RESEARCH ARTICLE

Nanocurcumin Enhances Antioxidant Defense through GPx Upregulation in Ovarian Granulosa Cells of Endometriosis Mouse Model

Haspita Rizki Syurya Handini[1], Hendy Hendarto[2],*, Bambang Purwanto[3], Widjiati[4]

[1]Doctoral Program in Medical Sciences, Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo No.47, Surabaya, Indonesia
[2]Department of Obstetrics and Gynaecology, Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo No.47, Surabaya, Indonesia
[3]Department of Physiology, Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo No.47, Surabaya, Indonesia
[4]Department of Veterinary Medical Sciences, Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Dharmahusada Permai No.1, Surabaya, Indonesia

*Corresponding author. Email: hendy.hendarto@fk.unair.ac.id

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Abstract

BACKGROUND: Endometriosis impairs female reproductive function through oxidative stress and apoptosis, reducing oocyte quality and causing infertility. Current therapies are limited by suboptimal efficacy and side effects, including ovulation suppression. Curcumin offers antioxidant and anti-inflammatory benefits but has low bioavailability and poor solubility, which can be improved through nanoparticle formulation. Although nanocurcumin is suggested to act through multiple pathways, its mechanisms remain unclear. This study was conducted to evaluate the antioxidant and anti-apoptotic effects of nanocurcumin in a mouse model of endometriosis.

METHODS: Thirty-five mice were allocated into five groups and induced to develop endometriosis using cyclosporine A, ethinyl estradiol, and human endometrial tissue. Nanocurcumin was formulated at three particle sizes (3.71; 3.98; and 25.60 nm) and administered orally at doses of 50, 100, or 200 mg/kg/day for 14 days. After treatment, the mice were euthanized, and ovarian tissues were collected for immunohistochemical analysis of glutathione peroxidase (GPx) and B-cell lymphoma-2 (Bcl-2) expression.

RESULTS: The highest GPx expression was observed in the group receiving 50 mg/kg/day nanocurcumin (mean±SD= 6.31±1.97; p=0.042). The lowest expression of Bcl-2 was observed in control group with no treatment (mean±SD=4.15±2.48; p=0.582). Nanocurcumin administration significantly increased GPx expression in a dose-dependent manner compared with the untreated group, while no significant differences were found in Bcl-2 expression.

CONCLUSION: Nanocurcumin increases GPx expression, particularly at 50 mg/kg/day, indicating its potential as an antioxidant in reducing oxidative damage associated with endometriosis. However, nanocurcumin did not significantly influence Bcl-2 expression. These findings support nanocurcumin's role as an effective antioxidant agent in protecting ovarian granulosa cells in endometriosis.

KEYWORDS: nanocurcumin, GPx, Bcl-2, endometriosis, granulosa cells

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Introduction

Endometriosis is a progressive, estrogen dependent disease with a high recurrence rate, characterized by the implantation of endometrial tissue outside the uterus, which triggers chronic inflammatory reactions.(1) Among women of reproductive age, the prevalence of endometriosis is estimated at 5–10%.(2) Endometriosis causes chronic pain and long-term consequences to infertility. Women with endometriosis have more difficulty conceiving. Thirty percent of women with primary infertility have endometriosis and 70-80% of idiopathic infertility cases are associated with endometriosis.(3)

Endometriosis impacts all components of the female reproductive system, including oocyte quality, embryo development and implantation, ovarian function, uterine function, and endocrine system regulation. These disruptions can lead to infertility and spontaneous abortion.(4) Early detection and timely management of endometriosis are therefore crucial to reduce pain, prevent disease progression and organ damage, and preserve fertility.(5)

The pathophysiology of endometriosis involves three key dysfunctions: immune system dysregulation, impaired apoptotic signalling, and increased oxidative stress. (6) Elevated reactive oxygen species (ROS) levels are a triggering factor in the development of endometriosis.(7) Several studies have suggested that ROS and free radicals are a significant contributing factor to infertility in women with endometriosis.(8) The most important endogenous antioxidant enzyme that scavenges free radicals is glutathione peroxidase (GPx), which breaks down hydrogen peroxides into water and lipid peroxides into alcohol.(9)

