decreasing the survival and proliferation of endometrial-like tissues. Gabapentinoids, including gabapentin and pregabalin, bind to the alpha-2-delta subunit of voltage-gated calcium channels, decreasing excitatory neurotransmitter release and modifying glutamatergic signaling, which alleviates neuropathic pain (Fan *et al*., 2021).

Several traditional medicinal plants show promise in managing endometriosis symptoms through their anti-inflammatory and hormonal activities. For example, turmeric (*Curcuma longa*) contains curcumin, which exhibits anti-inflammatory properties by inhibiting mediators like COX-2, IL-6, and TNF-α while modulating the NF-κB and MAPK pathways. Ginger (*Zingiber officinale*) contains gingerols and shogaols, which reduce inflammation by inhibiting pro-inflammatory cytokines and COX enzymes. Vitex (Chaste Tree, *Vitex agnus-castus*) may help restore hormonal balance by modulating dopamine receptor activity, influencing prolactin and progesterone levels (Balan *et al*., 2021; Meresman *et al*., 2021). Evening primrose oil (*Oenothera biennis*), rich in gamma-linolenic acid (GLA), has anti-inflammatory properties that modulate arachidonic acid metabolism, impacting prostaglandin synthesis. Lastly, fennel (*Foeniculum vulgare*) exerts anti-inflammatory effects through compounds like anethole and fenchone, and may influence estrogenic activity via phytoestrogens, potentially affecting the growth of endometrial-like cells (Neykhonji *et al*., 2024). This multifaceted approach to treating endometriosis, combining hormonal therapies with non-hormonal medications and the complementary use of medicinal plants, underscores the importance of understanding the underlying molecular mechanisms to develop targeted therapies and highlights the need for ongoing research into more effective, personalized treatment strategies.

Isoflavonoids, such as Biochanin A, a class of phytoestrogens predominantly found in soybeans and other legumes, have garnered attention for their potential role in the treatment of endometriosis due to their various biological activities, including estrogenic effects, antioxidant properties, and the ability to modulate cell signaling pathways (Cao *et al*., 2024). They can bind to estrogen receptors (ERα and ERβ), influencing the proliferation and survival of endometrial cells, which is crucial in endometriosis (Bartiromo *et al*., 2021). Additionally, isoflavonoids can inhibit the aromatase enzyme involved in estrogen synthesis, decreasing local estrogen levels in endometrial tissue and possibly alleviating symptoms associated with endometriosis (Gardella *et al*., 2023). Certain isoflavonoids also induce apoptosis in endometrial cells, potentially helping to reduce the size of endometrial lesions and improve symptoms. Their anti-inflammatory properties can reduce chronic inflammation associated with endometriosis by modulating inflammatory cytokines (e.g., TNF-α and IL-6) and inhibiting the NF-κB pathway. Furthermore, the antioxidant activities of isoflavonoids reduce oxidative stress within endometrial tissues, which is particularly beneficial given the oxidative damage common in endometriosis. These compounds can influence several signaling pathways involved in cell proliferation and apoptosis, including the PI3K/Akt pathway, where they may inhibit Akt signaling, leading to reduced survival of endometriotic cells, and the MAPK pathway, which affects cell proliferation, migration, and invasion in endometrial cells (Sutrisno & Destikatari, 2023). Some clinical studies suggest that dietary intake of soybean isoflavones can decrease the risk and severity of endometriosis symptoms, although results vary. Animal studies support their beneficial effects in managing endometriosis, including reducing lesion size and alleviating pain. The effectiveness of isoflavonoids also depends on their form (e.g., supplements or dietary sources) and dosage (Golabek *et al*., 2021). While generally considered safe, high doses may lead to hormonal disturbances or gastrointestinal issues, so individuals should consult healthcare providers before starting any supplementation, especially in hormone-sensitive conditions. Overall, isoflavonoids show promise as a complementary approach in managing endometriosis due to their multifaceted actions on estrogen modulation, inflammatory response, and cell signaling; however, more extensive clinical trials are needed to establish their efficacy and safety comprehensively. This study aims to investigate the therapeutic effects of Biochanin A on endometriosis by examining its biochemical, molecular, and histopathological impacts in a rat model. Specifically, we seek to elucidate the mechanisms through which Biochanin A influences estrogen receptor modulation, inflammatory pathways, oxidative stress, and cellular apoptosis, as well as assess its potential efficacy in reducing the severity of endometrial lesions and associated symptoms.

MATERIALS AND METHOD

Animal care and experimental groups.

Endometriosis (EM) induction. In all experimental groups, laparotomy was performed along the ventral midline of the animals. Due to the delicate and inseparable nature of the mouse endometrium, the entire uterine structure—including the endometrium, myometrium, and perimetrium was excised and transplanted as an allograft. In contrast, for rats, the endometrial layer was isolated from the rest of the uterine tissue and transplanted into recipient mice using a xenograft approach. Two distinct anatomical sites were selected for transplantation: the anterior abdominal wall and the mesentery of the small intestine. At both locations, the uterine epithelium was directly sutured to the peritoneal surface (Abdolmaleki *et al*., 2021).

Animal grouping. Fifty adult BALB/c mice, aged 9 ±1 weeks and weighing around 32±5 grams, were kept in propylene cages under standardized laboratory conditions. Environmental parameters were maintained at a temperature of 22±3 °C, with a relative humidity of 33±3 %, and a 12-hour light/dark cycle. The animals were given 72 hours to acclimate to the laboratory setting before the initiation of the experiment. Throughout the study, they had unrestricted access to tap water and a standard rodent diet. All experimental procedures were conducted in compliance with internationally accepted ethical guidelines and were approved by the institutional ethics committee of the Longgang Maternal and Child Health-Care Hospital. The study included fifty female rats, which were randomly divided into five groups: a sham group receiving normal saline, an endometriosis group (EM), and three treatment groups. The treatment groups consisted of one group receiving danazol (EM+DZ) at a dosage of 5 mg/kg/day via intraperitoneal injection for 28 days, a second group receiving BCA (EM+BCA) at 40 mg/kg/day orally for 28 days, and a third group receiving a combination of both danazol and BCA (EM+DZ+BCA) (Yo *et al*., 2022; Feng *et al*., 2024).

