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ARTICLE

Effect of active vitamin D on proliferation, cell cycle and apoptosis in endometriotic stromal cells

BIOGRAPHY

Ali-Akbar Delbandi was born in Tehran in 1974 and graduated in immunology at Mashhad University of Medical Sciences. He has been a faculty member at the Iran University of Medical Sciences since 2014. His research focuses on reproductive immunology with an emphasis on endometriosis.

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KEY MESSAGE

The effects of $1,25(OH)_2D_3$ (active vitamin D) on endometriosis were assessed; it prevented the proliferation of endometriotic stromal cells, and simultaneously induced apoptosis. Increased sensitivity of endometriotic cells to apoptosis might prevent the distribution and implantation of endometriotic stromal cells to ectopic sites. Vitamin D might prevent the progression of endometriosis by reducing the proliferation of these cells.

ABSTRACT

Research question: What is the effect of vitamin D3 ($1,25(OH)_2D_3$) on proliferation, cell cycle and apoptosis of endometrial stromal cells (ESC) in endometriotic patients?

Design: ESC isolated from 10 women with endometriosis and 10 healthy controls were treated with $1,25(OH)_2D_3$. The proliferation of control endometrial stromal cells (CESC), eutopic endometrial stromal cells (EuESC) and ectopic endometrial stromal cells (EESC) was analysed 72 h after the treatment using methyl thiazolyl tetrazolium assay. Propidium iodide staining and flow cytometry were used to determine the cell cycle distribution in ESC. Annexin V/propidium iodide double staining was used to evaluate apoptosis in ESC.

Results: In the presence of oestrogen, $1,25(OH)_2D_3$ treatment inhibited the proliferation of ESC from all three origins ($P = 0.009$ for CESC, $P = 0.005$ for EuESC and $P < 0.001$ for EESC). The percentage of S phase cells in EESC was higher than in EuESC and CESC ($P = 0.002$ and $P = 0.001$, respectively). The percentage of S phase cells in EuESC was higher than in CESC ($P = 0.005$). The percentage of G1 phase cells in EESC was lower than that of EuESC and CESC ($P = 0.003$ and $P = 0.002$, respectively) and the percentage of G1 phase cells in EuESC was lower than that of CESC ($P = 0.007$). Moreover, $1,25(OH)_2D_3$ inhibited cell cycle regardless of cell type ($P = 0.002$ in EESC, $P = 0.001$ in EuESC and $P = 0.014$ in CESC), but in the absence of oestrogen, inhibited cell cycle only in EuESC ($P = 0.012$).

Conclusions: Although $1,25(OH)_2D_3$ increased apoptotic and necrotic cells and decreased live cells in the EuESC and EESC, it did not affect apoptosis in CESC and only increased necrotic cells. These findings indicate that $1,25(OH)_2D_3$ potentially has a growth-inhibiting and pro-apoptotic effect on ESC from endometriotic patients.

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KEYWORDS

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INTRODUCTION

Endometriosis is a prevalent, oestrogen-dependent and chronic gynaecological condition that affects 5–10% of women of reproductive age (*Giudice and Kao, 2004*; *Taylor* et al., *2021*). The major symptoms of the disorder include chronic pelvic pain, dysmenorrhoea, dyspareunia and infertility, which can impair young women's quality of life (*Giudice and Kao, 2004*). Although there are various theories to explain the causes of endometriosis, the most widely accepted is Sampson's retrograde menstrual theory (*Sampson, 1927*). According to Sampson's theory, retrograde menstruation induces the entry of endometrial cells/tissue into ectopic sites (peritoneum), where the overexpression of integrins facilitates their implantation. The endometrial cells in the peritoneum have high proliferation and the ability to implant and survive. Also, these cells have lost their spontaneous sensitivity to apoptosis, which plays an important role in their abnormal implantation and growth in ectopic tissue (*Harada* et al., *2004*).

Several pieces of evidence have shown that cell growth is precisely controlled by the cell cycle (*Shiozawa* et al., *1998*). Various cell cycle-related components, such as cyclins and cyclin-dependent kinases, regulate the cell cycle (*Draetta, 1994*). Also, changes in these molecules are regulated by sex hormones, such as oestrogen and progesterone, which are lost in endometriosis (*Pellegrini* et al., *2012*). So, a characteristic of the endometrium in patients with endometriosis is an incomplete transition from the oestrogen-dependent proliferative phase to the progesterone-dependent secretory phase. Continuous contact with oestrogen leads to the expression of genes involved in DNA synthesis and cellular mitosis, such as cyclins in endometriotic lesions (*Velarde* et al., *2009*; *Yotova* et al., *2011*). Studies have shown that cyclin B1 and D1, as key cell cycle regulators, are up-regulated during endometriosis and are responsible for proliferative events in endometriosis (*Pellegrini* et al., *2012*; *Tang* et al., *2009*). The endometrium of women with endometriosis shows fundamental differences from healthy women. One of these differences is resistance and tolerance to apoptosis in endometriotic stromal cells. Apoptosis is a special type of programmed cell death that results in adequate cell removal without inducing an inflammatory response (*Taniguchi* et al., *2011*). Impaired sensitivity of endometrial tissue to spontaneous apoptosis contributes to abnormal implantation and growth of endometrium at ectopic sites, which is associated with increased expression of anti–apoptotic factors (Bcl–2) and decreased expression of pre–apoptotic factors (BAX) (*Meresman* et al., *2000*).

Therapeutic strategies for endometriosis are limited to surgical procedures and hormonal interventions, the former of which is associated with high recurrence (approximately 22% at 2 years and 40–50% at 5 years) and the latter of which cannot be performed for those who wish to conceive (*Guo, 2009*; *Vercellini* et al., *2014*). Considering the inflammatory conditions, high proliferative capacity and resistance to apoptosis in endometriosis, immunomodulatory compounds are used for its treatment today. Therefore, due to the proven antiproliferative and pro-apoptotic effects of vitamin D in tumour settings, it is considered for endometriosis therapy (*Crosignani* et al., *2006*, *Liu* et al., *2018*). $1,25(OH)_2D_3$ is a physiologically active metabolite of vitamin D3, a pleiotropic hormone, and a key regulator of calcium and phosphorous homeostasis (*Bouillon* et al., *2008*; *Grzesiak, 2020*; *Holick, 2003*). In addition to the classic actions, a growing body of evidence suggests that vitamin D plays a role in reproductive health (*Cermisoni* et al., *2018*). So vitamin D, along with sex and steroid hormones as classic regulators of reproduction, regulates reproductive processes in women and men (*Lerchbaum and Obermayer-Pietsch, 2012*). It exerts its effect by binding to the vitamin D receptor (VDR), which is found in the female reproductive system, including the placenta, ovaries and endometrium (*Keane* et al., *2017*).

