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Original Article

Correlation Between Vitamin D Administration and Reduced Expression of VEGF and MMP-9 in Abnormal Endometrial Stroma (Experimental in Mice Models with Endometriosis)

Windarti Windarti*[,1], Ashon Sa'adi[2], Widjiati Widjiati[3]

[1]*Master of Reproductive Health, Airlangga University, Surabaya, Indonesia*
[2]*Faculty of Medicine, Airlangga University, Airlangga, Indonesia*
[3]*Faculty of Veterinary Medicine, Airlangga University, Airlangga, Indonesia*

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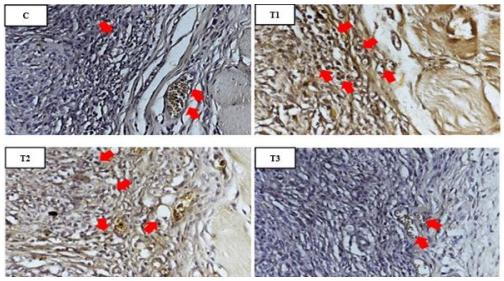
ABSTRACT

Background: Women with vitamin D deficiency are at higher risk of having endometriosis. Endometriosis is a benign gynecological disease that occurs in women of reproductive age with multifactorial etiopathogenesis with high migratory and invasive potential. Invasion requires angiogenesis derived from vascularization mediated by VEGF and initiated by MMP. Vitamin D acts on women reproduction in target gene regions by inhibiting cell proliferation in various cancer cells through induction of apoptosis and G0/G1 arrest, suppression of angiogenesis, and modulation of growth factor receptor expression.
This study aims to prove the role of graded vitamin D to decrease the expression of VEGF and MMP-9 administered to endometriosis model mice.
Methods: Experimental study with ethical due diligence certificate no. 2.KE.144.12.2021 used 24 endometriosis model mice divided into 4 groups; 1 Control Group and 3 treatment groups that were administered vitamin D orally at a dose of 8 iu, 16 iu and 24 iu. The expression of VEGF and MMP-9 was assessed from the peritoneal tissue of mice in all groups.
Results: It was found that there was a decreased average value of the expression of VEGF and MMP-9 of treated endometriosis model mice compared to that of untreated endometriosis model mice. The decrease in VEGF expression was not statistically significant, while the decrease in MMP-9 expression was statistically significant with a P value of 0.027. Therefore, there was a significant relationship between VEGF expression and MMP-9 expression with doses in the negative direction, the higher the dose, the lower the value of VEGF expression and MMP expression.
Conclusion: Vitamin D can suppress angiogenesis by reducing the expression of VEGF and MMP-9 in endometriosis model mice at a dose of 24 iu.

GRAPHICAL ABSTRACT



* Corresponding author: Windarti Windarti
✉ E-mail: windarti-2019@fk.unair.ac.id

Introduction

Endometriosis is a complex gynecological disorder which occurs in women of reproductive age caused by a combination of several genetic and environmental factors characterized by a high migratory and invasive potential such as tumor metastasis [1, 2]. The prevalence of endometriosis is 10 percent of women of reproductive age regardless of ethnicity and social status [3]. Various data on the prevalence of vitamin D deficiency which occurs in women of reproductive age in European, American and Asian countries vary from 42%-90% vitamin D: An overview of vitamin D status and intake in Europe [4, 5], whereas the rate of vitamin D deficiency in Indonesia reaches 63% [6]. The features of women of reproductive age who have endometriosis are chronic type, thus affecting their quality of life with the main symptoms of pain and infertility [7], which interfere with psychological and social functions [8, 9]. Increased activity of cytokines and steroid hormones in endometrial tissue increases Matrix Metalloproteinase-9 (MMP-9) expression and plays an important role in invasion and metastasis [10]. Vascular Endothelial Growth Factor (VEGF) expression plays a role to form the vascular tissue cycle by stimulating the degradation of the extracellular matrix around endothelial cells [11]. MMP-9 is a group of proteolytic enzymes that degrade components of the extracellular matrix in their progression [12]. Vitamin D can suppress cyclooxygenase 2 (COX 2) expression, thereby reducing levels of IL-6, TNF, and PG [13, 14]. Vitamin D can reduce the inflammatory response induced by IL-1β- or TNF-α so that the expression of MMP-2 and MMP9 decreases through inhibition of nuclear factor-κB [15]. Vitamin D also reduces VEGF-A expression in the stroma of endometriosis lesions, and can reduce VEGF levels in diseases such as PCOS [16-18]. Research on nutrition and food groups that correlate with endometriosis is limited to determining potential of disease risk factors in the endometriosis pathogenesis to food groups and nutrition. Vitamins A, C, and E have been widely researched to be correlated with endometriosis. However, the results show that there is no statistically significant relationship, and only vitamin D shows significant results [13]. The researchers are interested in conducting research on endometriosis model mice that are administered vitamin D supplements at doses of 8, 16, and 24 iu a decrease in the expression of MMP-9 and VEGF and the relationship of the decreased expression with the administered graded doses.