At the end of treatment (29th day), all animals were anesthetized via intraperitoneal injection using a dose of 25 IU ketamine-xylazine per 25 grams of body weight (comprising 10 IU ketamine and 90 IU xylazine). Euthanasia was subsequently performed through cervical dislocation. The harvested tissues were immediately transferred into DMEM/F12 medium supplemented with 5 % FBS to maintain cell viability. Circular grafts, 3 mm in diameter, were prepared using a biopsy punch and then sutured appropriately. Following laparotomy, the uterus and endometriotic lesions were excised. A thoracotomy was also conducted to aspirate blood from the right ventricle, which was then centrifuged at 3000 g for 15 minutes to separate the serum. All collected biological samples were either snap-frozen in liquid nitrogen for later biochemical and genetic analyses or fixed in 10 % formaldehyde for histopathological examination. The animals' total body weights were also measured and recorded (Jamali *et al*., 2021).

Assay of serum FSH, LH, T, progesterone (P), and estrogen (E2) hormones. On the 29th day, at the conclusion of the study, blood was drawn from the heart after sacrifice. Serum samples were obtained by centrifuging the blood at 10,000 g for 15 min. Hormone levels were measured using commercial ELISA kits from Novus Biologicals (Centennial, Colorado, US) for the following hormones: estradiol (E2) (Cat. No. KA1907), progesterone (P) (Cat. No. NBP2-60127-1), testosterone, LH (Cat. No. NBP2-68054), and FSH (Cat. No. KA2330) (Cat. No. NBP2-42044), and. All procedures adhered to the manufacturer's guidelines and protocols (Zhang *et al*., 2025).

Assay of peritoneal concentrations of interleukin-6 (IL-6), IL-1b, C-reactive protein (CRP), and tumor necrosis factor-α (TNF-α). An injection of 1 mL of sterile distilled water was administered into the peritoneal cavity. After a two-minute interval, peritoneal fluid was collected by aspiration. The concentrations of specific inflammatory markers were measured using commercially available ELISA kits, including CA-125 (Abcam, Cat. No. ab108653, USA), IL-37 (Abcam, Cat. No. ab213798, USA), and VEGF-A (Abcam, Cat. No. ab193687, USA). All analyses were conducted in strict accordance with the manufacturer's instructions and protocols (Zhang *et al*., 2025).

Serum levels of CA-125, and IL-37, and VEGF-A. Serum concentrations of CRP (R&D Systems, Cat. No. DY999B, Colorado, USA), IL-6 (Cat. No. R6000B, Colorado, USA), IL-1β (Cat. No. RLB00, Colorado, USA), and TNF-α (Cat. No. RTA00, Colorado, USA) were assessed using commercial ELISA kits from R&D Systems, Inc. (USA). All measurements were performed in strict accordance with the protocols and instructions provided by the manufacturer (Abdolmaleki *et al*., 2021).

Uterine tissue total antioxidant capacity (TAC), lipid peroxidation (TBARS), and thiol levels. The total antioxidant capacity (TAC) of uterine tissue was evaluated using the ferric reducing ability of plasma (FRAP) assay. Uterine tissues were carefully dissected to remove surrounding adipose tissue, and the samples were homogenized. For the assay, 100 µL of the homogenate was rinsed with 200 µL of cold phosphate-buffered saline and transferred into a 2 mL polyethylene tube. Then, 10 µL of

FRAP reagent (Catalog No. EIAFECL2; Thermo Fisher Scientific Inc., Lonsee, Germany) was added, and the mixture was incubated at 25°C for 15 min. Following incubation, the samples were centrifuged at 12,000 *g* for 10 min, and the absorbance was measured using a UV-visible spectrophotometer according to the established protocol (Kumar *et al*., 2024).

Lipid peroxidation levels in the uterine tissue were assessed using the thiobarbituric acid reactive substances (TBARS) assay. For this, 100 µL of uterine tissue homogenate was placed into a 2 mL polyethylene tube and mixed with 100 µL of TBARS reagent (Catalog No. EEA021; Invitrogen Inc., Lonsee, Germany). The mixture was incubated at 37 °C for 30 min, and absorbance was recorded with a UV-visible spectrophotometer as previously described.

To quantify total thiol content in the uterine tissue, 250 µL of the homogenized sample was mixed with 12 µL of Tris-EDTA buffer and incubated at 25 °C for 10 min. The initial absorbance (A1) was measured at 412 nm using an ELISA plate reader (Awareness Technology, Stat Fax ELISA reader, Model 303, USA). Subsequently, 20 µL of DTNB (Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid)) was added, and the sample was further incubated for 15 min at 25 °C. The second absorbance (A2) was recorded at 412 nm, and a DTNB-only solution served as the blank control (B). The concentration of thiol groups was then calculated using the following formula:

Total thiol concentration (µM) = (A2 - A1 - B) × 1.07 / (0.05 × 13.6) (Kumar *et al*., 2024).

RT-qPCR analysis. Alterations in HOX genes, particularly HOXA10 and HOXA11, are well-documented markers of endometrial receptivity and embryo implantation in fertility research. To investigate the potential pathological impact of endometriosis on the uterus, the expression levels of several key genes were analyzed: p53 (Forward: CAGCACATGACGGAGGTTGT, reverse: TCATCCA AATACTCCACACGC), Caspase-3 (Forward: GAACTGGA CTGTGGCATTGAG, reverse: CGTACCAGAGCGA GATGACA), HOXA10 (Forward: GCCCTTCCGAGA GCAGCAAAG, reverse: AGGTGGACGCTGCGGCTA ATCTCTA), and HOXA11 (Forward: GATTTCTCCAGCCT CCCTTC, reverse: AGAAATTGGACGAGACTGCG). For this purpose, the left uterine horn was excised, and total RNA was isolated using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen Scientific, Union City, CA, USA). RNA purity and concentration were assessed by determining the absorbance ratio at 260/280 nm using a UV spectrophotometer (Model UV1240, Shimadzu, Kyoto, Japan). Complementary DNA (cDNA) was synthesized using the Takara PrimeScript RT reagent kit (Catalog No. RTK0104; Takara Bio, Tokyo, Japan). Quantitative real-time PCR (qRT-PCR) was conducted using the High ROX BioFact™ 2X Real-Time PCR Smart Mix SYBR Green Master Mix. The β-actin gene (Forward: GGCACCACACCTTCTACAATG, Reverse: GGGGTG TTGAAGGTCTCAAAC) served as an internal control. Relative gene expression levels were calculated using the comparative Ct (2^{-DDCt}) method and expressed as fold changes.