In addition to oxidative stress, impaired apoptosis plays an important role in the pathogenesis of endometriosis. (6) In mammals, mitochondria serve as central regulators of the apoptotic pathway, which is modulated by the Bcl-2 family of proteins. It has been demonstrated that eutopic endometrial tissues in women with endometriosis show increased expression of the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and reduced expression of the pro-apoptotic protein, Bcl2-associated X protein (Bax), when compared to healthy endometrial tissues.(10)

Current treatments for endometriosis, such as surgical intervention and hormonal therapy, are limited in their effectiveness and are associated with various potential side effects such as inhibition of ovulation and menstruation and a downstream decrease in inflammation. Consequently, there is a growing interest in developing more effective and safer therapeutic strategies. Curcumin, a lipophilic orange polyphenol derived from turmeric (*Curcuma longa*), has been traditionally used as a herbal remedy. Curcumin has significant roles in the prevention and treatment of a variety of diseases, including cancer, cardiovascular disease, diabetes, and autoimmune disorders, due to its potent antioxidant, anti-inflammatory, and anticancer properties. (11) However, curcumin's clinical application is hindered by its low oral bioavailability and biodegradability, and its poor solubility in water, especially under acidic and neutral pH conditions. Furthermore, its instability in bodily fluids and rapid metabolism limit its therapeutic efficacy.(12)

To address these limitations, nanotechnology-based approaches have been employed to deliver curcumin into nanoparticles. It expected to enhance curcumin solubility and bioavailability. Nanoparticles are known to successfully improve the solubility and dissolution rates of natural substance.(12) Additionally, their small size prolongs systemic circulation time, modifies drug distribution, improves targeting ability, and facilitates the crossing of biological barriers.(13)

Current treatments for endometriosis cannot yet be integrated with infertility therapies. There is no standardized formula for the most effective and stable treatment of endometriosis, especially in cases associated with infertility. Nanocurcumin is an alternative treatment to improvement folliculogenesis profile that can be used to treat infertility caused by endometriosis.(14) However, the specific molecular mechanism of nanocurcumin remain unclear. This study aimed to investigate the effectiveness of nanocurcumin as antioxidant especially in GPx enzyme activity and anti-apoptotic marker of granulosa cells in a mouse model of endometriosis. This research seeks to contribute to the exploration of nanocurcumin's therapeutic potential as an effective and safe strategy to prevent infertility associated with endometriosis.

Methods

Preparation of Nanocurcumin

Nanocurcumin was prepared using a chitosan-tripolyphosphate (TPP)-curcumin composition ratio of 5:1:1. Chitosan solution was first placed in a beaker and stirred using a magnetic stirrer. Subsequently, curcumin solutions at concentrations of 1.25%, 2.5%, and 5% were added to the chitosan solution to form the F1, F2, and F3 formulations, respectively. The mixtures were stirred at 1000 rpm for 30 minutes. Afterward, the TPP solution was gradually added dropwise while stirring continued for an additional 3 hours at the same speed.

Particle size and polydispersity index, both critical parameters for nanoparticle characterization, were assessed. Measurements were conducted at 20°C using a refractive index of 1.333 and a viscosity of 1.002E-03 Pas. Particle size and polydispersity index were analyzed using a Particle Size Analyzer (PSA), Biobase BK-802N (BioBase, Jinan, China), capable of measuring particle sizes ranging from 1 to 10000 nm. The PSA operates based on the principle of dynamic light scattering. The analysis results included the mean particle size, polydispersity index, and particle size distribution at the 10^{th}, 50^{th}, and 90^{th} percentiles (Table 1). The polydispersity index decreases when the chitosan-to-

Table 1. PSA results of nanocurcumin.

Nanocurcumin Dosage	Average Diameter (Dav, Xav)	D10 (nm)	D50 (nm)	D90 (nm)	Polydispersity Index (PDI)
50 mg	3.71 nm	1.87 nm	2.93 nm	4.62 nm	0.1324
100 mg	3.98 nm	2.11 nm	3.22 nm	4.91 nm	0.1148
200 mg	25.60 nm	11.14 nm	18.90 nm	32.12 nm	0.1850

According to the FDA, nanoparticles range in size from 1-1000 nm.