The antiproliferative and anti-apoptotic properties of vitamin D, low serum and peritoneal fluid concentrations of vitamin D in patients with endometriosis compared with the control group, as well as the expression of VDR in reproductive tissues, mean that vitamin D may play a role in the aetiology of endometriosis (*Delbandi* et al., *2021*). VDR signalling in cells induces growth arrest, differentiation and/or induction of death, which especially reveals the role of vitamin D signalling in cell growth suppression (*Halder* et al., *2013*). Various studies have shown the antiproliferative and pro-apoptotic properties of vitamin D3 on various cells (*Bläuer* et al., *2009*; *González-Pardo* et al., *2014*; *Lange* et al., *2007*; *Narvaez and Welsh, 1997*; *Sharan* et al., *2011*). Few studies have investigated the pro-apoptotic and antiproliferative effects of $1,25(OH)_2D_3$ on endometrial stromal cells (ESC). However, no studies have been conducted on the regulatory effects of $1,25(OH)_2D_3$ on ESC proliferation, cell cycle and apoptosis in the presence of oestrogen. In this study, it was postulated that $1,25(OH)_2D_3$ could modulate proliferation, cell cycle and apoptosis in human endometriotic and non-endometriotic cells in the presence or absence of oestrogen.

MATERIALS AND METHODS

Patient selection

Ten women with laparoscopically and histologically confirmed endometriosis (mean age: 26–36 years old) and 10 women without any evidence of endometriosis despite careful evaluation by an experienced laparoscopic surgeon (mean age: 22–34 years old) in the gynaecology ward of Erfan and Omid Hospital, Tehran, Iran, were enrolled in the study. All eligible women had regular menstrual cycles and were at the proliferative phase of the menstrual cycle. Subjects had not received any hormone or vitamin D supplements for at least 3 months before the sampling. Patients with a history of pelvic inflammatory diseases, adenomyosis, autoimmune disorders and malignancies were excluded from the study. All endometriotic patients were classified at stage III–IV according to the revised American Fertility Society system (rAFS) (*American Society for Reproductive Medicine, 1997*). Ectopic endometrial biopsies were collected during laparoscopy, and eutopic samples from endometriotic patients and control subjects were obtained through a biopsy curette. A small portion of the excised ectopic tissues was evaluated histologically for approval of endometriosis. All tissue samples from different sources were put in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) (Gibco, Germany) containing 1% penicillin-streptomycin antibiotics (Gibco, Thermo Fisher Scientific, USA) and immediately transferred to the laboratory for the stromal cell isolation. All participants provided signed written informed consent and the study protocols were approved by the Ethics Committee of the Iran University of Medical Sciences

(Ethics Code: IR.IUMS.FMD.REC.1401.280, 21 November 2017).

Isolation, culture and purification of ESC

In order to perform proliferation, cell cycle and apoptosis assays, ESC were isolated from fresh ectopic (n = 10) and eutopic (n = 10) endometrial samples from endometriotic patients and eutopic tissues (n = 10) from non-endometriotic controls (*Delbandi* et al., *2013*). Briefly, tissues were minced into small pieces and digested with 2 mg/ml collagenase D (Sigma, USA) and 300 mg/ml DNase (Roche, Germany) for 1.5 h with vortexing every 10 min at 37°C. To remove undigested tissue fragments, the cell suspension was passed through a 100-μm mesh (BD Biosciences, USA). Then, the isolated cells were seeded in a T25 culture flask for 12 h in 5% CO_2 in DMEM-F12 culture media containing 1% penicillin-streptomycin antibiotics. Subsequently, after two passages, the adherent cells were harvested and immunofluorescent labelling and flow cytometry were used to assess the purity of the isolated ESC. Then ESC were defined as nestin$^+$, CD10$^+$, cytokeratin$^-$ and CD45$^-$ cells (*Arablou* et al., *2019*).

Treatment of ESC with oestrogen and $1,25(OH)_2D_3$

To evaluate the optimal time point for the effect of oestrogen on the proliferation of ESC, the cells were treated with 10 nmol/l oestrogen (*Al-Hendy* et al., *2015*) for 24, 48 and 72 h. After that, the cells were stimulated with or without oestrogen (10 nmol/l and 10 μmol/l) for 72 h. Furthermore, the oestrogen-induced (10 nmol/l and 10 μmol/l) and non-induced ESC were treated in the presence or absence of 10 nmol/l $1,25(OH)_2D_3$.

Cell proliferation assay

The methyl thiazolyl tetrazolium (MTT) proliferation assay was used to evaluate the proliferative potential of ESC. To investigate the basal proliferative activity of ESC, the ESC were seeded in a 96-well plate (7.5×10^3 cells/well) in DMEM-F12 supplemented with 5% fetal bovine serum (FBS) (Gibco). Also, to investigate the effect of $1,25(OH)_2D_3$ on cell proliferation in the absence and presence of oestrogen, ESC were seeded in a 96-well plate (7.5×10^3 cells/well) and were treated with 10 nmol/l $1,25(OH)_2D_3$ in the presence or absence of 10 nmol/l oestrogen for 72 h. Then, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) assay solution was added to each well and incubated at 37°C in 5% CO_2. After 4 h, the MTT-containing medium was removed, and 100 μl of dimethylsulphoxide (DMSO, Sigma-Aldrich) was added to each well and the plate was shaken for 15 min to solubilize formazan crystals. Finally, the optical density (OD) was measured at 570 nm using a microplate reader (Biohit, Finland). All experiments were performed in triplicate.