$\Delta\Delta CT = [(CT_{Sample} - Ct\ GAPDH) - (CT_{Sample} - CT_{Control})]$, Fold change of genes=$2^{-\Delta\Delta CT}$ (Shilpasree *et al*., 2022).

Stereological analysis. Uterine and ovary tissues fixed in 10 % formalin were dehydrated through a graded ethanol series and subsequently embedded in paraffin. The resulting paraffin blocks were sectioned into 5 µm slices and stained with hematoxylin and eosin (H&E) for histological examination. Microscopic images were acquired using a BX61TRF light microscope (Olympus, Tokyo, Japan). To estimate the original tissue volumes, the immersion method was employed. Following this, tissue shrinkage was determined, and isotropic uniform random (IUR) sections were generated using the orientator technique. After embedding in paraffin, 5-µm sections were again obtained with a microtome (Leica Microsystems) and stained with H&E. Tissue shrinkage was quantified by measuring the final diameter of circular sections post-processing, which was then used in the following formula:

$$\text{Volume shrinkage} = 1 - (AA/AB)1.5$$

The volume densities of specific uterine components—namely, uterine volume (UV), lumen volume (LV), mucosal volume (MV), lamina muscularis volume (LMV), and adventitial volume (ADV)—were quantified in 5-µm tissue sections using the point-counting technique based on Delesse's principle.

$$Vv(structure) = \sum_{i=1}^{n} p\,(structure) / \sum_{i=1}^{n} (reference)$$

"$\sum_{i=1}^{n} p\,(structure)$" was the number of test points that settled on the targeted structures, and "$\sum_{i=1}^{n} p\,(structure)$" was the total number of points that fell on the lung sections. The following formula was used to determine the total targeted structural volume.

$$V\ (structure) = V(total\ uterine) \times Vv)structure)$$

The coefficient of error (CE) for the Cavalieri volume estimate was calculated using the following formula:

$$CE(V)=\frac{1}{\sum P}\left(\frac{1}{12}\{3a+c-4b\}\right)^{1/2}$$

were, $a=\sum_{i=1}^{m} Pi.Pi$

$b=\sum_{i=1}^{m} Pi.Pi+1$

$c=\sum_{i=1}^{m} Pi.Pi+2$

subsequently, the coefficient error for fractional volume was estimated as follows:

$$CE(Vv)=\left[\frac{k}{k-1}\left\{\frac{\sum u^2}{\sum u.\sum u}+\frac{\sum v^2}{\sum v.\sum v}-2\frac{\sum uv}{\sum u.\sum v}\right\}\right]^{1/2}$$

where, k was the number of sections, Σu was the total points hitting the reference space and Σv was tha sum of points hitting the desired structure.

To assess follicle differentiation and the histopathological characteristics associated with cystic follicles (CF), and antral follicles (AF), secondary follicles (SF), and corpus luteum (CL) as well as the inner and outer theca layers and granulosa/corona radiata cells at 100X magnification, an optical microscope (model No. BX61TRF; Olympus, Japan) paired with ImageJ software was utilized. The average counts of CF, AF, SF, and CL were recorded from 10 random fields of view for each sample (Lohrasbi *et al*., 2022).

Immunohistochemistry (IHC) assay for ovary. The assessment of Ki-67 positive cells acts as markers for apoptotic differentiation in ovarian follicular and parenchymal cells. For this evaluation, ovarian tissues were rinsed with PBS and processed using standard tissue processing techniques. Paraffin blocks were created from the ovarian samples, and 5 µm sections were mounted onto slides. The slides were incubated overnight at 95 °C, followed by a 1h incubation at 25 °C with primary antibodies for Ki-67 (1:1000; Cat. No. AF7649) from R&D Systems, Inc. (US). Tween-20 served as the washing buffer, while bovine serum albumin (5 %) was used to block any unbound antibodies. Subsequently, the slides were treated with 3 % hydrogen peroxide (H_2O_2) at 25 °C for 20 min and then stained with 3, 3'-diaminobenzidine (DAB). All slides were counterstained with hematoxylin. The evaluation was conducted using an optical microscope (model No. BX61TRF; Olympus, Japan) connected to ImageJ software at a magnification of 100X, and the percentage of Ki-67 positive cells relative to the total cell count was calculated from 10 random fields of view per sample (Khazayel *et al*., 2025).

Statistical analyses. Following data extraction, the Kolmogorov–Smirnov test was initially performed to assess the normality of the data distribution. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by the Tukey post hoc test to identify significant differences among the groups. Data analysis was conducted using the Statistical Package for the Social Sciences, version 16 (SPSS Inc., Chicago, IL). Results are reported as mean ± standard deviation (SD), with statistical significance set at $p < 0.05$.

RESULTS

Serum levels of hormones. In the EM group, serum levels of FSH, progesterone, and estrogen were significantly decreased, while LH and testosterone levels also showed significant reductions ($p < 0.05$) compared to the Sham group. In the DZ and BCA-only treatment groups, serum levels of FSH, progesterone, and estrogen increased, whereas LH and testosterone levels decreased compared to the EM group; however, these changes were not statistically significant. In contrast, the co-treatment group (EM + BCA + DZ) exhibited significant increases in FSH, progesterone, and estrogen levels ($p < 0.05$), along with significant decreases in LH and testosterone levels ($p < 0.05$) compared to the EM group (Fig. 1).