TPP ratio was lowered. TPP concentration significantly influenced the occurrence of aggregation during nanoparticle formation.(15)

Experimental Animals

Each group contains seven female mice (*Mus musculus*). The mice selected for the study were 2-3 months old, weighed 20-30 g, and exhibited clean fur, clear eyes, and no physical defects. Mice were acclimatized in the laboratory for seven days with temperatures maintained at 24-26°C and a 12-hour dark/light cycle. Mice were provided feed and mineral water ad libitum. This study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga (Approval No.: 2.KEH.172.11.2023). The study was designed in full compliance with animal welfare principles.

Development of Endometriosis Mouse Model

All mice were administered cyclosporine A at a dose of 10 mg/kg body weight (equivalent to 1.82 mg per mouse). Cyclosporine A was injected intramuscularly (IM) into the posterior thigh muscle of the right leg using a 1 mL syringe. This injection was intended to induce immunodeficiency. On the posterior side of the left thigh, ethynyl estradiol was injected IM. Ethynyl estradiol was administered at a dose of 30 µg/kg body weight (equivalent to 5.4 µg per mouse), and the injection was repeated on day-5.

Endometrial tissue was obtained from benign tumor surgeries and stored in PBS. The tissue was washed by centrifugation at 2500 rpm, and the supernatant was discarded. PBS, 200 IU/mL penicillin, and 200 µg/mL streptomycin were added to the tissue. Each mouse required 1 cm^3 of endometrial tissue. The tissue was subsequently injected intraperitoneally at a volume of 0.1 mL using a 3 mLsyringe and a 16-gauge needle, ensuring that the tissue was easily introduced into the peritoneum.

Thirty-five female mice were divided into five groups: the pre-experiment control group with no treatment (C1), the post-experiment control group receiving placebo (C2), and three experimental groups receiving nanocurcumin at doses of 50 mg/kg (T1), 100 mg/kg (T2), and 200 mg/kg (T3), respectively. The mice were then allowed to rest and be fed and watered regularly until day-14. On day-15, the C1 group was euthanized to evaluate the development of endometriosis. The C2 group received a placebo in the form of a chitosan solution, while the T1, T2, and T3 groups were given their respective doses of nanocurcumin orally for 2 weeks. After the 2 weeks treatment period, all mice were euthanized with ketamine. The ovarian tissue was processed for immunohistochemistry examination (Figure 1).

Immunohistochemistry Procedure

The mouse ovaries were cleaned of connective tissue and then washed with 0.9% physiological NaCl. The fixation was carried out with 4% formaldehyde solution for 18 hours. The removed ovarian tissues were embedded in paraffin blocks. Immunohistochemical examination was conducted to detect the expression of GPx and Bcl-2 in the granulosa oocyte cells of endometriosis mouse model at 400x magnification. The primary antibodies Gpx2 antibody (bs-13396R) and Bcl-2 antibody (bs-4563R) (Bioss Antibodies, Woburn, MA, USA) were used. The presence of GPx and Bcl-2 expression was marked by the intensity of dark brown staining. Quantitative observations were performed by counting the number of positive cells per visual field using manual assessment. The positive cells were counted in 10 fields.

Data Analysis

Research data were presented with mean±standard deviation (SD). Normality test used Shapiro wilk and oneway Anova to determine the difference between groups. Results were considered statistically significant at $p<0.05$.