Materials and Methods

Experimental animals

The experimental animals were obtained from the Airlangga University Pusvetma. The research was carried out at the Laboratory of Veterinary Medicine Faculty, Airlangga University after obtaining a certificate of ethical due diligence no. 2.KE.144.12.2021.

Making endometriosis model mice

An endometriosis model mouse was mde by injecting cyclosporin A on the mice, and then intraperitoneal injection of 0.1 cc endometrial biopsy tissue was carried out on them and on the 5th day, the mice were administered intramuscular injection of estrogen with a dose of 5.4 µg/mouse. After 14 days, endometriosis model mice were obtained.

Research design and stages

This type of research is true experimental using a completely randomized design posttest only control group design. Using the randomization technique, the samples were categorized into 4 groups, specifically the control group (C) which was not given vitamin D, the group treated with dose I (T1), dose II (T2), and dose III (T3). The need for vitamin D was calculated according to the Endocrine Society (21) and sampling was carried out so that the mice in group T1 were administered as much as 8 iu vitamin D that equals to 0.2 cc; those in group T2 were administered as much as16 iu Vitamin D that equals to 0.4 cc and those in group T3 received 24 iu vitamin D equals to 0.6 cc. Vitamin D was administered individually once daily. Treatment began on day 15 after the occurrence of

endometriosis lesions by administering vitamin D according to the dose per group.

Examination on the expression of VEGF and MMP-9

Samples were taken from the peritoneal tissue of mice on day 37. The peritoneal tissue with endometriosis lesions was fixed in 10% formalin [19, 20]. VEGF expression was analyzed using immunohistochemical techniques. Staining was carried out using DAB Kit with Coomasie brilliant blu counterstaining. MMP-9 expression analysis was performed by immunohistochemical staining using MMP-9 polyclonal antibody. The sample was evaluated using the Remmele scale index (Immuno Reactive Score/IRS) in a semi-quantitative manner. The assessment involved multiplying the percentage score of immunoreactive cells by the color intensity score on these cells, as listed in Table 1. For each sample, the data represented the average IRS value observed across ten different field of views using the magnifications of 100x and 400x.

Results and Discussion

Effect of vitamin D on VEGF expression

Figure 1 displays the impact of administering vitamin D supplements to mice with an endometriosis model. This figure shows that there is VEGF expression in cell tissue of endometriosis lesions taken from the peritoneum of endometriosis model mice. Accordingly, group C has stronger expression and it is clustered, compared to group P1, P2, and P3 which have weaker expression and it is dispersed. The figure on VEGF expression is in accordance with the graph on average values which tends to decrease in Figure 2. VEGF expression in the control group had an average value of 5.6; the intervention group 1 with a dose of 8 iu had an average value of 5.5, intervention group 2 with a dose of 16 iu had an average value of 4.3, and the lowest was the intervention group 3 with a dose of 24 iu that had an average value of 3.8.

Table 1: Post Hoc Bonfferoni test of MMP-9 expression conducted on Endometriosis Model mice supplemented with vitamin D

	Average value difference	P-value
C vs. T1	1.67	0.213
C vs. T2	1.75	0.168
C vs. T3	2.37	0.027
T1 vs. T2	0.08	1.000
T1 vs. T3	0.70	1.000
T2 vs. T3	0.62	1.000

C: A group that serves as a control; T1: A group that receives an intervention with 8 iu of vitamin D; T2: A group that receives an intervention with 16 iu of vitamin D; T3: A group that receives an intervention with 24 iu of vitamin D.