Cell cycle analysis

To investigate the cell cycle distribution, 2×10^5 cells were cultured in 24-well culture plates in DMEM-F12 supplemented with 5% FBS. Also, to evaluate the effect of $1,25(OH)_2D_3$ on cell cycle phases in the absence and presence of oestrogen, 2×10^5 cells were treated with 10 nmol/l $1,25(OH)_2D_3$ in the presence or absence of 10 nmol/l oestrogen. After 72 h, the supernatants were collected and the harvested cells were rinsed with ice-cold phosphate-buffered saline (PBS) and fixed by gently adding ice-cold 70% ethanol to the cell suspension. Then, the cells were centrifuged and the cell pellet was carefully resuspended in 1x DNA-staining solution (propidium iodide [BD Biosciences] in PBS) and maintained at 37°C for 20 min. After that, DNA content was determined using a flow cytometer (BD FACSCalibur, BD Biosciences) and the distribution of cells in different cell cycle phases was assessed. Finally, the data were analysed using FlowJo 7.6.1 software.

Apoptosis analysis

To investigate the apoptosis in ECS, 2.0×10^5 ESC were cultured in 24-well culture plates in culture medium supplemented with 1% FBS for 96 h in the presence of 10 nmol/l oestrogen. Also, to evaluate the effect of $1,25(OH)_2D_3$ on apoptosis in ESC, 2.0×10^5 ESC were cultured in 24-well culture plates and treated with 10 nmol/l $1,25(OH)_2D_3$ in the presence or absence of 10 nmol/l oestrogen in culture medium supplemented with 1% FBS for 96 h. Then, the supernatants were collected, and the harvested cells were incubated for 15 min with annexin V and propidium iodide (IQ Products, USA). The BD FACSCalibur was used to assess cell apoptosis. The data were analysed using FlowJo 7.6.1 software. The percentage of cells in each population was presented as Q1 = necrotic cells; Q2 = late apoptotic cells; Q3 = early apoptotic cells; Q4 = live cells.

Statistical analysis

The data were statistically analysed using GraphPad Prism software, version 8. Data are shown as mean ± SD. Based on the non-parametric distribution of data, Mann–Whitney U-test was used to compare variables between the control and treated groups ($1,25(OH)_2D_3$ and oestrogen) in the proliferation test, differences between different phases in the control and treated groups in the cell cycle test, and differences between the percentage of each cell population in the apoptosis assay. P-values <0.001 (***), <0.01 (**) and <0.05 (*) were considered statistically significant.

RESULTS

Basal proliferation of ESC and effect of $1,25(OH)_2D_3$ on proliferation in ESC

The MTT proliferation test was used to investigate the proliferative ability of ESC from three different origins (FIGURE 1). Based on these findings, the basal proliferation capacity of ectopic endometrial stromal cells (EESC) and eutopic endometrial stromal cells (EuESC) was significantly higher than that of endometrial stromal cells from control subjects (CESC) (P = 0.003 and P = 0.006, respectively) (FIGURE 1A). The results showed that $1,25(OH)_2D_3$ inhibited EuESC proliferation in the absence of oestrogen (P = 0.006). Such treatment also reduced the proliferation of EESC, but the effect was not statistically significant (P = 0.08) (FIGURE 1B). In the presence of oestrogen, $1,25(OH)_2D_3$ reduced the proliferation capacity of EESC, EuESC and CESC (P < 0.001, P = 0.005 and P = 0.009, respectively) (FIGURE 1C).

Cell cycle distribution of ESC and the effect of $1,25(OH)_2D_3$ on cell cycle distribution in ESC

To evaluate the cell cycle distribution, a DNA content analysis was performed (FIGURE 2). The results showed that the percentage of S phase cells in EESC was higher than that in EuESC and CESC (P = 0.002 and P = 0.001, respectively). Also, the percentage of S phase cells in EuESC was higher than that of the CESC (P = 0.005). Additionally, the percentage of G1 phase cells in EESC was lower than that of EuESC and CESC (P = 0.003 and P = 0.002, respectively), and the percentage of G1 phase cells in EuESC was lower than that of CESC (P = 0.007) (FIGURE 2A and 2B). $1,25(OH)_2D_3$ treatment significantly decreased the percentage of