Results

Histological Results in Mice Ovarium

Mice models of endometriosis exhibit impaired folliculogenesis, characterized by a decrease in the number

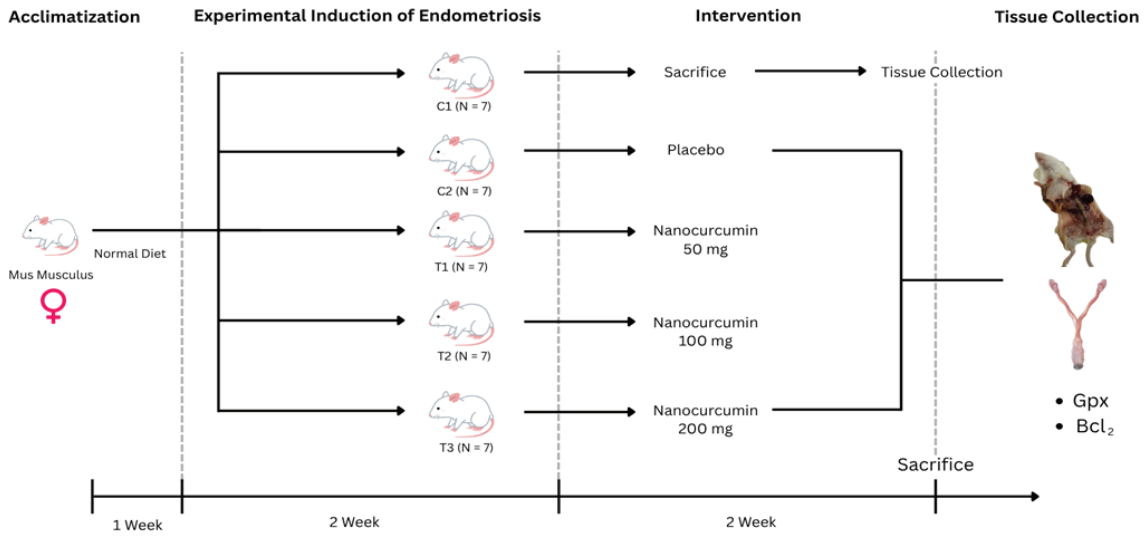

Figure 1. Experimental procedure according to the assigned group.

of primary, secondary, and tertiary follicles (Figure 2). This was due to oxidative damage to oocytes due to excessive ROS production.

Nanocurcumin Enhances GPx Expression in Granulosa Cells

GPx is an enzyme with peroxidase activity that plays a crucial role in protecting organisms from oxidative damage. Higher expression of GPx represented a higher capacity of antioxidant in the cell. The comparative expression of GPx in granulosa cells from endometriosis mouse model, across various treatment groups using nanocurcumin (Figure 3).

The GPx expression were significant difference between groups. The highest GPx expression was found at the T1 group (treated with 50 mg/kg body weight of nanocurcumin). In contrast, the lowest GPx expression was found at C1 control group (no treatment). Nanocurcumin treatment altered the GPx expression of ovaries granulosa cell among endometriosis mice models (Figure 4).

The group with the highest average GPx expression was the experimental group T1 (treated with 50 mg/kg body weight of nanocurcumin), with an average of 6.31 cells per field of view. In contrast, the group with the lowest average GPx expression was the pre-experiment control group C1 (no treatment), which had an average of 3.21 cells per field of view (Figure 4). Statistical analysis revealed a $p<0.05$, indicating a significant difference in GPx expression between the nanocurcumin-treated groups and the untreated group in the granulosa cells of the ovaries in the endometriosis mouse model.

Effect Nanocurcumin on Bcl-2 Expression

Bcl-2 is an anti-apoptotic (pro-survival) protein that plays an important role in the regulation of programmed cell

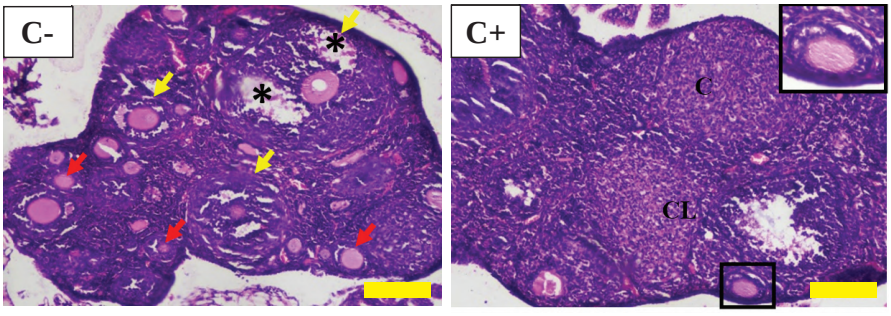

Figure 2. Histopathological images of the ovaries of mice in groups without endometriosis (C-) and with endometriosis (C+). C- shows the presence of several secondary follicles (red arrows) and tertiary follicles (yellow arrows) which are marked by the formation of Cal-Exner bodies (*), while C+ appears to be dominated by the corpus luteum (CL) with few secondary follicles (black boxes) and no tertiary follicles. Yellow bar: 10 μm.