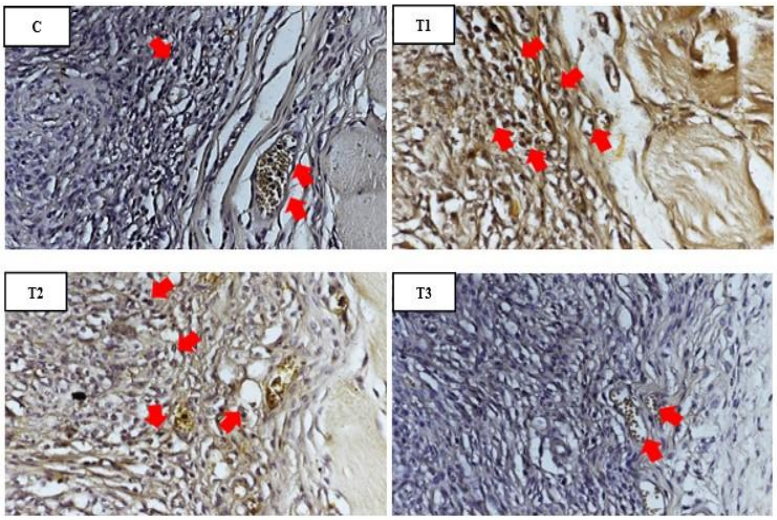


Figure 1: VEGF expression on endometriosis model mice tissue shown by red arrows

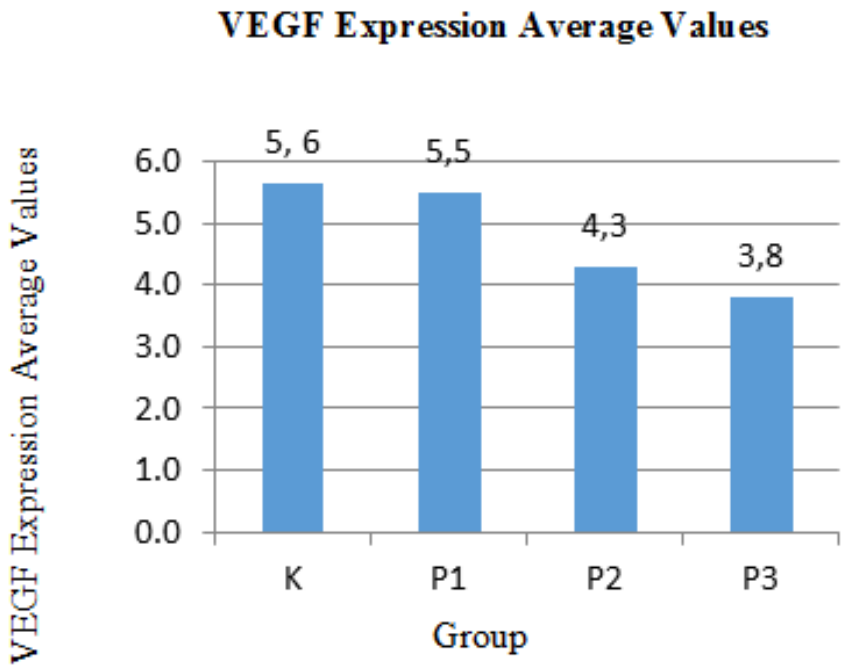


Figure 2: Distribution of VEGF expression average values in endometriosis model mice

Based on the graph, mice with an endometriosis model that received vitamin D supplementation at doses of 8 iu, 16 iu, and 24 iu showed decreased VEGF expression compared to mice with the endometriosis model without vitamin D supplementation. Among the different groups, the mice receiving 24 iu of vitamin D supplementation demonstrated the least amount of VEGF expression

Effect of Vitamin D on MMP-9 Expression

Figure 3 demonstrates the impact of vitamin D on the MMP-9 expression. In addition, Figure 4 displays the MMP-9 expression in the tissue of mice with an endometriosis model.

The depicted graphs and figures illustrate the effects of vitamin D supplementation at doses of 8 iu, 16 iu, and 24 iu on mice with an endometriosis model had lower MMP-9 expression compared to that of endometriosis model mice that were not supplemented with vitamin D and after the One-way ANOVA test was carried out, P = 0.027 was obtained; thus at the real level we reject Ho. Hence, there is a significant difference in the average values of MMP-9 expression in the four groups. These results are in line with Befferoni's Post Hoc analysis presented in Figure 4.

The association between varying doses of vitamin D supplementation and the variables of VEGF expression and MMP-9 expression in lesions of mice with an endometriosis model was examined using Spearman's Rho test, and the findings are presented in Table 2.