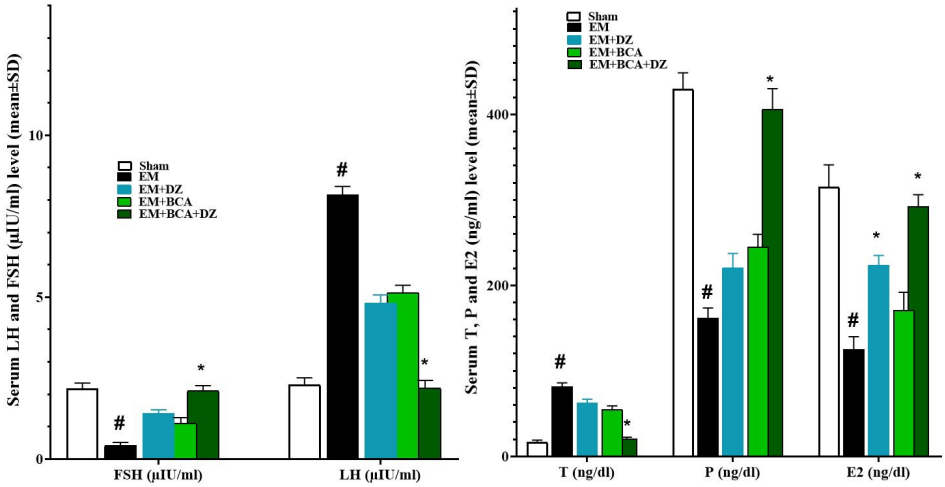

Fig. 1. The mean serum levels of FSH, LH, T, P, and E2 in experimental groups. Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups. Estradiol (E2), progesterone (P), testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH).

Uterine tissue FRAP, TBARS, and thiol. In this study, the antioxidant capacity and oxidative stress markers were assessed across various treatment groups. The FRAP levels in the Sham group were significantly higher ($p < 0.05$), averaging 6.61 µmol/mg, compared to the EM group, which showed a marked decrease ($p < 0.05$) with values of 1.22 µmol/mg. The EM + DZ group had FRAP levels of 1.29 µmol/mg, while the EM + BCA group recorded values of 3.94 µmol/mg, indicating a partial recovery. In terms of TBARS, the Sham group had an average of 1.91 nmol/mg, which was significantly reduced ($p < 0.05$) in the EM group to 0.32 nmol/mg. The EM + DZ and EM + BCA groups showed TBARS levels of 0.41 and 1.22 nmol/mg, respectively, with the EM + BCA + DZ group significantly increasing ($p < .05$) to 2.11 nmol/mg. Thiol levels reflected similar trends, with the Sham group averaging 3.91 µmol/mg, which decreased significantly ($p < 0.05$) in the EM group to 0.94 and 1.15 µmol/mg. The EM + BCA group showed a significant increase ($p < 0.05$) to 3.29 µmol/mg, while the EM + BCA + DZ group significantly increased ($p < 0.05$) to 4.11 mmol/mg (Fig. 2).

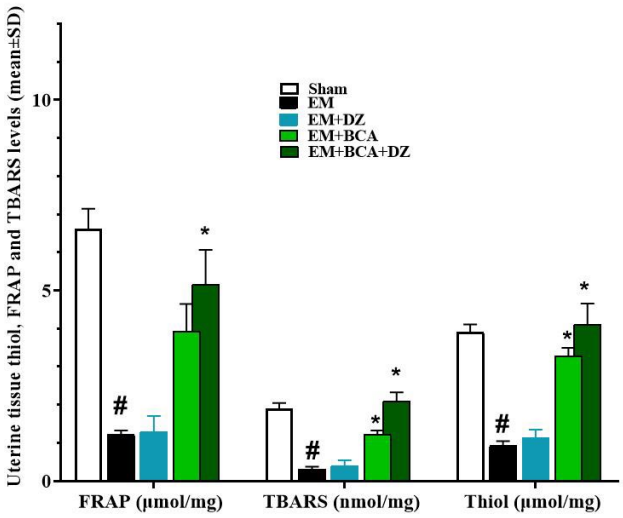

Fig. 2. The mean uterus tissue levels of FRAP, thiol, and TBARS in experimental groups. Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups.

Serum and peritoneal cytokine levels. The impact of oral exposure to BCA on serum and peritoneal inflammatory markers and growth factors in experimental groups is detailed in Tables I and II. In Table I, the sham group exhibited baseline peritoneal levels of CRP, TNF-α, IL-8, and IL-1β. The EM group showed significantly elevated levels across all markers ($p < 0.05$). In contrast, groups administered BCA (EM+BCA and EM+DZ+BCA) demonstrated significant reductions ($p < 0.05$) in CRP, TNF-α, IL-8, and IL-1β, indicating potential anti-inflammatory effects. Table II presents the effects on serum levels of VEGF-A, CEA-125, and IL-37. The sham group had baseline values for all three markers. The EM group exhibited a significant increase ($p < 0.05$) in VEGF-A, IL-37, and CEA-125. In comparison, groups treated with BCA showed a notable decrease ($p < 0.05$) in VEGF-A, CEA-125, and IL-37. Overall, these results suggest that BCA treatment may modulate inflammatory and growth factor responses in the experimental context (Tables I and II).

Table I. Effects of oral exposure to BCA on peritoneal CRP, TNF-α, IL-8, and IL-1β in experimental groups.

Group	CRP (ng/L)	TNF-α (ng/L)	IL-8 (ng/mL)	IL-1β (ng/mL)
Sham	3.1±0.6	19.1±1.3	2.2±0.2	0.91±0.06
EM	6.1±0.6$^{\#}$	69.1±2.2$^{\#}$	5.1±0.5$^{\#}$	2.14±0.09$^{\#}$
EM+DZ	5.2±0.6	44.2±2.7	4.1±0.4	1.91±0.1
EM+BCA	4.9±0.8	39.1±2.9	4.0±0.3	1.56±0.16*
EM+DZ+BCA	4.1±2.4*	22.1±6.2*	2.6±1.1*	1.12±0.23*

Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups.