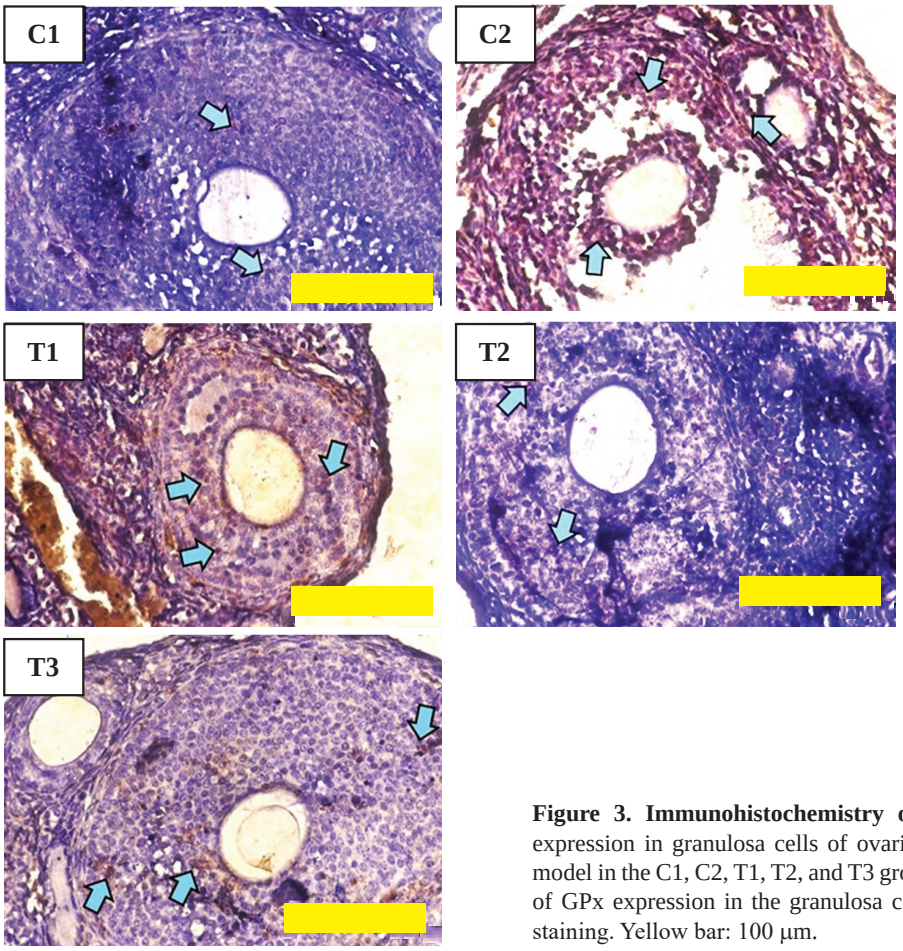

Figure 3. Immunohistochemistry of GPx expression. Comparison of GPx expression in granulosa cells of ovarian follicles from the endometriosis mouse model in the C1, C2, T1, T2, and T3 groups. The blue arrows highlight the presence of GPx expression in the granulosa cells, as indicated by the brown chromogen staining. Yellow bar: 100 µm.

death (apoptosis). The comparative expression of Bcl-2 in granulosa cells from the endometriosis mouse model is presented (Figure 5).

The expression of Bcl-2 was mostly found at the experimental group T2 (administered 100 mg/kg body weight of nanocurcumin). The lowest expression of Bcl-2 was found at pre-experiment control group C1 (no treatment). Dose of nanocurcumin had no effect on the expression of Bcl-2 as marker of apoptotic granulose cell of ovaries (Figure 6).