Based on the results of the Spearman's Rho test on VEGF expression with graded dose of vitamin D, p-value of 0.022 and MMP-9 of 0.007 with graded doses of vitamin D are obtained. Based on these results, it can be concluded that there is a significant relationship between the two variables. Whereas, judging from the correlation coefficient, it is shown that the two variables have a moderate correlation and negative direction, as shown in the following scatter plot graph (Figure 5).

The scatter plot graph depicted above exhibits a distinct pattern wherein the data points align in a straight line, extending from the lower right corner to the upper left corner. This pattern indicates a linear and negative correlation between the dosage of vitamin D and the expression levels of VEGF and MMP-9. In simpler terms, as the dosage of vitamin D increases, the expression of VEGF and MMP-9 decreases, and vice versa.

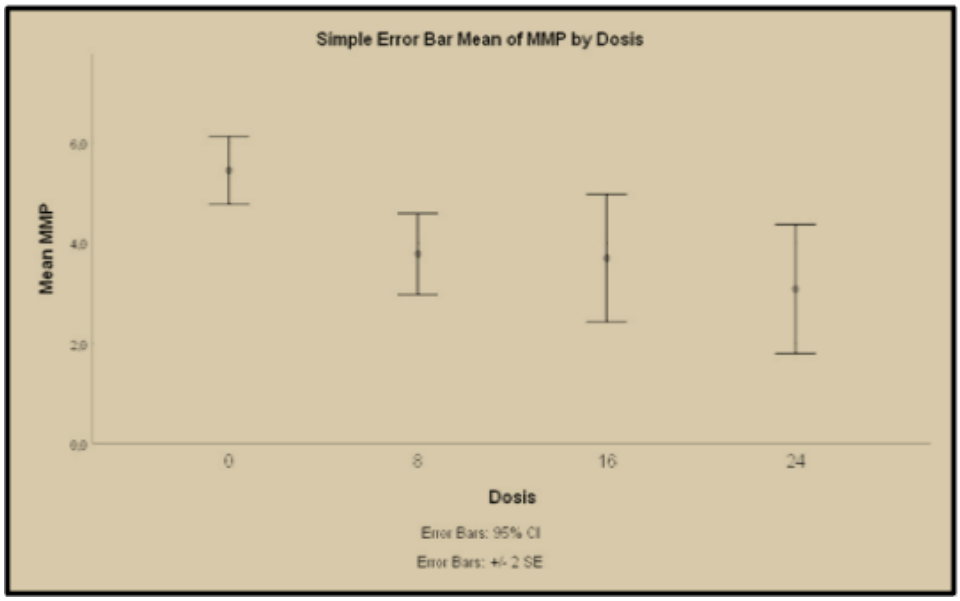


Figure 3: Graph on MMP-9 expression value distribution on endometriosis model mice Control group (C) zero dose. Intervention Group 1 (T1) with a dose of vitamin D 8 iu, Intervention Group 2 (T2) with a dose of vitamin D 16 iu, and Intervention Group 3 (T3) with a dose of vitamin D 24 iu