Table II. Effects of oral exposure to BCA on serum VEGF-A, CEA-125, and IL-37 in experimental groups.

Group	VEGF-A (pg/mL)	CEA-125 (pg/mL)	IL-37 (pg/mL)
Sham	190.1±11.2	6.2±1.9	22.4±1.4
EM	449.2±22.1$^{\#}$	39.1±5.2$^{\#}$	59.1±2.1$^{\#}$
EM+DZ	291.1±16.2*	26.4±4.1	44.2±3.9
EM+BCA	316.2±21.4	20.2±3.1*	38.1±4.2*
EM+DZ+BCA	226.2±14.6*	11.4±2.1*	25.1±2.2*

Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups.

Expression of uterine p53, Cas-3, HOXA10, and HOXA11 genes. The results from the analysis of gene expression levels across different experimental groups are summarized as follows. In the EM group, the expression of the HOXA10, HOXA11, p53, and Caspase-3 genes significantly decreased ($p < 0.05$) compared to the sham group. Treatment with DZ in the EM+DZ group and BCA in the EM+BCA group resulted in further increases across all genes; however, these changes were not significant. Finally, the co-treatment with BCA and DZ in the EM+BCA+DZ group exhibited significantly ($p < 0.05$) higher expression levels, with HOXA10 at 0.64, HOXA11 at 0.84, Caspase-3 at 0.71, and p53 at 0.79 compared to the EM group (Fig. 3).

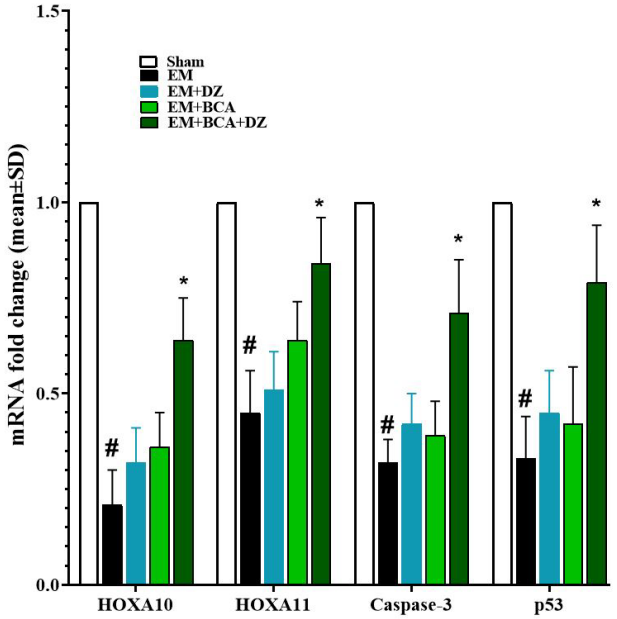

Fig. 3. The mean uterus ratio of p53, Cas-3, HOXA10, and HOXA11 genes expression in experimental groups. Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups.

Uterine stereological parameters. The effects of oral exposure to BCA on uterine stereological parameters are summarized as follows. In the sham group, the values for UV, LV, MV, LMV, ADV, and UGL were recorded at 34, 9.4, 14.2, 12.21, 3.21, and 113.21 (mm^3), respectively. In the EM group, significant ($p < 0.05$) changes were observed, with UV decreasing to 56.2 ($p < 0.05$), LV to 5.6, and MV to 15.21. The EM+DZ group showed further increases in MV (20.6) and LMV (15.21), while ADV remained stable at 4.2. In the EM+BCA group, UV decreased to 39.2 ($p < 0.05$), LV to 6.1 ($p < 0.05$), and MV significantly increased to 28.23 ($p < 0.05$). Finally, the EM+BCA+DZ group exhibited values of 30.1 for UV, 7.9 for LV, 13.21 for MV, 11.12 for LMV, and 3.12 for ADV, indicating significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM group ($p < 0.05$). These findings suggest that BCA treatment may have a significant impact on uterine stereological parameters (Figs. 4 and 5).

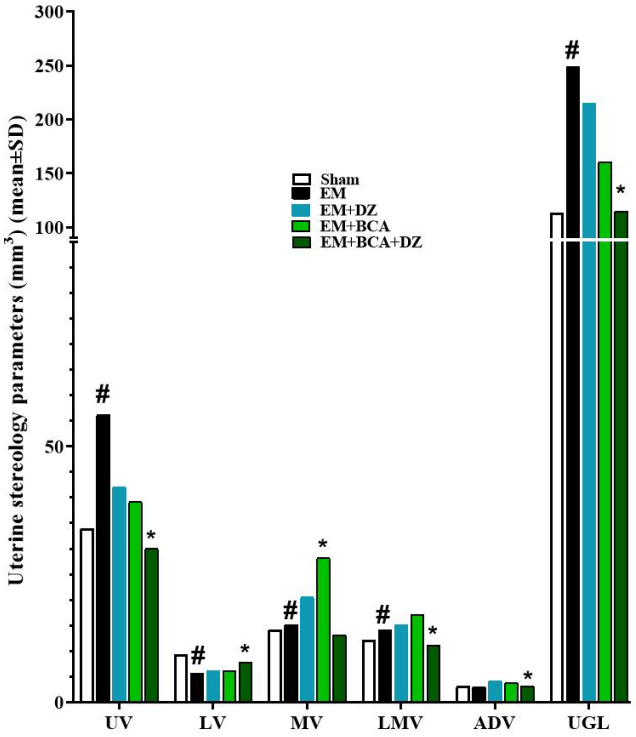

Fig. 4. The mean uterus stereological in experimental groups. Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups. UV, uterine volume; LV, lumen volume; MV, mucosa volume; LMV, lamina muscularis volume; ADV, adventitia volume; Uterine glands length (UGL).

