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The Impact of Endometriosis on Intracellular Calcium Levels, Cyclic Dependent Kinase 1 (Cdk1) Expression, and Cyclin B Expression in Post-Ovulation Oocytes of Mice Model

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Abstract

Background: Since endometriosis causes a decrease in oocyte quality, the success rate of in vitro fertilization cycles decreases. The purpose of the current study was to analyze the effect of endometriosis on intracellular calcium levels, Cdk1 expression, and cyclin B expression in oocytes.

Methods: Thirty-two mice (Mus musculus) were divided into control and endometriosis groups. The cumulus oocyte complex (COC) were obtained in all groups. Denudated cells were assessed for calcium levels by calorimetric examinations. Complex oocytes were examined for Cdk1 and cyclin B expression by immunecytochemistry and were read under a microscope.

Results: Intercellular calcium levels, Cdk1, and cyclin B expression were significantly lower in the endometriosis group than in the control group. There was a significant relationship between calcium levels and Cdk1 expression ($p<0.05$, $r=0.659$), a significant relationship between calcium levels and cyclin B expression ($p<0.05$, $r=0.885$), and also a significant correlation between Cdk1 and cyclin B expression ($p<0.05$, $r=0.537$).

Conclusion: The data presented in this study suggested that the intracellular oocyte calcium level, Cdk1 expression, and cyclin B expression were lower in mice with endometriosis. A positive correlation was observed between calcium levels and the expression of Cdk1 and cyclin B. Furthermore, a positive correlation was also found between Cdk1 and cyclin B expression.

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Introduction

Endometriosis causes infertility, but the exact mechanism is still not fully understood. According to a study, approximately 25% to 50% of women with infertility are diagnosed with endometriosis and 30% to 50% of women diagnosed with endometriosis face challenges with fertility. In endometriosis, there is a decrease in oocyte quality (1). The rate of oocyte fertilization and embryo cleavage is lower in women with endometriosis undergoing in vitro fertilization cycles (2, 3).

Possible mechanisms that can cause infertility in endometriosis are pelvic adhesions and endometrioma, as well as excessive production of inflammatory factors in the pelvic environment, that affect fertility potential of women thus changing fertility function (4).

Endometriosis induces macrophages to release IL-1, tumor necrosis factor-α (TNF-α), and other cytokines that not only inhibit gonadotropin-induced progesterone production by granulosa cells but also androgen production by theca cells. How-

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ever, even cytokines secreted by peritoneal fluid macrophages inhibit the process of steroidogenesis (5).

In women with endometriosis, there is an increased concentration of cytokines such as TNF-α, Interleukin-8, Interleukin-10, Interleukin-6, macrophage-derived growth factor, and macrophage colony-stimulating factor (M-CSF). Macrophages in women with endometriosis have been reported to secrete fibronectin in large amounts, and mild endometriosis is associated with decreased activity of platelet-activating factor acetylhydrolase (6).

The quality of oocytes in endometriosis patients is thought to have changed. In a typical IVF or ICSI cycle, approximately 70% of mature oocytes are expected to be fertilized (7). Most laboratories assign a key performance indicator of 50–60% of fertilization of all oocyte yield per cycle. Lower fertilization rate of less than 30% after ICSI or 10–40% after conventional IVF can sometimes be observed for reasons such as oocyte immaturity, low overall oocyte count, abnormal oocytes, and severe male factor (8, 9).

Endometriosis causes the release of inflammatory cytokines such as TNF-α. The cytokine TNF-α is a pleiotropic cytokine that mediates a variety of biological responses, including inflammation, infection, cell injury and apoptosis, in which TNF-α levels in the peritoneal fluid of women with endometriosis were shown to be significantly higher than in controls. TNF-α can cause impaired gap junction expression (10, 11). Endometriosis affects the gap junction in the granulosa cells surrounding the oocyte. Calcium within the intracellular environment can be transported from the endoplasmic reticulum (ER) to the mitochondria and lysosomes. Calcium is released from the endoplasmic reticulum (ER) through the IP3 receptor (IP3R) and the ryanodine receptor (RyR). Alterations in calcium levels can impact mitochondrial function, leading to an increase in cytochrome c levels. Consequently, there is a decrease in ATP pro-duction and impaired maturation promoting factor (MPF) activity. These disturbances interfere with the oocyte maturation process and result in a dec-line in oocyte quality. MPF is a complex composed of cyclin B and cyclin-dependent kinase 1 (Cdk1) (12).