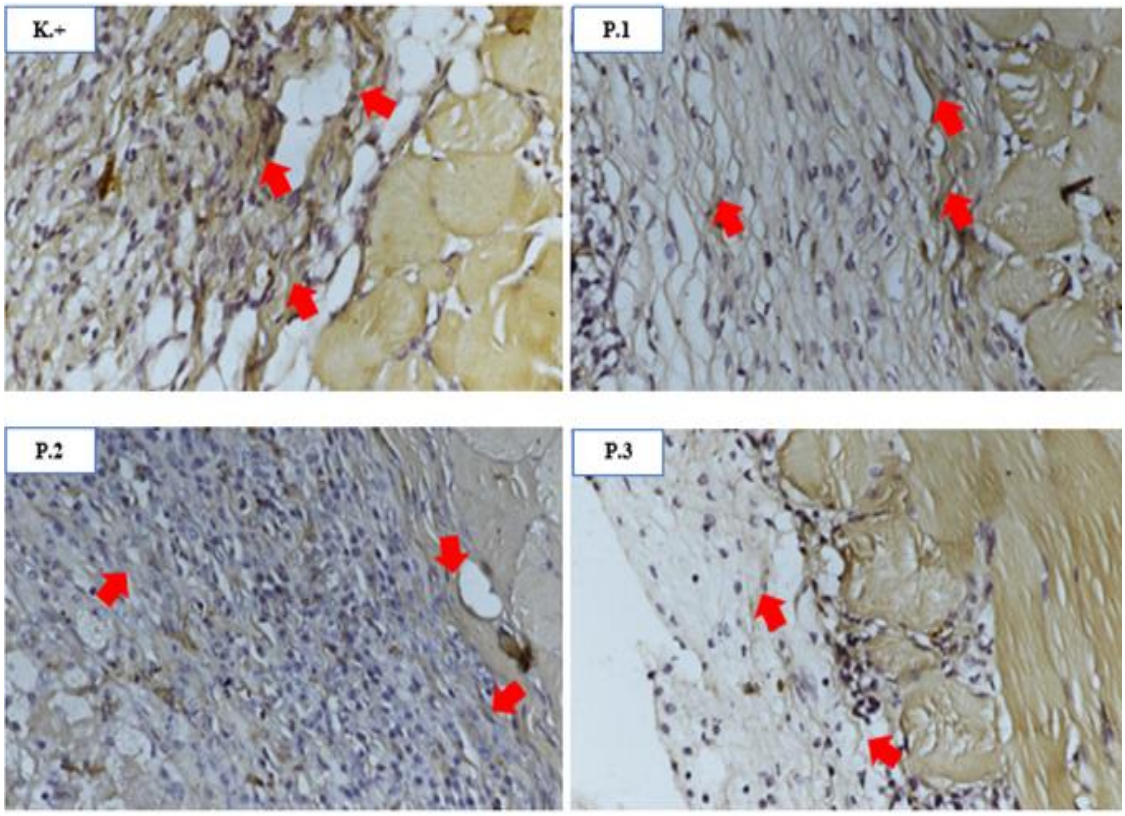


Figure 4: Images on MMP-9 expression at endometriosis model mice tissue shown by red arrows

Table 2. The relationship on vitamin D supplementation at various doses with the expression of VEGF and MMP-9 at Endometriosis Mice Model

No.	Relationship	Correlation coefisient	P-value
1	VEGF and Dose	-0.467*	0.022
2	MMP and Dose	-0.539**	0.007

Notes: Relationship on vitamin D supplementation at various doses with the expression of VEGF and MMP-9

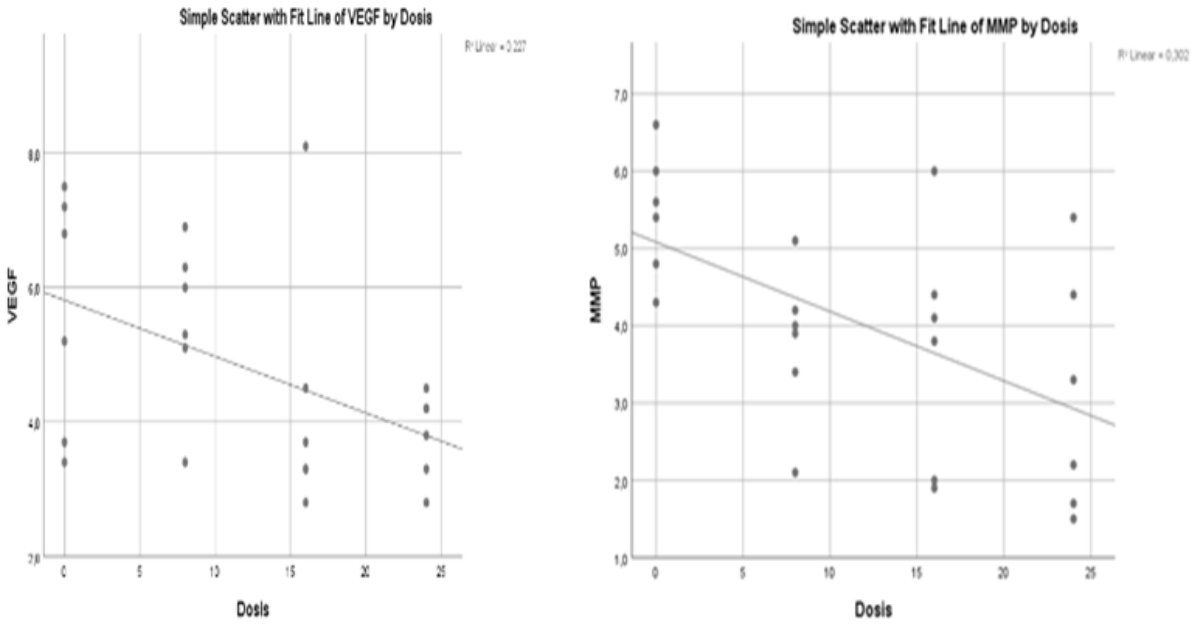


Figure 5: Scatter plot graph on expression of VEGF dan MMP -9 at Endometriosis Model mice supplemented with vitamin D