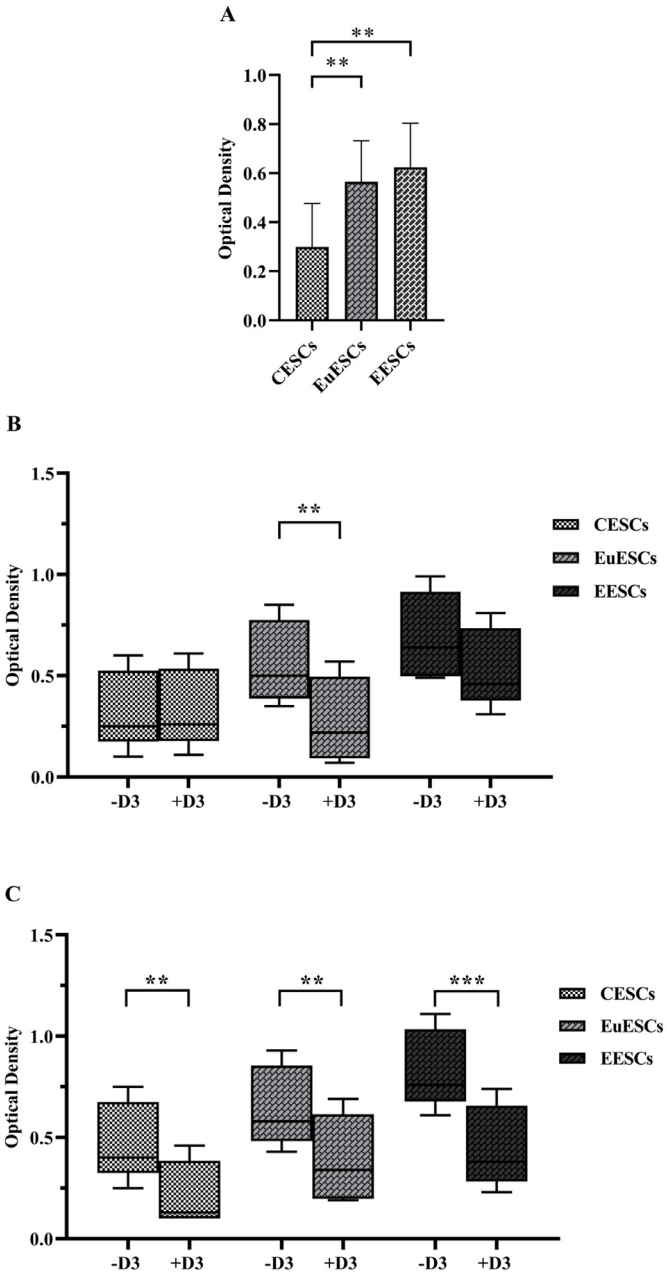

FIGURE 1 The effect of $1,25(OH)_2D_3$ on proliferation in ESC. Comparison of basal proliferation between CESC from non-endometriotic controls ($n = 10$) and EuESC and EESC ($n = 10$) from endometriotic patients measured by MTT assay. EESC versus CESC ($P = 0.003$) and EuESC versus CESC ($P = 0.006$) (A). Also, the effect of $1,25(OH)_2D_3$ on the proliferation of CESC from non-endometriotic controls ($n = 10$) and EuESC and EESC ($n = 10$) from endometriotic patients measured at 72 h. In the absence of oestrogen: EuESC ($P = 0.006$) (B) and in the presence of oestrogen: EESC ($P < 0.001$), EuESC ($P = 0.005$) and CESC ($P = 0.009$) (C). Data are represented as mean ± SD. Data for optical density are presented as box and whisker graphs. $^{**}P < 0.01$; $^{***}P < 0.001$. CESC = control endometrial stromal cells; D3 = $1,25(OH)_2D_3$; EESC = ectopic endometrial stromal cells; ESC = endometrial stromal cells; EuESC = eutopic endometrial stromal cells; MTT = methyl thiazolyl tetrazolium.

EESC in the G0/G1 and G2/M phases ($P = 0.002$ and $P = 0.02$, respectively). Furthermore, $1,25(OH)_2D_3$ induces apoptosis in the EESC by increasing the sub-G1 phase ($P = 0.001$). Moreover, such treatment significantly decreased the EuESC population in the S phase ($P = 0.012$). However, the percentage of EuESC in the G1 and G2/M phases was not significantly affected by treatment with $1,25(OH)_2D_3$. The $1,25(OH)_2D_3$ had no significant effects on the cell cycle of the CESC (FIGURE 2C and 2D). Moreover, this study investigated the effects of $1,25(OH)_2D_3$ on oestrogen-induced ESC. Cell cycle analysis of oestrogen-induced ESC treated with $1,25(OH)_2D_3$ revealed that such treatment decreased the percentage of EESC, EuESC and CESC in the S phase ($P = 0.002$, $P = 0.001$ and $P = 0.014$, respectively), which was accompanied by an increase in the percentage of EuESC and CESC in the G0/G1 phase of the cell cycle ($P = 0.008$ and $P = 0.021$, respectively). Interestingly, $1,25(OH)_2D_3$, by increasing the sub-G1 phase, induces apoptosis in the oestrogen-induced EESC ($P = 0.006$) (FIGURE 2E and 2F).

Apoptosis of ESC and the effect of $1,25(OH)_2D_3$ on apoptosis in ESC

To evaluate the apoptosis in ESC, annexin V and propidium iodide staining was performed (FIGURE 3). In the basal state, the percentage of necrotic cells in EESC and EuESC was significantly higher than that in CESC ($P = 0.002$ and $P = 0.025$, respectively). Also, early apoptotic cells in EuESC were lower than CESC ($P = 0.033$) (FIGURE 3A and 3B). The results showed that $1,25(OH)_2D_3$ increased the number of early and late apoptotic and necrotic cells, which was accompanied by a decrease in the live cell populations in EESC ($P = 0.003$, $P = 0.001$, $P = 0.002$ and $P = 0.001$, respectively) and EuESC ($P = 0.001$, $P = 0.003$, $P = 0.002$ and $P = 0.001$, respectively). Moreover, $1,25(OH)_2D_3$ did not affect the total apoptotic population or lived CESC. However, such treatment significantly increased necrotic cells in this cell population ($P = 0.049$) (FIGURE 3C and 3D).

DISCUSSION

Endometriosis is a common chronic inflammatory disorder characterized by the settlement of endometrial fragments consisting of stroma and glands outside the uterus that may be the consequence of either increased cell proliferation or decreased cell apoptosis (*Ferrero* et al., *2018*). Recently, some therapeutic approaches have focused on the induction of apoptosis and cell cycle arrest and cell proliferation inhibition for endometriosis treatment (*Chuang* et al., *2009*; *Hirakawa* et al., *2016*; *Pellegrini* et al., *2012*). Increased cellular proliferation or decreased apoptosis in response to suitable stimuli could cause endometriosis (*Agic* et al., *2009*). Implanted lesions have significantly higher proliferative activity