The purpose of the current study was to analyze the effect of endometriosis on intracellular calcium levels, Cdk1 expression, and cyclin B expression in oocytes. Considering that the human research for determining the influence of endometriosis on oocytes is ethically constrained, an endometriosis model of mice was used in the current study. Mice have been extensively utilized as a research model to study endometriosis, and the findings obtained from these studies have been published in previous scientific literature (3). Other studies prove the existence of endometriosis growth in mice using endometriosis models (13). Mouse models of endometriosis have also been used to assess the quality of oocytes based on their maturation levels (3).

Methods

Animals: This study is a simple experimental research (pretest-posttest randomized experimental design) using experimental female Balb/c mice aged two months, with a body weight of 25-30 *gr* at the model of endometriosis. Mice were housed at room temperature under a 12 *hr* light/ 2 *hr* dark cycle with free access to water and food. The sample size calculated by the Lemeshow formula (14) resulted in a total of 32 mice, which were divided into two groups (n=16) randomly, a treatment group of the endometriosis model (T1) and a control group (T0) as placebo. In the control group (T0), injections of placebo aqua dest were administered on days one and five. In the treatment group (T1), endometriosis was induced in the mice by suppressing their immune response through the administration of Cyclosporin A injections. Additionally, intraperitoneal injections of human endometrium were administered to further facilitate the modeling of endometriosis. To stimulate endometrial growth, estrogen injections were administered. Following intraperitoneal injection of endometrial tissue, the mice were expected to develop an endometriosis model within a duration of fourteen days. Red nodule or neovascularization was found in the peritoneal cavity. The control and treatment mice groups were superovulated by administering pregnant mare serum gonadotropin (PMSG) on the 15th day and hCG injections on the 17th day and they were mated with male mice castrated. The occurrence of ovulation in mice was triggered by mating with male mice. Seventeen hours after hCG injection, all mice that exhibited positive vaginal plugs were euthanized to collect zygote complexes. This research was conducted in August–September 2022. All procedures in this study were approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Airlangga University,

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Surabaya, Indonesia and certificate of ethical approval (Approval no: 2.KEH.116.09.2022) was obtained.

Oocytes collection: Samples were taken immediately after sacrificing the mice using ketamine HCL 100 *mg/kgBW*. The oocyte cumulus complex was obtained by tearing the fallopian tubes. Calcium ions were measured by immersing the samples in PBS media (GIBCO-BRL, USA) supplemented with 3% bovine serum albumin (BSA) and 0.1% hyaluronidase (Sigma Co., USA) to release granulose cells (denudation).

Calorimetric examination of intracellular calcium levels: The primary experimental outcome evaluated in the study was intracellular calcium levels—the procedure for measuring calcium levels using the calcium (Ca) colorimetric assay kit following the guidelines. The working principle is that the calcium ions in the sample will bind to methylthymol blue (MTB) in the alkaline solution and form a blue complex. There are four reagent kits: reagent 1 contains MTB, reagent 2 contains alkaline reagent, reagent 3 contains fractional solution, and reagent 4 contains 2.5 *mmol/L* calcium standard. The process of measuring calcium ion levels is the preparation of reagents and examination steps. Stage 1 of reagent preparation involves the following steps; reagent 3, which is solid at a temperature of 2–8°*C*, needs to be heated to 37°*C* before use. After that, reagent 1, reagent 2, and reagent 3 should be mixed in a ratio of 10:20:1 to prepare the fresh solution for further use. In Stage 2, the examination steps are as follows; first, the 2.5 *mmol/L* calcium standard is diluted with deionized water to create a gradient of concentrations at 0.2, 0.3, 0.4, 0.6, 0.8, 1, and 1.2 *mmol/L*. For the standard solution, 10 μl of each concentration was taken for observation. Similarly, 10 μl of the sample solution was used for observation. Next, 250 μl of working solution was added to each sample. The solutions were then mixed for 30 *s* using a microplate reader and allowed to stand for 5 *min* at room temperature. Consequently, using a microplate reader, the optical density (OD) value was measured at 610 *nm*. The concentrations of the samples and standards were calculated using the provided formula specific to the assay kits, which incorporates the respective OD values of the standards and samples (Elabscience Biotechnology Co., USA).