In this study, a heterologous endometriosis induction method was employed to create the endometriosis model. The process involved the injection of human endometrial tissue into the peritoneal cavity of mice. The levels of VEGF and MMP-9 expression were assessed in the resulting endometriosis lesions. This method provides several benefits for investigating the initial phases of the disease, encompassing factors like angiogenesis, abnormal cell death (apoptosis), proliferation of endometrial cells, and inflammation [21].

Furthermore, this approach closely mimics the pathophysiology of retrograde menstruation in humans, making it a valuable tool for studying endometriosis lesions in humans [22]. According to Sampson's theory of retrograde menstruation, endometriosis develops when shed endometrial tissue during menstruation migrates to the common attachment sites on the peritoneal wall, invades the extracellular matrix (ECM), and proliferates to form endometriotic lesions. Endometriosis is characterized by the presence of eutopic and ectopic endometrial stromal cells (ESC), which exhibit invasive, adhesive, and proliferative properties. Women diagnosed with endometriosis exhibit decreased apoptosis and elevated expression levels of vascular endothelial growth factor (VEGF), urokinase plasminogen activator, and matrix metalloproteinase-3 (MMP-3) in their endometrial tissues [23]. VEGF is a heparin-binding glycoprotein secreted in the form of a homodimer (45 kDa). Heparin interacts with VEGF through the formation of the Heparin-VEGF complex which leads to changes in molecular conformation so that VEGF becomes more stable, and is resistant to inactivation and has a longer half-life. The formation of the Heparin-VEGF complex also leads to an increase in the affinity of VEGF receptors that exist on the cell surface so that intracellular signals are formed as a means of proliferation to activate. Under normal circumstances, VEGF is expressed to varying degrees by different tissues, including the brain, kidney, liver, and spleen. Exposure to hypoxic conditions induces rapid expression of VEGF. In contrast, under the conditions of normal oxygen levels (normoxia), VEGF expression decreases and stabilizes [24]. The results of the study on VEGF expression of endometriosis model mice supplemented with various doses of Vitamin D showed a decreasing trend when compared to that of endometriosis mice that were not supplemented with vitamin D. These findings indicate that the vitamin D receptor (VDR) is consistently expressed in all types of endometrial cells, and specifically in stromal cells. The endometrium, being responsive to

vitamin D, possesses the capacity to transform 25(OH) vitamin D3 into its active state. These discoveries provide support for the involvement of vitamin D in the reproductive hormonal processes [16]. Research has exposed that vitamin D plays a role in inhibiting cell growth in various cancer cells. This effect is achieved through the induction of apoptosis (cell death) and G0/G1 arrest, as well as through the suppression of angiogenesis (the formation of new blood vessels) and the modulation of expression of growth factor receptors. These findings highlight the significance of vitamin D in controlling cellular processes related to cancer development [25]. VEGF expression of the treatment group supplemented with vitamin D at various doses, sequentially from the highest to the lowest was shown in group T3, T2, T1, and C, in the T3 group, which received a 24 iu dose of vitamin D supplementation, the VEGF expression in the lesions was found to be the lowest. Dalbandi's study additionally demonstrated that treatment with vitamin D enhanced the attachment of endometrial stromal cells (ESC) from various sources to the extracellular matrix (ECM). In contrast, it was observed that the VDR agonist, elocalcitol, reduced the capacity of endometrial cells in mice to bind to collagen.