Ovarian tissue histopathological evaluations. The histopathological evaluation of ovaries across different groups indicated that the EM group significantly increased the mean number of cystic follicles (CF) while decreasing the mean number of antral follicles (AF), secondary follicles (SF), and corpora lutea (CL) compared to the sham group ($p < 0.05$). Although both DZ and BCA alone were effective in reducing CF relative to the EM group and increasing AF, SF, and CL, these changes were not statistically significant ($p > 0.05$). In contrast, in the co-treatment group of DZ and BCA (EM+BCA+DZ), there was a significant decrease in CF ($p < 0.05$) and a significant increase in AF, SF, and CL ($p < 0.05$) compared to the EM group (Figs. 6 and 7).

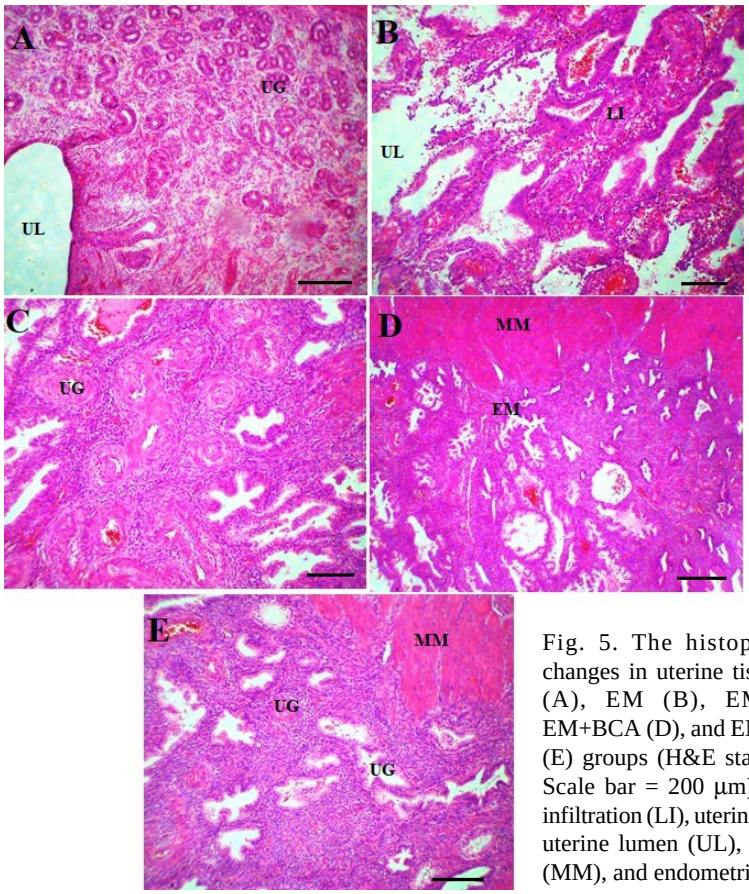

Fig. 5. The histopathological changes in uterine tissue in sham (A), EM (B), EM+DZ (C), EM+BCA (D), and EM+DZ+BCA (E) groups (H&E staining × 100, Scale bar = 200 μm). Lymphatic infiltration (LI), uterine gland (UG), uterine lumen (UL), myometrium (MM), and endometrium (EM).

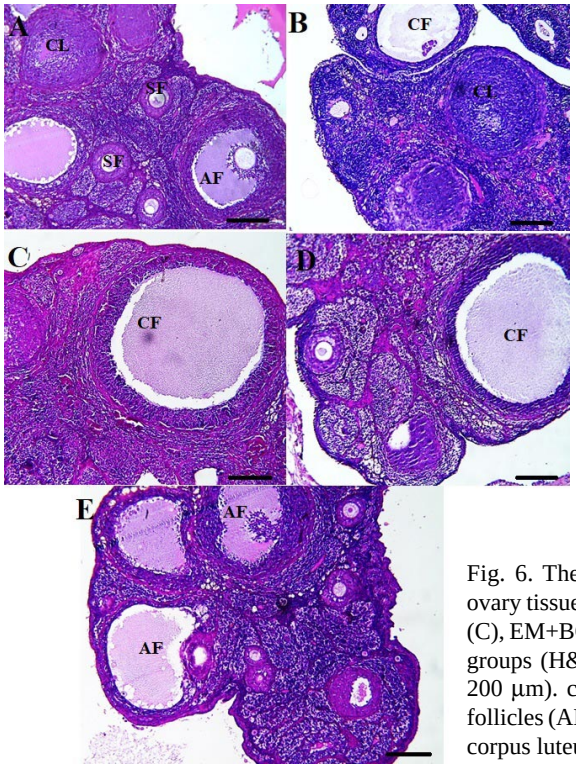

Fig. 6. The histopathological changes in ovary tissue in sham (A), EM (B), EM+DZ (C), EM+BCA (D), and EM+DZ+BCA (E) groups (H&E staining × 100, Scale bar = 200 μm). cystic follicles (CF), and antral follicles (AF), secondary follicles (SF), and corpus luteum (CL).

HC assay for expression of ovarian tissue of Ki-67 proteins. The analysis of results concerning the expression of the tissue protein Ki-67, an important marker of granulosa cell apoptosis and dominant follicle differentiation, revealed a significant increase in Ki-67 protein expression in the EM group compared to the sham group ($p < 0.05$). Both DZ and BCA, when administered alone, resulted in a decrease in Ki-67 protein levels relative to the EM group; however, these changes were not statistically significant ($p > 0.05$). In contrast, the co-treatment with BCA and DZ (in the EM+BCA+DZ group) led to a significant reduction ($p < 0.05$) in Ki-67 protein expression compared to the EM group (Fig. 8).