Immunocytochemical (ICC) examination to evaluate the expression of Cdk1 and cyclin B: The secondary experimental outcomes evaluated in the study were Cdk1 and cyclin B expression. The examined oocyte cells were placed on the object glass covered with a coverslip sterilized using a laminin coating. Then, they were washed and rinsed with PBS for 2 *min* at room temperature, fixed with 4% paraformaldehyde in PBS for 10-20 *min* at room temperature, and washed with PBS for 2 *min* at room temperature. The sample was mobilized with a permeabilization agent of 0.1%-0.4% solution of Triton X-100 in PBS for 10-15 *min* and the coverslips were blocked with a typical 1%-5% serum solution in PBS for 1 *hr* at room temperature. The immunostaining process started with incubating oocyte cells with a labeled Cdk 1 or cyclin B antibody in a humidity-controlled room for 1 *hr* at room temperature. Next, the antibody solution was poured onto the sample and the sample was washed three times with PBS for 5 *min*. Following this, counterstaining was performed by administering DAPI. The coverslips were removed, placing the sample cells in the glass object. The sample cell was given a drop of DAPI-added media solution and the sample cell was hardened. The sample cells were already mounted on a slide. Subsequently, they were examined under a microscope, and readings were taken for further analysis (Novus Biologicals, USA).

Using cytopathological examination to determine the expression of Cdk1 and cyclin B, each sample data was assessed semiquantitatively according to the modified Remmele method where the Remmele Scale Index (IRS) is the result of multiplication between the immunoreactive cell percentage score and the color intensity score on immunoreactive cells. Each sample's data was the average IRS value observed in ten fields of view at magnifications of 100x and 400x (15).

Semi-quantitative scale IRS resulted from multiplying the positive cell percentage score with the color intensity score. The scoring system for the positive cell percentage is as follows: score 0 indicates no positive cells, score 1 represents positive cells comprising less than 10% of the sample, score 2 corresponds to positive cells ranging from 11% to 50%, score 3 indicates positive cells ranging from 51% to 80%, and score 4 represents positive cells comprising more than 80% of the sample. Moreover, the scoring system for the color reaction intensity is as follows: score 0 indicates no color reaction, score 1 represents low

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color intensity, score 2 corresponds to medium color intensity, and score 3 represents strong color intensity (15).

Statistical analysis: The obtained data were tested for normality using the Shapiro-Wilk test. To analyze the differences between the control and treatment groups, the Mann-Whitney U test was utilized when the data exhibited abnormal distribution. Additionally, the Spearman test was employed to assess the correlation between variables. All statistical analyses were performed using the SPSS version 20 (IMB, USA), and statistical significance was set at $p<0.05$.

Results

The characteristics of mice prior to treatment exhibited a normal distribution based on the data analysis results. The statistical test yielded a p-value of 0.442, indicating that there was no significant difference in the characteristics of the study subjects before treatment. This suggests that the groups of mice were homogeneous. Both the control group, consisting of 16 mice, and the treatment group, also consisting of 16 mice, exhibited ovulation.

Oocyte calcium level and expression of Cdk1 and cyclin B: The analysis of oocyte calcium levels, Cdk1 expression, and cyclin B expression is presented and detailed in table 1.

Intracellular calcium levels of the treatment group had a median value of 0.29 *mmol/L* with IQD of 0,01 and the control group median value was 0.59 *mmol/L* with IQD of 0.12. In table 1, the results of Mann-Whitney U test showed a significant difference ($p<0.05$), where the intracellular calcium levels of the treatment group (endometriosis) were significantly lower than the control group.