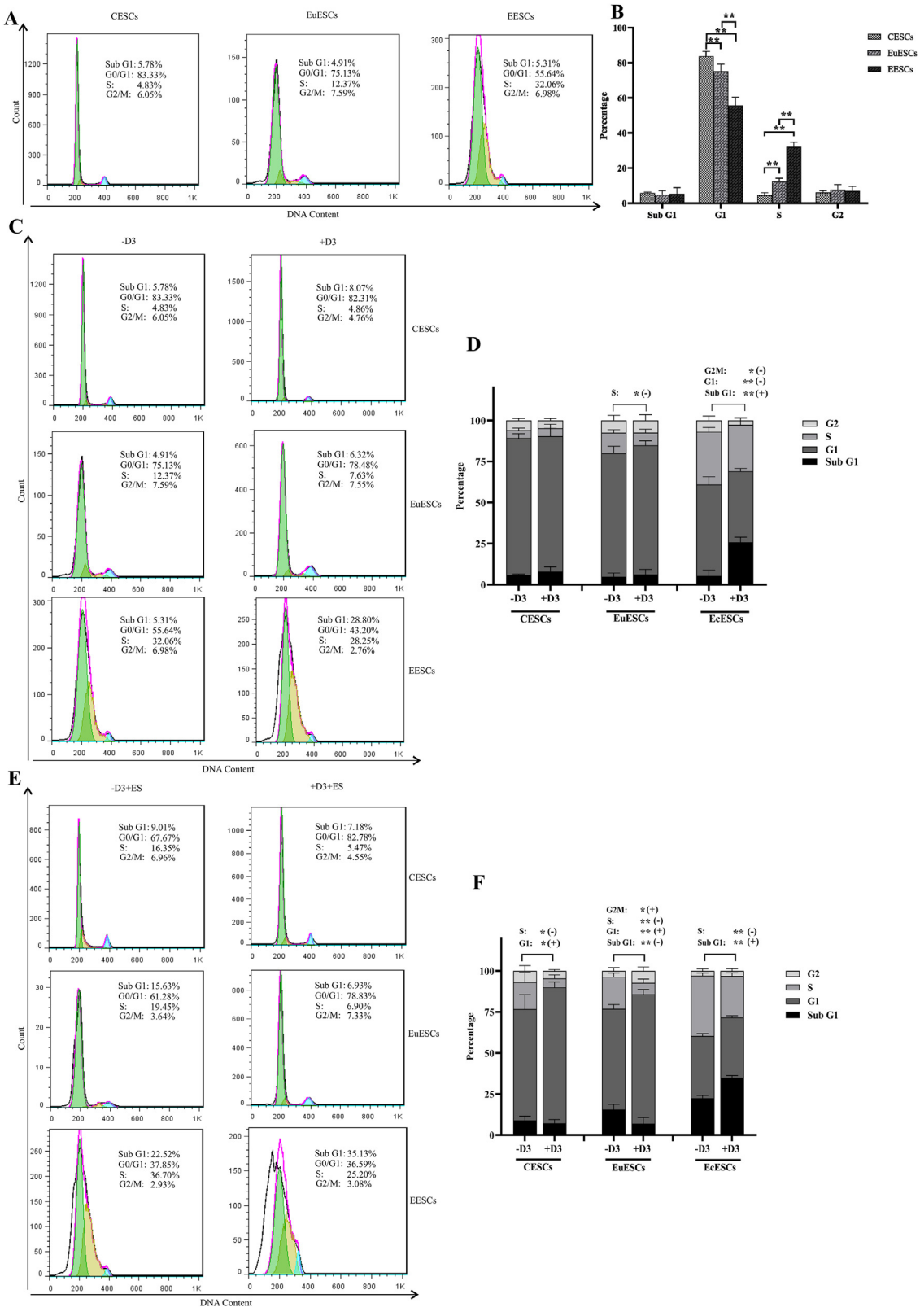

FIGURE 2 The effect of 1,25(OH)$_2$D$_3$ on cell cycle in ESC. CESC from non-endometriotic controls ($n = 10$) and EuESC and EESC ($n = 10$) from endometriotic patients were plated and incubated in DMEM with 2% FBS for 72 h in the absence of 1,25(OH)$_2$D$_3$ and oestrogen. Also, ESC treated with 1,25(OH)$_2$D$_3$ in the presence or absence of oestrogen. Finally, the cells were stained with propidium iodide and their distribution in the cell cycle was analysed by flow cytometry of DNA content. In the absence of 1,25(OH)$_2$D$_3$ and oestrogen: S phase cells: EESC versus EuESC ($P = 0.002$), EESC versus CESC ($P = 0.001$) and EuESC versus CESC ($P = 0.005$); G0/G1 phase cells: EESC versus EuESC ($P = 0.003$), EESC versus CESC ($P = 0.002$) and EuESC versus CESC ($P = 0.007$) (A and B). In the presence of 1,25(OH)$_2$D$_3$: G0/G1 phase cells: EESC ($P = 0.002$), G2/M phase cells: EESC ($P = 0.02$), sub-G1

than eutopic and normal endometrium, associated with higher production of proliferating cell nuclear antigen as a cell proliferation marker (*Jiang and Wu, 2012*). Due to the importance of proliferation in the pathogenesis of endometriosis, cell proliferation in CESC and EESC was studied and compared with the control group. The results demonstrated that endometriotic stromal cells had greater proliferative activity than the control group.

Endometrial proliferation is carried out by the cell cycle. Also, changes in important cell cycle molecules such as cyclins and cyclin-dependent kinases are regulated by the sex hormones, which are lost in endometriosis (*Jiang and Wu, 2012*; *Pellegrini* et al., *2012*). Besides, growth factors such as epidermal growth factor (EGF) and midkine (MK), a member of the heparin-binding growth factor family, play a role in the high proliferative potential of cells derived from endometriotic lesions (*Chegini* et al., *1992*; *Chung* et al., *2002*; *Klemmt* et al., *2007*). Also, recurrent bleeding in patients with endometriosis causes thrombin production, which eventually causes endometriotic cells to proliferate through protease-activated receptor 1 (PAR1). Downstream signalling of PAR1 induces MCP1, TNF, IL, COX-2, MMP, HGF and TF expression, all of which may lead to increased proliferation in endometriotic patients (*Jiang and Wu, 2012*). In this study, $1,25(OH)_2D_3$ inhibited the proliferation of EuESC in the absence of oestrogen. Interestingly, this vitamin also inhibited the proliferation of ESC from three different cellular sources in the presence of oestrogen. Various studies have shown that the $1,25(OH)_2D_3$ prevents the growth of different lymphoma cell lines and Hodgkin's lymphoma cells (*Gharbaran* et al., *2019*; *Han* et al., *2019*; *Hickish* et al., *1993*). Also, it was previously shown that $1,25(OH)_2D_3$ in the presence of fibronectin prevents the proliferation of EuESC and EESC but has no effect on CESC (*Delbandi* et al., *2016*). A recent study showed that vitamin D3, along with sulindac (a non-steroidal anti-inflammatory drug, NSAID), inhibits MCF-7 cell line proliferation through the AMPK/Akt/β-catenin axis (*Poursoltani* et al., *2021*).