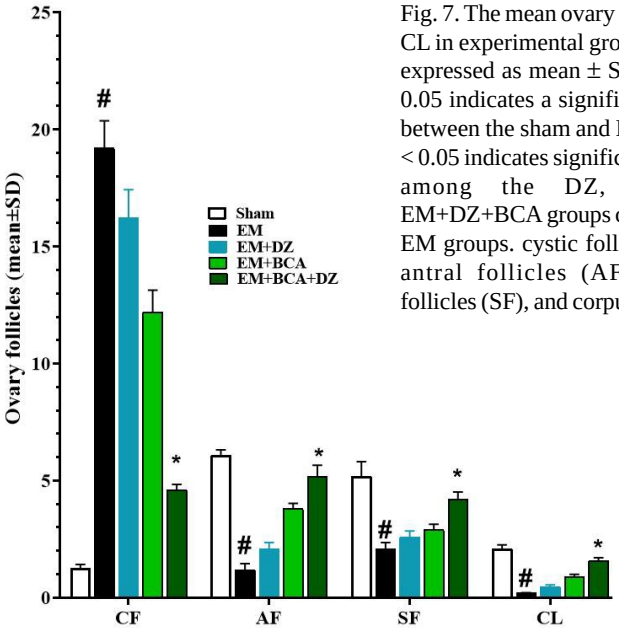

Fig. 7. The mean ovary CF, AF, SF, and CL in experimental groups. Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups. cystic follicles (CF), and antral follicles (AF), secondary follicles (SF), and corpus luteum (CL).

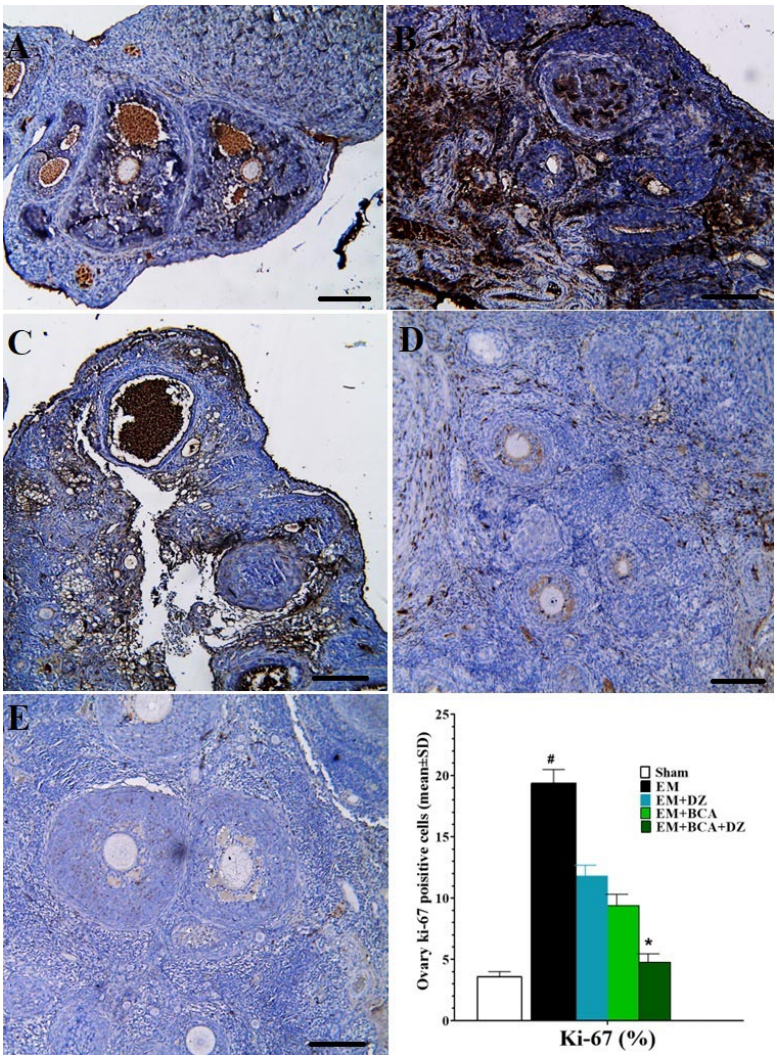

Fig. 8. The mean ovary Ki-67 positive cells in sham (A), EM (B), EM+DZ (C), EM+BCA (D), and EM+DZ+BCA (E) groups. Values are expressed as mean ± SD (n=6). # $p < 0.05$ indicates a significant difference between the sham and EM groups; * $p < 0.05$ indicates significant differences among the DZ, BCA, and EM+DZ+BCA groups compared to the EM groups (IHC/DAB staining × 100, Scale bar = 200 μm).

DISCUSSION

Endometriosis, a chronic and often debilitating condition, involves complex interactions among hormonal, immune, genetic, and environmental factors. Its multifaceted etiology complicates treatment approaches, necessitating the exploration of novel and adjunct therapies. Recent research emphasizes the critical role of oxidative stress and inflammation in the progression of endometriosis, with inflammatory cytokines such as IL-1β, IL-6, TNF-α, and markers like CRP and VEGF-A being elevated in patients. These markers not only fuel disease progression but also contribute to pain and fertility issues. Conventional therapies frequently come with significant side effects, highlighting the need for safer, more targeted options. In this context, the potential of bioactive natural compounds, particularly isoflavonoids like BCA, has garnered considerable interest due to their anti-inflammatory, antioxidant, and hormonal modulating properties.

The therapeutic promise of BCA and other isoflavonoids lies in their multifaceted mechanisms of action. Not only do they exhibit strong anti-inflammatory effects, reducing cytokine levels and systemic inflammation—as evidenced by decreased serum CRP, TNF-α, and IL-1β levels—but they also enhance antioxidant capacity, as reflected by increased plasma ferric reducing ability concentrations. These properties are crucial in creating a microenvironment that counters oxidative damage and suppresses inflammatory cascades that perpetuate endometriotic lesions. Animal studies consistently reveal that treatment with these compounds leads to declines in inflammatory markers and lesion size,

translating into symptom relief (Szukiewicz, 2023). Moreover, such compounds can influence angiogenesis, a fundamental process in lesion development, by lowering levels of VEGF-A, subsequently impairing neovascularization essential for lesion sustenance (Akkol *et al*., 2022).