The Cdk1 expression in the treatment group had a median value of 1 *mmol/L* with an interquartile range (IQR) of 1.0. In comparison, the control group exhibited a median value of 9 *mmol/L* with an IQR of 2.25. The results of the Mann-Whitney U test showed a significant difference ($p<0.05$), where the expression of Cdk 1 in the treatment group (endometriosis) was significantly lower than that of the control group (Figure 1).

Cyclin B expression of the treatment group had median value of 2 *mmol/L* with IQD of 0.00 in comparison to the median value of 12 *mmol/L* and IQD of 4.00 in the control group. The results of Mann-Whitney test showed a significant difference ($p<0.05$), where the expression of cyclin B oocytes in the treatment group (endometriosis) was significantly lower than that of the control group (Figure 2).

Table 1. Oocyte calcium level, Cdk1, and cyclin B expression (*mmol/L*)

Group	Median	IQD	p value
Oocyte calcium level			
Treatment (T1)	0.29	0.01	p<0.05 *
Control (T0)	0.59	0.12	
Cdk1 expression			
Treatment (T1)	1	1.0	p<0.05 *
Control (T0)	9	2.25	
Cyclin B expression			
Treatment(T1)	2	0.00	p<0.05 *
Control (T0)	12	4.00	

(*) The analysis test of oocyte calcium levels, Cdk1 and cyclin B expression using the Mann-Whitney U test ($p<0.05$; IQD: interquartile deviation; 95% CI:95% confidence interval; T0: control group; T1: treatment group)

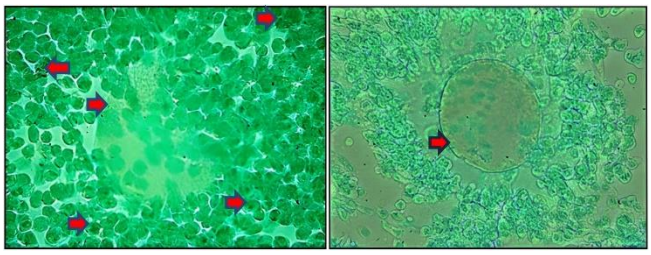

Figure 1. Comparison of Cdk 1 expression images in the oocyte. (Left) control group (T0); (Right) treatment group (T1). The red arrow indicates the presence of Cdk 1 expression in oocytes characterized by brown chromogen in the cytoplasm and cell nucleus. ICC. Original magnification at x1000

Correlation of variables: There was a correlation between calcium levels in the treatment group (endometriosis) and Cdk1 expression. A statistically significant result was obtained using the Spearman correlation test ($p<0.05$; $r=0.659$). Moreover, the correlation test revealed a significant relationship ($p<0.05$) between intracellular calcium levels and the expression of cyclin B in oocytes, with an exceptionally high correlation coefficient of 0.885. Table 2 shows the relationship between the expression of Cdk1 and the expression of cyclin B. The Spearman correlation test yielded a significant result ($p<0.05$), indicating a strong relationship with a positive correlation coefficient of 0.537.

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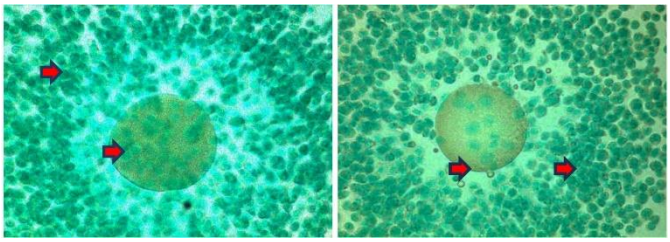

Figure 2. Comparison of cyclin B expression images in the oocyte. (Left) control group (T0); (Right) treatment group (T1). The red arrow indicates the presence of cyclin B expression in oocytes characterized by brown chromogen in the cytoplasm and cell nucleus. ICC. Original magnification at x1000