All eukaryote organisms grow by undergoing mitotic cell division. The process of cell growth is highly regulated and tightly controlled by the cell cycle, which consists of four sequential phases, including G1, S, G2 and M (*Williams and Stoeber, 2012*). Given the importance of the cell cycle in the growth of different cells and by hypothesizing that the cell cycle in endometriotic cells is different from normal endometrial cells, this study evaluated the cell cycle in ESC of endometriotic patients compared with healthy controls. The results of this study showed that the percentage of cells in the S phase of the cell cycle in EESC is higher than that in EuESC and CESC. Moreover, the percentage of cells in the G1 phase of the cell cycle in EESC is lower than in EuESC and CESC. Collectively, these findings indicate that EESC have greater proliferation capacity than EuESC and CESC. It suggests cyclins may be the main regulator of the cell cycle and play a key role in the proliferation and differentiation of endometrial cells (*Milde-Langosch* et al., *2001*). B1 and D1 cyclin overexpression has been observed in endometriosis, which is probably responsible for the promoted proliferation of endometriotic stromal cells. Furthermore, improper regulation of cyclins during the cell cycle leads to uncontrolled growth and ectopic cell function (*Pellegrini* et al., *2012*; *Tang* et al., *2009*).

Numerous studies have shown that vitamin D3 and its derivatives decrease cell cycle progression in various cells and tumour types such as malignant keratinocytes, breast and prostate cancer cells, and in an experimental skin cancer model and the Pfeiffer cell line of DLBCL (*Bhoora and Punchoo, 2020*; *González-Pardo* et al., *2014*; *Han* et al., *2019*). It has been shown that vitamin D3 by G0/G1 arrest leads to cell cycle regulation (*Bhoora and Punchoo, 2020*).

This study showed that $1,25(OH)_2D_3$ had no significant effects on different cell cycle phases in CESC. However, this vitamin effectively induced G0/G1 arrest in EuESC and increased sub-G1 in EESC, accompanied by a reduction of the S phase of the cell cycle. In contrast to the results of the current study, *Bao* et al. *(2014)* showed that $1,25(OH)_2D_3$ does not lead to G0/G1 arrest or G2/M phase change in BGC-823 cells. It is noteworthy that the type of cells (BGC-823 human gastric cancer cells) in this group, as well as the treatment time with $1,25(OH)_2D_3$, were different from the current study, and these factors possibly caused differences in the results of the two studies. It has been shown that the predominant anti-cancer action of vitamin D is due to reducing cell proliferation and G0/G1 cell cycle arrest by increasing the expression of p21 and p27, which are associated with the inhibition of cyclin E and D activity. In an in-vitro study, vitamin D3 caused G0/G1 arrest by decreasing C-MYC expression and increasing retinoblastoma protein concentrations. Nevertheless, few results of G2/M arrest with unknown mechanisms have been reported by $1,25(OH)_2D_3$ (*Bhoora and Punchoo, 2020*).

Steroid hormones regulate proliferation in normal endometrial and endometriotic cells. Oestrogen secreted by the ovaries before ovulation causes the endometrial stroma proliferation and gland development, while in turn, progesterone prepares the tissue lining of the uterus so that the fertilized egg can be implanted. In other words, oestrogen is the predominant hormone in the ovulatory phase of the menstrual cycle (*Maybin and Critchley, 2012*). The proliferative function of oestrogen in the endometrium begins with binding to superficial, cytosolic and nuclear (ER) receptors (*Press* et al., *1989*; *Song and Santen, 2006*), which in turn leads to the formation of the oestrogen–ER complex. This complex reacts directly with specific sequences in DNA (oestrogen receptor element, ERE) and the promoter of genes associated with the progression of the G1 phase of the cell cycle, which mainly leads to the transcription of cyclins and cyclin-related kinases, which play a role in endometrial

phase cells: EESC ($P = 0.001$) and S phase cells: EuESC ($P = 0.012$) (C and D). In the presence of $1,25(OH)_2D_3$ and oestrogen: S phase cells: EESC ($P = 0.002$), EuESC ($P = 0.001$) and CESC ($P = 0.014$); G0/G1 phase cells: EuESC ($P = 0.008$), CESC ($P = 0.021$) and sub-G1 phase cells: EESC ($P = 0.006$) (E and F). Representative cell cycle cytometry was shown in A, C and E and the percentages of each phase of the cell cycle, shown in bar graphs, were statistically analysed with Mann–Whitney *U*-test (B, D and F). Data are represented as mean ±SD. *$P < 0.05$; **$P < 0.01$. (+) indicates an increase in cell populations and (−) indicates a decrease in cell populations. CESC = control endometrial stromal cells; D3 = $1,25(OH)_2D_3$; EESC = ectopic endometrial stromal cells; ES = oestrogen; ESC = endometrial stromal cells; EuESC = eutopic endometrial stromal cells; G0/G1 = cells in G0/G1 cell cycle phase; G2/M = cells in G2/M cell cycle phase; S = cells in S cell cycle phase.