Excess VEGF in abnormal endometrial stroma: (1) VEGF stimulates the formation of new blood vessels, so excess VEGF in abnormal endometrial stroma can cause an increase in blood vessels in the tissue, (2) VEGF can also stimulate the proliferation of endothelial cells (cells lining blood vessels), which can contribute to the abnormal growth and expansion of endometrial stroma, (3) VEGF can increase vascular permeability, which can facilitate the movement of cells and substances into tissues, including cells involved in inflammatory or disease processes [26]. VEGF deficiency in abnormal endometrial stroma: (1) Deficiency of VEGF can inhibit the formation of new blood vessels needed to supply nutrients and oxygen to tissues. This can lead to disturbances in growth and abnormal endometrial stroma function and (2) VEGF also plays a role in the wound healing process. VEGF deficiency can inhibit the healing process in damaged or abnormal endometrial stroma [27]. Vitamin D exerts its effects at an optimal concentration dose of 10-7 M, which is crucial for cellular maintenance and corresponds to the physiological levels of reproductive hormones [16]. The expression level of VEGF is also influenced by the presence of inflammatory cytokines and growth hormone, including Epidermal Growth Factor (EGF), Interleukin-1β, platelet derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-1β)[24]. MMP normally regulates macrophage activity by degrading the extracellular matrix of cells to be phagocytosed [28]. Excess MMP-9 in abnormal endometrial stroma:

(1) MMP-9 is an enzyme involved in extracellular matrix degradation. MMP-9 excess may lead to increased extracellular matrix degradation in abnormal endometrial stroma, which may contribute to structural changes and tissue softness, (2) MMP-9 can facilitate the invasion of cells into healthy tissue. Excess MMP-9 in abnormal endometrial stroma can increase the ability of cells to spread beyond their normal area and invade surrounding tissue [29]. MMP-9 deficiency in abnormal endometrial stroma: (1) Deficiency of MMP-9 may interfere with the extracellular matrix remodeling process required for normal changes in the endometrial stroma during the menstrual cycle. This can contribute to abnormal stromal development or interference in the tissue regeneration process and (2) MMP-9 deficiency can inhibit the ability of cells to move and spread to new areas. This may affect the ability of the abnormal endometrial stroma to invade surrounding tissue or participate in proliferation and healing processes [30]. Changes in the expression of MMP family members in the endometrium with endometriosis may represent a key mechanism which relates to the invasive potential of endometrial reflux to have this disease [31]. Regulation of MMP expression in the endometrium is mediated by steroids that specifically influence the formation of ectopic lesions. Increased MMP expression in endometriosis occurs due to a decrease in the progesterone action and an increase in proinflammatory cytokine exposure, which leads to an increase in the ability to build ectopic

growths in endometriosis [31]. This study significantly showed that the endometriosis model mice that were supplemented with vitamin D had lower MMP-9 expression compared to that of the endometriosis model mice that were not supplemented with vitamin D. Among the different groups, the administration of vitamin D at a dosage of 24 iu exhibited the lowest MMP-9 expression in the lesions when compared to the other groups. The results of this study corroborate the research of Garcia-Gomes *et al.*, where MMP-9 had decreased expression (mRNA and protein) and enzyme activity, caused by the presence of prostaglandins (PGE2) in the peritoneal fluid [32]. In addition, PGE2 activity takes place by the EP2/EP4 (receptor for MMPs) which depends on PKA pathway. Another study reported that inactivation of phagocytosis by PGE2 via CD36 inhibition is mediated by EP-2 [28]. With the decrease in MMP-9 expression, the ability to develop invasive ectopic growth also does not occur [31]. Vitamin D affects angiogenesis, changes cell adhesion, migration, and reduces cancer cell invasion. Vitamin D not only reduces expression and secretion of metalloproteinase (MMP) 2 and 9, but also decreases cathepsin K activity, increases tissue inhibitory MMP1 (TIMP1) and regulates various components of the plasminogen activator system [33]. Based on the results of the dose correlation test for VEGF expression in this study, it was concluded that there was a significant relationship between VEGF expression and dose, while the correlation coefficient showed that the two variables had a negative correlation/relationship. Decreased expression of VEGF gene after treatment with Vitamin D is a possible beneficial effect of this hormone in patients with endometriosis. In a recent study by Yildirim *et al.*, it was observed that endometriosis mice treated with vitamin D exhibited lower VEGF immunoreactivity compared to the control group. Decreased expression of VEGF (Vascular Endothelial Growth Factor) and MMP-9 (Matrix Metalloproteinase-9) in abnormal endometrial stroma can be correlated with the given dose of vitamin D because vitamin D has a role in regulating gene expression and enzyme activity. Several factors that can explain this correlation are as follows:

(1) Genetic regulation: Vitamin D can interact with vitamin D receptors (VDR) present on target cells, including endometrial stromal cells. VDR activation by vitamin D will affect gene expression, including genes involved in the production of VEGF and MMP-9. High doses of vitamin D can produce different gene regulatory effects than low doses or vitamin D deficiency [34].