The group with the highest average Bcl-2 expression was found to be the experimental group T2 (administered 100 mg/kg body weight of nanocurcumin), with an average of 6.51 cells per field of view. The group with the lowest average Bcl-2 expression is the pre-experiment control group C1 (no treatment), with an average of 4.16 cells per field of view. The analysis revealed a $p>0.05$, indicating that there was no significant difference in Bcl-2 expression between the groups that were given nanocurcumin and those that were not, in the granulosa cells in the endometriosis mice model.

Discussion

This study found that nanocurcumin can enhance GPx expression as an antioxidant, however administration of nanocurcumin at various did not result in significant difference in Bcl-2 expression between treated and untreated groups in

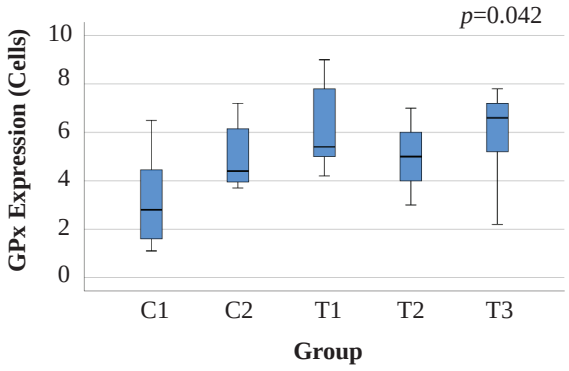

Figure 4. Comparison GPx expression level in control and experiment groups. Values are presented as mean±SD and analyzed with one-way ANOVA test.