Table 2. Correlation of variables

Variables	p-value	Correlation coefficient (r)
Calcium and Cdk 1	p<0.05 *	0.659
Calcium and cyclin B	p<0.05 *	0.885
Cdk1 dan cyclin B	p<0.05 *	0.537

r: correlation coefficient

Discussion

This study demonstrated the effect of endometriosis on intracellular calcium levels, cyclin dependent kinase 1 (Cdk1) expressions, and cyclin B in a mouse model of post-ovulation oocytes with endometriosis. The results showed that intracellular calcium ion levels in the endometriosis treatment group were significantly lower compared to the control group (p<0.05). This may be attributed to an upregulation of TNF-α cytokines, which affect the expression of gap junction proteins, connexin 37 (Cx37) and connexin 43 (Cx43), (16).

Tacheau et al. stated that the cytokine TNF-α represses the expression of the CX 43 gene in HaCaT cells. TNF-α cytokines working through c-Jun N-terminal kinase (JNK) signal pathway decrease junction gaps between keratinocyte cells and decrease the expression of all connexins in the epidermis. Previous research investigating the effects of TNF-α cytokines has demonstrated a reduction in endothelial cell junction gaps in human umbilical veins, hepatocyte cell gap junctions in mice, rat heart muscle cell gap junctions, and human corneal fibroblast cell gap junctions. TNF-α cytokines will affect gap junction expression. The disruption of gap junction function can affect the ion channeling capability, including calcium, within the junction (16).

Intracellular calcium is stored in the endoplasmic reticulum, where the endoplasmic reticulum is a place for the synthesis and transport of several types of biomolecules. The endoplasmic reticulum has several calcium channels, including inositol 1, 4, 5 triphosphate (IP3) receptors (IP3R) and ryanodine receptors, which are responsible for the release of calcium from RE to the cytoplasm where the calcium concentration in the cytoplasm is low. If there is a calcium leakage in the RE, the calcium can be returned to the RE with sarcoplasmic reticulum (SR) Ca2+-ATPase (SERCA) pump. Calcium reserves in the endoplasmic reticulum (ER) can be depleted through the inositol trisphosphate receptor (IP3R) channel. However, there is a mechanism known as store-operated calcium entry (SOCE) that allows for the replenishment of calcium (17).

The study measured serum IL-6 and TNF-α levels in the peritoneum, which showed a significant increase in endometriosis. TNF-α cytokines play a crucial role in the pathogenesis and manifestation of symptoms associated with endometriosis in the reproductive system. TNF-α mediates various biological responses, including inflammation, imperfection, and cell damage, and also affects the gap junction (18). TNF-α cytokines generated by the endometriosis process cause repression in the gap junction (connexin) expression through the c-Jun N-terminal kinase (JNK). C-Jun N-terminal kinase is a family of protein kinases that play an essential role in stress signaling and have implications for gene expression, regeneration, and cell death. There are three types of JNK, namely JNK1, JNK2, and JNK3. JNK1 and JNK2 are distributed over a vast network. Activation of the JNK pathway will cause the interaction of the JNK complex with proteins that cause DNA connexin damage (19, 20).

The results of this study demonstrated that the expression of Cdk 1 in the endometriosis treatment group (T1) was significantly lower than the control group (T0) (p<0.05). This could be due to a decrease in intracellular calcium ion levels which results in the decrease of CDC 25 phosphatase enzyme levels, affecting the activity of threonine 14 and tyrosine 15. Dephosphorylation of threonine 14 and tyrosine 15 will affect the Cdk1/cyclin B complex, causing a decrease in the maturation of oocyte nucleus. Oocytes develop through the process of folliculogenesis. In this phase of development, the follicle becomes dependent on gonadotropin. Under the influence of

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FSH, follicles develop from early antral to pre-ovulatory follicles. The late follicular phase (mid-menstrual cycle) of LH spikes induces the formation of germinal vesicle breakdown (GVB) and chromosomal changes (21).