(2) Immunomodulatory effect: Vitamin D also has an immunomodulatory effect, which can affect the activity of immune system cells, including endometrial stromal cells. VEGF and MMP-9 can be produced by immune cells, including macrophages and endometrial stromal cells. High doses of vitamin D can alter the immune response and inhibit the production of VEGF and MMP-9 by these cells.

(3) Inhibition of angiogenesis: Studies have shown that vitamin D can inhibit the process of angiogenesis by inhibiting the VEGF expression. High doses of vitamin D can amplify this inhibitory effect, potentially reducing VEGF expression in the abnormal endometrial stroma.

(4) Inhibition of enzyme activity: Vitamin D can affect enzyme activity, including MMP-9 enzyme activity. High doses of vitamin D can inhibit the activity of this enzyme, which can reduce MMP-9 expression in the abnormal endometrial stroma [35].

Conversely, according to Dalbandi *et al.*, vitamin D did not have an impact on the proliferation of endometrial stromal cells (ESC) in any of the groups studied in the absence of fibronectin. These findings emphasize the potential therapeutic use of vitamin D3 in preventing the dissemination of endometriosis. Consistent with Dalbandi's findings, Liu *et al.* provided evidence that the signaling process facilitated by extracellular fibronectin played a pivotal role in the proliferative reaction of thyroid cancer cells to vitamin D. In another study by Dalbandi *et al.*, it was found that vitamin D administration led to a decrease in VEGF expression in endometrial epithelial cells (EESC) at 18 and 48 hours after treatment, depending on the specific time points. However, no significant effects of vitamin D were

observed in other cell groups across all time intervals [16]. While the conclusion of the correlative test results for MMP-9 expression at various doses in this study was significantly strong in the medium category and in the negative direction, the higher the dose, the lower the MMP-9 value.

In a pilot study conducted by Dalbandi, it showed that Vitamin D exerts its effects that depends on a dose. Predetermined optimal concentration of 10-7 M Vitamin D for cell maintenance equals to physiological levels of reproductive hormones. According to [16], vitamin D exhibits a biphasic effect on cell proliferation, where it suppresses cell growth at concentrations higher than 10-8 M.

In their study, [10] discovered that they could regulate the levels of VEGF, MMP-9, and tissue inhibitor of metalloproteinase-2 (TIMP-2) in mice with an endometriosis model, it was possible to induce apoptosis in endometriotic lesions. Elevated 1α-hydroxylase in patients with endometriosis leads to local production of the active form of vitamin D, 1.25 (OH)2D3 that reduces endometriosis cell growth. The high expression of key vitamin D enzymes in the endometrium of patients with endometriosis underlines the potential for local autocrine or paracrine responses rather than the classic endocrine effects of vitamin D [35].

This suggests that Vitamin D has the ability to inhibit the growth of ectopic endometrial cells. [15] provided evidence to support the notion that vitamin D hampers the nf-κB activation, as a result, there was a decrease in the quantity and DNA replication of abnormal endometrial cells. Furthermore, the administration of vitamin D to the mice with the endometriosis model caused a decline in the expression of VEGF-A, VEGF, and MMP-9 in the abnormal endometrial stroma, effectively suppressing the angiogenic potential associated with endometriosis [25],[16]. Delbandi *et al.* (2016) also demonstrated the positive effects of vitamin D on endometriosis stromal cells by decreasing the rates of invasion and proliferation of the abnormal endometrial cells.

Moreover, [36] concluded that vitamin D played a role in regressing the extent of endometriosis lesions through the angiogenesis modulation, this was accomplished by reducing the VEGF expression and inhibiting the MMP-9 activity.

Conclusion

The finding of this research provide support for the pathogenesis theory of endometriosis and confirm the findings of Vitamin D to treat targeted mediators and molecules related to inflammation in the endometriosis treatment. The decreased expression of VEGF and MMP-9 significantly correlated with dose, and with higher dose of Vitamin D, the expression of VEGF and MMP-9 in the abnormal endometrial stroma was diminished more significantly so that the capacity for endometriosis angiogenesis in endometriosis stromal cells decreased its level of invasion and proliferation.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

ORCID

Windarti Windarti
https://orcid.org/0000-0002-6264-2418
Ashon Sa'adi
https://orcid.org/0000-0003-2682-3139
Widjiati Widjiati
https://orcid.org/0000-0002-8376-1176

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