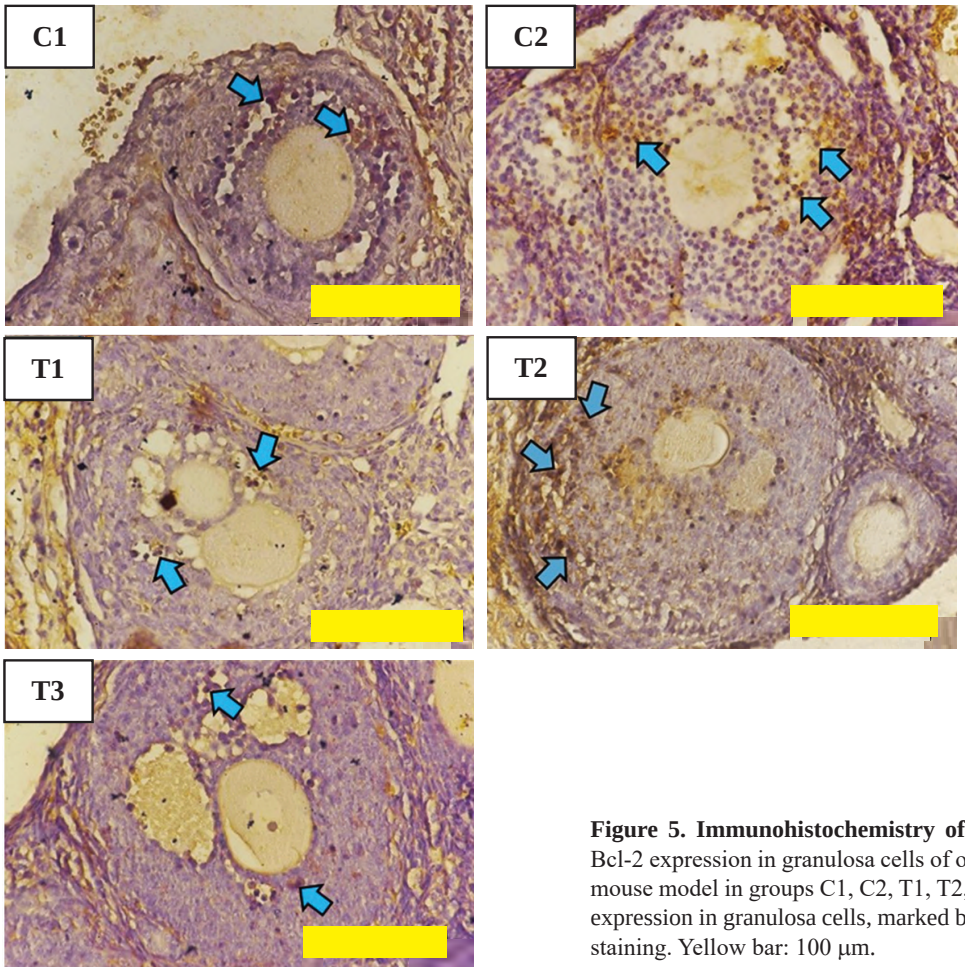

Figure 5. Immunohistochemistry of Bcl-2 expression. Comparison of Bcl-2 expression in granulosa cells of ovarian follicles in the endometriosis mouse model in groups C1, C2, T1, T2, and T3. Blue arrows indicate Bcl-2 expression in granulosa cells, marked by the presence of brown chromogen staining. Yellow bar: 100 µm.

the granulosa cells of the mice in the endometriosis model (Figure 4 and Figure 6). Endometriosis is a multifactorial and systemic disease that directly and indirectly impacts the reproductive system. However, the clinical relationship between endometriosis and infertility, along with the mechanisms involved, is not fully understood. Evidence suggests that oxidative stress plays an important role in the development of endometriosis and that endometriosis itself may be a manifestation of high oxidative stress, which directly contributes to infertility.(7) When free radicals accumulate uncontrollably, they adversely affect fertility, causing disruptions in folliculogenesis, oocyte freezing, tubal function, ovarian steroidogenesis, and more.(16)

Studies in infertile patients have shown that excessive ROS can induce granulosa cell aging by increasing endoplasmic reticulum stress, thereby contributing to infertility in endometriosis. Oxidative stress also negatively affects folliculogenesis, oocyte maturation, and embryogenesis.(17) Excessive ROS disrupts redox homeostasis in the endoplasmic reticulum, leading to endoplasmic reticulum stress, which triggers an unfolded protein response by activating genes involved in protein folding and antioxidant sources to restore endoplasmic reticulum homeostasis.(18) To counteract the harmful effects of ROS, there is a complex antioxidant defence system. The most efficient first-line defence mechanisms

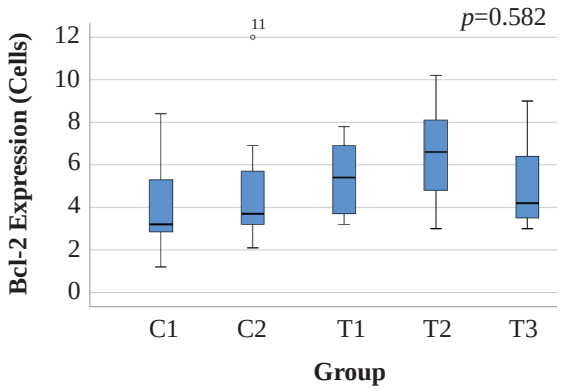

Figure 6. Comparison Bcl-2 expression level in control and experiment groups. Values are presented as mean±SD and analyzed with one-way ANOVA test.

involve antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and GPx.(19)

Curcumin, a polyphenol from turmeric, has gained attention as a therapeutic candidate due to its ability to modulate key biochemical pathways involved in various diseases, including cancer.(20) Research that has analyzed the protective effects of curcumin on diabetic rat models shows that curcumin is a natural antioxidant that can improved fertility in diabetic patients.(21) However, curcumin has limitations in therapeutic application due to its poor solubility in water, low bioavailability, and rapid metabolism. Nanocurcumin formulations have been developed to significantly enhance curcumin's biological and pharmacological benefits.(22)

Curcumin is well known as antioxidant activity, which has been widely studied in laboratory and animal experiments. Chemical structure of curcumin containing C=C double bonds, β-diketone groups and phenyl rings with hydroxyl and o-methoxy groups. These features enable curcumin to neutralize free radicals by donating hydrogen atoms, transferring electron or directly interacting with reactive molecules.(23,24) Antioxidant systems play essential roles in protecting oocytes from oxidative injury throughout folliculogenesis. ROS can interact with cellular components and impair vital organelles and functions. Sustained ROS overproduction can disrupt multiple signalling pathways critical for normal follicular development.(14)