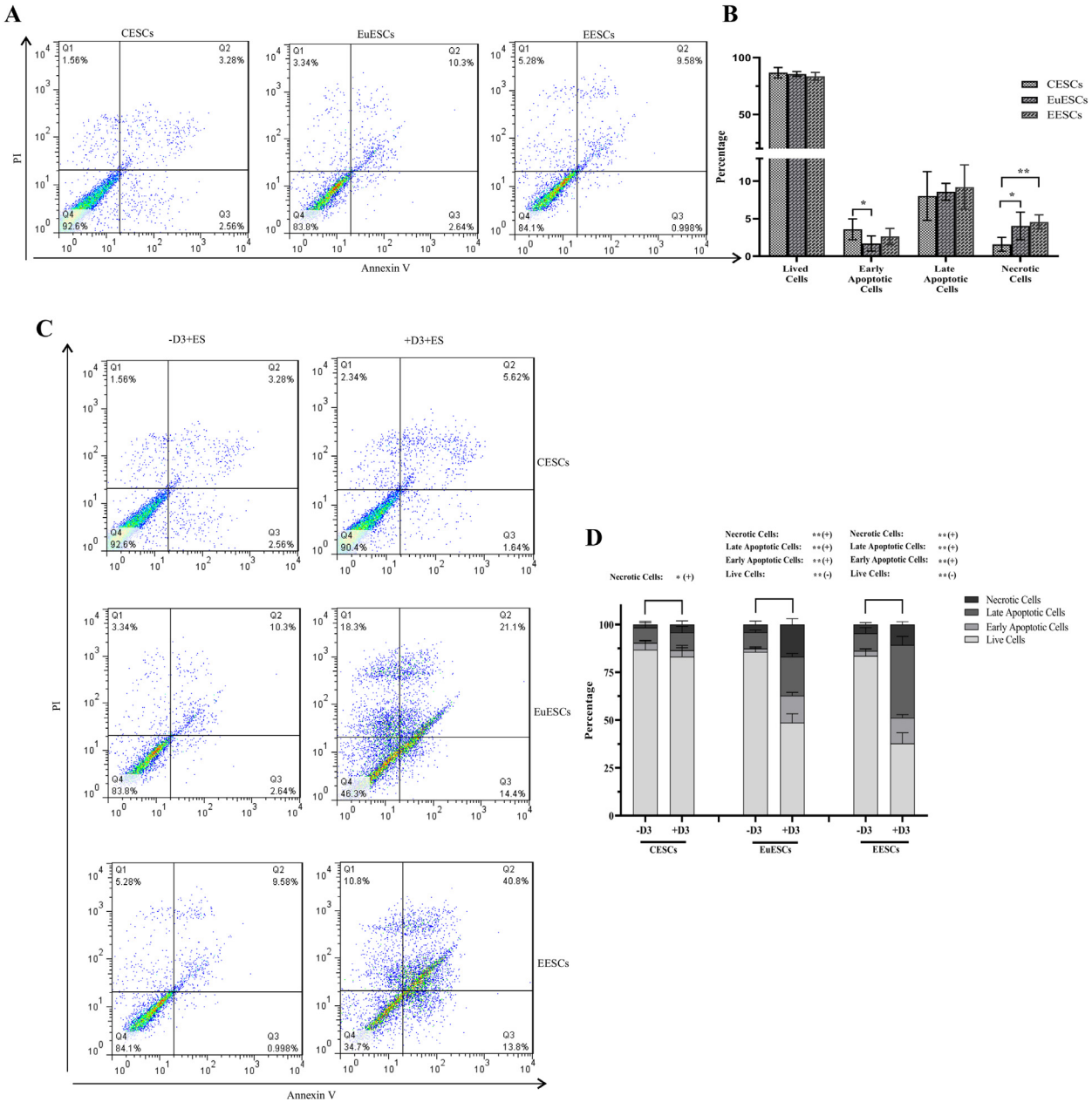

FIGURE 3 The effect of $1,25(OH)_2D_3$ on apoptosis in ESC. CESC from non-endometriotic controls ($n = 10$) and EuESC and EESC ($n = 10$) from endometriotic patients were plated and incubated with oestrogen for 96 h in the absence or presence of $1,25(OH)_2D_3$. The cells were then stained with propidium iodide and their distribution was analysed by flow cytometry. In the absence of $1,25(OH)_2D_3$: necrotic cells: EESC versus CESC ($P = 0.002$), EuESC versus CESC ($P = 0.025$) and early apoptotic cells: EuESC versus CESC ($P = 0.033$) (A and B). In the presence of $1,25(OH)_2D_3$: early apoptotic cells: EESC ($P = 0.003$), late apoptotic cells: EESC ($P = 0.001$), necrotic cells: EESC ($P = 0.002$) and live cells: EESC ($P = 0.001$); early apoptotic cells: EuESC ($P = 0.001$), late apoptotic cells: EuESC ($P = 0.003$), necrotic cells: EuESC ($P = 0.002$) and live cells: EuESC ($P = 0.001$) and necrotic cells: CESC ($P = 0.049$) (C and D). Representative flow cytometry is shown in A and C, and the percentage of each cell population, shown in bar graphs, was statistically analysed by Mann–Whitney *U*-test (B and D). Data are represented as mean ± SD. Annexin V− PI+ represents necrotic cells (upper-left quadrant, Q1). Annexin V+ PI+ represents late apoptotic cells (upper-right quadrant, Q2). Annexin V+ PI− represents early apoptotic cells (lower-right quadrant, Q3). Annexin V− PI− represents live cells (lower-left quadrant, Q4). *$P < 0.05$; **$P < 0.01$. (+) indicates an increase in cell populations and (−) indicates a decrease in cell populations. CESC = control endometrial stromal cells; D3 = $1,25(OH)_2D_3$; EESC = ectopic endometrial stromal cells; ES = oestrogen; ESC = endometrial stromal cells; EuESC = eutopic endometrial stromal cells; PI = propidium iodide.

cell proliferation (*Planas-Silva and Weinberg, 1997*).

On the other hand, oestrogen signals are transmitted in endometriotic tissue by genomic and non-genomic methods. In the genomic pathway, oestrogen binds to the nuclear ER and ERE region after interaction with the SRC1 protein, leading to up-regulation of cyclin E p27 and cyclin B1 (*da Costa e Silva Rde* et al., *2016*). In the other pathway, oestrogen binds to the JUN region in the AP-1 binding region after binding to transcription factors, which ultimately leads to cyclin D1 up-regulation (*Shiozawa* et al., *2004*). B1 and D1 cyclin up-regulation has been observed in endometriosis, which is associated with

abnormal cell cycle regulation and proliferative events in the disease (*Pellegrini* et al., *2012*; *Tang* et al., *2009*). In the non-genomic pathway, the GPR30 receptor, which is highly expressed in endometriosis, triggers two important pathways, MAPK and PI3K, after binding to oestrogen and activating the c-Src protein (e, e1 and e2). Both these pathways eventually lead to the proliferation of endometriotic cells (*da Costa e Silva Rde* et al., *2016*; *Yuguchi* et al., *2013*). Oestrogen also stimulates the PI3K pathway by degrading PTEN (an internal inhibitor of the PI3K pathway, which is naturally expressed in endometrial glandular cells), eventually binding NF-κB to DNA and the continuous proliferation of endometrial cells responsible for the pathogenesis and endometriosis progression (*Zhang* et al., *2010*). Oestrogen increases the expression of cyclins and eventually proliferates endometriotic stromal cells by activating all the above-mentioned pathways. In other words, any imbalance in oestrogen and progesterone is associated with high concentrations of oestrogen and progesterone resistance in patients with endometriosis. As shown, $1,25(OH)_2D_3$ in the CESC and EuESC increases the G0/G1 phase in the presence of oestrogen, which is associated with the S phase reduction. This study is the first to investigate the effect of $1,25(OH)_2D_3$ on cell cycle progression in ESC of patients with endometriosis and healthy groups either in humans or in animals, in the presence of oestrogen, and it is impossible to compare the results with similar data. Based on the experiments of previous studies and the current study, it is inferred that the largest effect of $1,25(OH)_2D_3$ on cell proliferation is likely due to a G0/G1 arrest-dependent mechanism of action.