The results of this study also found that the expression of cyclin B in the endometriosis treatment group (T1) was significantly lower than the control group (T0) ($p<0.05$). This can be caused by inhibition of protein synthesis and dephosphorylation of enzymes in oocytes, which decrease the complex activity of Cdk1/cyclin B. Maturation promoting factor (MPF) controls meiosis and cell cycle regulation. MPF was initially thought of as an activity in oocytes that induces maturation of the nucleus. Cyclin B is responsible for MPF activity. The activation of MPF in cells is regulated by the balance of activity of regulators Wee1/Myt1 kinases through phosphorylation inhibitions of Cdk1 on Thr14 and Tyr15 (maintaining heterodimers in an active state called pre-dimer) and threonine 161, causing activation of Cdk1 dephosphorylation at the same place. During meiosis of cells, cyclin B must be phosphorylated in cytoplasmic retention sequences, which leads to the rapid accumulation of cyclin B and MPF in the nucleus and induces nuclear envelope breakdown (NEB) (21, 22).

In this study, the correlation between calcium levels and Cdk1 expression was evaluated and significant correlation was observed. According to the theory, endometriosis that results in low calcium levels will also affect Cdk1. The study findings revealed that endometriosis leads to decreased calcium levels, which in turn affects cyclin B. Additionally, the correlation test demonstrated a significant relationship between Cdk1 expression and cyclin B expression, indicating that endometriosis not only causes low Cdk1 expression but also impacts cyclin B. Germinal vesicle breakdown (GVB) is preceded by a surge in the pre-ovulation LH hormone. Large amounts of cAMP and hypoxanthine can prevent GVB oocytes. The addition of LH to the culture medium can induce GVB. Induction likely occurs through an indirect action mediated by cumulus cells because there are no LH receptors in oocytes. The mechanism involving LH induces a disruption in the communication between oocytes and cumulus cells, resulting in the cessation of regulatory molecule flow into the oocytes. This induction is also mediated by the IP3/calcium pathway. Hormone signaling plays a crucial role in the transient mediation of calcium during GVB (23-25). Cdk1 undergoes phosphorylation at two critical regulatory sites. One of these phosphorylations occurs at threonine 161, which is necessary for the kinase activity of Cdk1, while the second phosphorylation takes place at tyrosine15 and threonine 14. The phosphorylation of tyrosine 15, catalyzed by a protein kinase known as Wee1, inhibits the activity of Cdk1 and promotes the accumulation of inactive Cdk1/cyclin B complexes (26).

When activated, the protein kinase Cdk1 promotes the phosphorylation of various target proteins that drive phase M events. In addition, Cdk1 activity triggers the degradation of cyclin B through ubiquitin-assisted proteolysis. This proteolytic degradation of cyclin B subsequently inhibits Cdk1, leading to the exit of the cell from meiosis (27).

C-MOS prevents the proteolytic degradation of cyclin B, resulting in the accumulation of cyclin B between meiosis I and II. This accumulation helps maintain high MPF activity during the metaphase arrest. Therefore, C-MOS kinase plays a crucial role in the proper progression of meiotic cleavage. Blocking C-MOS activity in mice oocytes by injecting antibodies into the C-MOS protein and antisense oligonucleotide can prevent meiosis II. In Xenopus, the sustained high MPF activity maintained by C-MOS leads to the suppression of DNA replication between meiosis I and II, facilitating cell division and preventing the parthenogenetic activation of oocytes (28, 29).

Two peaks of high MPF activity characterize oocyte maturation. The first occurs at the time of continuation of meiotic cleavage, and the second occurs during the cessation of meiosis at the MII stage. During the time interval between these two peaks, the MPF activity is sustained at a significant level (27-29).

Based on the findings of this study, a therapy targeting ion levels could be developed to enhance oocyte quality in patients with endometriosis, focusing on intracellular calcium, Cdk1, and cyclin B expression. Further research is required to assess changes in oocyte organelles, such as the endoplasmic reticulum and mitochondria, as well as to measure calcium levels, Cdk1 expression, and cyclin B expression specifically in relation to endometriosis.

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Conclusion

The data presented in this study suggested that the intracellular calcium level, Cdk1 expression, and cyclin B expression were lower in mice with endometriosis. A significant relationship exists between intracellular calcium ion levels with Cdk1 expression and cyclin B expression.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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