Central to endometriosis management is the hormonal dysregulation characterizing the condition. BCA's influence on the hypothalamus-pituitary-gonad (HPG) axis emerges as an important aspect of its therapeutic action. Isoflavonoids like BCA can bind to estrogen receptors, exerting estrogenic effects that help restore hormonal balances disrupted by endometriosis (Gade *et al*., 2022). Treatments involving BCA have demonstrated significant increases in serum FSH, progesterone, and estrogen levels, emphasizing its capacity to modulate reproductive hormones favorably. These hormonal adjustments are vital not only for mitigating symptoms but also for improving fertility outcomes. The modulation extends to molecular regulators of endometrial receptivity, notably HOXA10 and HOXA11, whose expression levels are significantly upregulated following BCA treatment. These genes are key to optimizing conditions for embryo implantation, thereby accentuating the fertility-enhancing potential of BCA in women suffering from endometriosis (Zhao *et al*., 2025).

On a cellular and molecular level, BCA influences endometrial cell proliferation and apoptosis, which are critical processes in the pathogenesis of endometriosis. The proliferation marker Ki-67, often elevated in endometriotic tissue, was notably reduced following BCA administration, indicating suppressed cell growth (Dong *et al*., 2021). Additionally, gene expression analysis reveals that BCA upregulates p53 and Caspase-3 (Cas-3), both integral to programmed cell death pathways. Studies in rats indicate that BCA suppresses p53 in a dose-dependent manner, inhibits EM, and prevents ovarian cyst formation by modulating the HPG axis via the PI3K/AKT and mitochondrial pathways. By promoting apoptosis of ectopic endometrial cells, BCA counters the resistance to cell death that characterizes endometriotic lesions (Cao *et al*., 2023). Furthermore, its effects extend to the regulation of key gene expressions associated with endometrial health and receptivity, like HOXA10 and HOXA11, which are essential for successful implantation. The combined influence on cell proliferation, apoptosis, and gene regulation suggests that BCA offers a comprehensive approach to controlling disease progression and restoring reproductive function, with fewer side effects than some traditional treatments.

Histopathological analyses further support the benefits of BCA in improving ovarian and endometrial health. Findings demonstrate increased numbers of healthy follicles—such as antral follicles and corpora lutea—and a reduction in cystic formations, indicating improved ovarian function. The proliferation marker Ki-67's decreased expression after treatment signals a reduction in abnormal cell growth within lesions. Additionally, BCA appears to impact signaling pathways involved in cell survival and proliferation, including PI3K/Akt and MAPK pathways, which are often overactive in endometriosis (Wang *et al*., 2024). By inhibiting these pathways, BCA not only reduces lesion size but also alleviates associated pain and infertility symptoms. The cumulative evidence from molecular, hormonal, and histological perspectives advocates for integrating BCA into therapeutic regimens for endometriosis. While these animal model findings are promising, translating them into clinical practice requires further validation through controlled human trials to confirm safety, efficacy, and long-term benefits. As research advances, BCA and similar phytochemicals may pave the way for more personalized, targeted, and side-effect-free therapeutic options, ultimately improving quality of life for women affected by this complex disease.

CONCLUSION

This study demonstrates the therapeutic potential of Biochanin A (BCA) in managing endometriosis through a rat model. BCA treatment, both alone and with danazol, effectively reduced inflammatory markers and restored hormonal balance, highlighting its role in addressing the condition's underlying mechanisms. Histopathological evaluations confirmed BCA's efficacy by reducing cystic follicles and promoting healthier ovarian structures. Additionally, molecular analysis showed positive effects on protective gene expression related to apoptosis and inflammation. These findings suggest that BCA could be a valuable addition to current treatment strategies, warranting further clinical trials to explore its long-term safety and effectiveness in humans.

Ethical Approval. The experimental protocols of this study were approved by Longgang Maternal and Child Health-care Hospital, Shenzhen Guangdong, 518000, China ethics committee.

ZHANG, L. & HE, Q. Efectos terapéuticos de la biocanina A en la endometriosis: Un estudio bioquímico, molecular e histopatológico exhaustivo en ratas. *Int. J. Morphol., 43(4)*:1458-1469, 2025.

RESUMEN: La endometriosis, caracterizada por el crecimiento de tejido endometrial fuera del útero, plantea importantes desafíos para la salud reproductiva femenina. Este estudio investiga el potencial terapéutico de la Biocanina A (BCA)

en un modelo de endometriosis en ratas. Cincuenta ratas hembra se dividieron en cinco grupos: un grupo de tratamiento simulado, un grupo de endometriosis (EM) y tres grupos de tratamiento que recibieron danazol (DZ), BCA o una combinación de ambos. Durante 28 días, se evaluaron diversos parámetros bioquímicos y moleculares, incluyendo los niveles séricos de marcadores inflamatorios como PCR, IL-6, IL-1β, TNF-α y VEGF-A, junto con los perfiles hormonales. Los resultados indicaron que el grupo EM presentó niveles elevados de citocinas inflamatorias y niveles hormonales reducidos en comparación con el grupo placebo, con alteraciones significativas en la expresión génica relacionada con la apoptosis y la endometriosis. Cabe destacar que el tratamiento con BCA, tanto solo como en combinación con DZ, moduló eficazmente estas respuestas inflamatorias y restableció los niveles hormonales, lo que demuestra su potencial para mejorar la expresión de genes protectores contra la endometriosis. Las evaluaciones histopatológicas revelaron que el BCA redujo significativamente los folículos quísticos, a la vez que promovió el desarrollo de los folículos antrales y los cuerpos lúteos. Estos hallazgos subrayan la eficacia del BCA para mitigar los efectos adversos de la endometriosis, lo que sugiere su papel como un agente terapéutico prometedor en el manejo de esta compleja afección.

PALABRAS CLAVE: Modelo experimental de endometriosis; Biocanina A; Danazol; Rata; Endometriosis.

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