The study of apoptosis here showed that there is not much difference between apoptosis in ESC derived from three different sources, and the number of necrotic cells in EESC and EuESC was higher than in CESC. Previous studies that examined molecules involved in apoptosis have shown there is resistance to apoptosis in endometriotic stromal cells, which was not proven in the current study (*Jones* et al., *1998*; *Meresman* et al., *2000*; *Nishida* et al., *2005*). One reason for this discrepancy may be the different methods of evaluating apoptosis used in these studies; they investigated apoptosis by examining pro- and anti-apoptotic molecules. Moreover, in some studies, apoptosis tests have been performed on whole endometriotic tissue, which in addition to stromal cells, contains different cell types. Also, different stimuli are used in different studies. All these differences can affect the results of apoptosis studies. Apoptosis is a natural process that removes unwanted cells without triggering an inflammatory response. It is a crucial regulator of eutopic endometrial function. Evidence suggests that apoptosis by removing aged cells from the uterine endometrium's active layer helps maintain cellular homeostasis during the menstrual cycle (*Agic* et al., *2009*). It has been revealed that endometrial cells from women with endometriosis vary from normal ones (*Sharpe-Timms, 2001*). Endometrial tissue-reduced sensitivity to spontaneous apoptosis contributes to aberrant endometrium implantation and development at ectopic sites (*Taniguchi* et al., *2011*). Increased expression of anti-apoptotic proteins (Bcl-2) and decreased expression of pre-apoptotic factors (BAX) are linked to the incapacity of endometrial cells to deliver a 'death' signal or their ability to escape cell death (*Meresman* et al., *2000*).

Also, it was observed that in the presence of oestrogen, $1,25(OH)_2D_3$ could increase the number of annexin-positive cells in EESC and EuESC associated with increased early and late apoptotic cells but did not affect CESC. It had been previously shown that $1,25(OH)_2D_3$ decreased Bcl-2 expression in EuESC and Bcl-xL expression in EESC (*Delbandi* et al., *2016*). Only one study investigated the effect of $1,25(OH)_2D_3$ on apoptosis in ESC by dual staining of annexin V and propidium iodide. Contrary to the current results, this study showed that $1,25(OH)_2D_3$ cannot induce apoptosis in ESC (*Miyashita* et al., *2016*). Although the exact reasons for this discrepancy are not fully clear, factors such as the stages of endometriosis, vitamin D3 concentration and time of treatment, and different stimuli may be effective. As will be noted below, $1,25(OH)_2D_3$ induces failure in resistance to apoptosis in endometriotic stromal cells in the presence of oestrogen.

Because VDR is associated with calcium channels, it appears that $1,25(OH)_2D_3$, by connecting to VDR, leads to increased intracellular calcium and eventually activates calcium-dependent apoptotic proteases such as caspase-12 (*Sergeev, 2014*). Studies on the effect of $1,25(OH)_2D_3$ on the cell cycle, proliferation and apoptosis have shown that ESC are more sensitive to $1,25(OH)_2D_3$ in the presence of oestrogen. Because studies have shown that oestrogen, through an ER-mediated mechanism, increases the sensitivity of cancer cells to $1,25(OH)_2D_3$ by increasing the VDR expression on them, it can be inferred that oestrogen affects ESC via a similar mechanism and increases the sensitivity of these cells to $1,25(OH)_2D_3$ (*Mahonen* et al., *1991*). Also, a result of binding $1,25(OH)_2D_3$ to VDR is the reduction of aromatase and ER expression. Therefore, $1,25(OH)_2D_3$ prevents oestrogen production and its effect on ESC and, ultimately, oestrogen-dependent proliferation in these cells (*Krishnan* et al., *2012*).

The current study had some limitations and advantages. As mentioned earlier, it was the first study to investigate the basal cell cycle progression and also the effect of $1,25(OH)_2D_3$ treatment on the cell cycle progression in ectopic (EESC) and eutopic (EuESC) endometrial stromal cells of women with endometriosis in comparison with non-endometriotic controls (CESC) in the presence and absence of oestrogen. Due to the small number of obtained ESC, the effect of $1,25(OH)_2D_3$ on the apoptosis of ESC was assessed only in the presence of oestrogen, and the lack of investigation in the absence of oestrogen is one of the limitations of the study; this should be evaluated in further studies. Also, it would have been preferable to analyse the effect of $1,25(OH)_2D_3$ on the gene and protein expression of cyclins, especially B1, D1 and E, as a key regulator of the cell cycle. This study only examined the effect of $1,25(OH)_2D_3$ on proliferation, cell cycle and apoptosis of endometriotic cells compared with the control group, so more research is needed to investigate the mechanisms involved in these processes.

The findings of the present study showed increased proliferation capacity in patients with endometriosis compared with the control group. Also, the rate of apoptosis in endometriosis patients is no different to the control group. It was shown here for the first time that $1,25(OH)_2D_3$ exerts many beneficial effects on cancer-like features of ESC from endometriotic patients, reflecting the potential usefulness of this hormone in endometriosis treatment. To the best of our knowledge, the present study demonstrates for the first time that $1,25(OH)_2D_3$ inhibited the proliferation of ESC by inducing cell cycle arrest and by stimulating apoptosis in endometriotic patients and healthy individuals. 1,25

$(OH)_2D_3$ may be used as a strategy to overcome resistance to apoptosis in ESC from patients with endometriosis and to improve routine therapy.

DATA AVAILABILITY

Data will be made available on request.

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