Curcumin plays a key role as an antioxidant in combating oxidative stress by enhancing the activity of SOD, CAT, and GPx.(25) A reduction in the activity of these enzymes can result from insufficient Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) activation, a consequence of increased nuclear factor-kappaB (NF-kB) activation by reactive oxygen and nitrogen species (RONS). Curcumin exhibits strong antioxidant activity and inhibits NF-kB activation, a crucial transcription factor activated by ROS and inflammatory cytokines.(26) Research examining the potential effects of nanocurcumin against the reduction of GPx scavenger enzyme in granulosa cells of mice suggests that nanocurcumin can enhance GPx expression. GPx is a vital endogenous antioxidant enzyme that neutralizes free radicals.(27)

Previous studies have also reported an increase in Bcl-2 expression in endometriosis patients.(10) Bcl-2 is a key protein that prevents apoptosis by inhibiting the activation of caspase-9 when mitochondrial membrane permeability decreases due to cytochrome c release. Excessive granulosa cell apoptosis can compromise oocyte quality.(28) Because oxidative stress is a major trigger of apoptosis, regulating ROS levels is essential to preventing apoptotic activation. In this study, the inhibition of apoptosis may have occurred through Nrf2 pathway activation, preventing the initiation of the intrinsic apoptotic cascade.(29)

Studies examining the effects of curcumin and nanocurcumin on oxidative stress, inflammation, and apoptosis induced by copper sulfate in rat brains indicate that both agents reduce pro-inflammatory cytokines while decreasing pro-apoptotic genes, increasing Bcl-2 levels, and enhancing antioxidants and DNA integrity.(30) Similar findings were reported in breast cancer mouse models, where nanocurcumin supplementation combined with aerobic exercise reduced Bax and malondialdehyde (MDA) levels while increasing Bcl-2 activity.(31)

In a different context, curcumin significantly and simultaneously decreases Bax expression while increasing Bcl-2 expression, thereby inhibiting apoptosis by modulating Bax/Bcl-2 expression in rat testis models of diabetes.(32) Studies on rat granulosa cells indicate that curcumin enhances granulosa cell proliferation and reduces ovarian cell apoptosis, positioning curcumin as a potential therapeutic for treating infertility.(33) From several studies that have been conducted, it can be concluded that nanocurcumin has dual effects that depend on cell type, dose, pathological conditions and nano formulation.

Bcl-2 is an antiapoptotic protein that can inhibit programmed cell death. However, the apoptosis mechanism is not solely controlled by Bcl-2. Other proteins such as Bcl-2 homologous antagonist/killer (Bak), Bax, caspase-3, and p53 also play a role in apoptosis. Therefore, changes in Bcl-2 expression alone are not significant enough to determine apoptosis in granulosa cells. Protein expression also depends on biological variability between subjects. Bcl-2 expression can vary depending on tissue type, so using animal models will result in different expression results compared to using human tissue.

Conclusion

Nanocurcumin increased GPx expression but did not significantly affect Bcl-2 expression. At a dose of 50 mg/kg, nanocurcumin effectively improved the histopathological structure of granulosa cells in mice with an endometriosis model, demonstrating its potent antioxidant activity. Although its role in modulating the anti-apoptotic protein Bcl-2 remains unclear, further investigation is required. Overall, these findings indicate that nanocurcumin

may serve as a promising therapeutic agent to enhance endogenous antioxidant activity and support reproductive health in endometriosis-associated infertility.

Authors Contribution

HRSH, HH, BP and WW were involved in concepting and planning the research. HRSH and WW performed the data acquisition/collection. HRSH, HH and BP calculated the experimental data and performed the analysis. HRSH, HH and BP drafted the manuscript and designed the figures. HRSH, HH, BP and WW aided in interpreting the results. HH and BP performed supervision and validation of the study. All authors took parts in giving critical revision of the manuscript.

Conflict of Interest

The authors declare no conflict of interest or competing interest related to the content of this manuscript.

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