VIA MEDICA

ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2019, vol. 90, no. 12, 669–674

ISSN 0017–0011

DOI: 10.5603/GP.2019.0115

Ca^{2+} channel subunit a 1D inhibits endometriosis cell apoptosis and mediated by prostaglandin E2

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ABSTRACT

Objectives: Endometriosis is considered as a chronic pelvic inflammatory disease and prostaglandin E2(PGE2) (a kind of the inflammatory cytokines) was increased in the endometriosis patient's peritoneal fluid . Ca2+ signal and Ca2+ channels play an important role in cell apoptosis. This study was to explore the L-type calcium channel (Cav1.3) expression and its biological function in endometriosis. Furthermore the molecular mechanism between Cav1.3 and PGE2 was also clarified.

Material and methods: The real-time PCR and immunohistochemical were used to detect the expression of Cav1.3. Apoptosis was detected by Flow cytometry assay and Western blot assay.

Results: Cav1.3 was high expression in endometriosis tissue and primary endometrial stromal cells (hEM15A). Treatment with PGE2 rapidly inhibited apoptosis and increased Cav1.3 expression in hEM15A . The silencing of Cav1.3 promoted apoptosis, which was unchanged after PGE2 treatment. Moreover, the inhibition of Cav1.3 by shRNA transfection activated cleaved PARP and cleaved caspase-3.

Conclusions: These available evidences suggest that Cav1.3 is required for PGE2 induction apoptosis and relates to the pathophysiology of endometriosis. Interference with Cav1.3 may offer a neo-therapeutic window in endometriosis treatment.

Key words: endometriosis; Cav1.3; apoptosis; PGE2

Ginekologia Polska 2019; 90, 12: 669–674

INTRODUCTION

Endometriosis (EM), which is characterized the presence and growth of endometrial glands and stroma outside the uterine cavity, affects about 10–15% of reproductive women and up to 24–50% in infertile women [1, 2]. Clinical manifestations of endometriosis include pelvic pain, dysmenorrhea, dyspareunia and infertility. For the majority of these patients, surgery and/or hormonal therapy remain the primary therapy of choice. However, hormonal therapy usually is associated with serious adverse effects and endometriosis lesions relapse in 30–50% women within 3–5 years after surgery [3]. Therefore, to explore the pathogenesis mechanism of endometriosis and find a novel therapy is imperative.

Generally, endometriosis is perceived as a chronic pelvic inflammatory process with systemic subclinical manifestations. Peritoneal fluid from women with endometriosis shows activated defective macrophages and natural killer cells which alter the recognition and clearance of endometrial cells. Macrophages secrete different products such as growth factors, enzymes, prostaglandins, and cytokines that stimulate and promote the progression of endometriosis [4] . Especially for prostaglandin E2 (PGE2), which high expresses in abdominal fluid and blood in endometriosis patient, induces a classical inflammatory responses [5]. PGE2 is a mediator of many biological functions as well as active inflammation, which promotes local vasodilatation with recruitment and activation of inflammatory cells [6]. PGE2 triggers a range of cellular responses through binding with receptors (EP1, EP2, EP3 and EP4) [7, 8], which causes activation of PKC accompanied by cytosolic Ca^{2+} mobilization [9].

Ca^{2+} channels tightly modulated Ca^{2+} homeostasis which regulates second messenger pathway. Ca^{2+} channels introduce Ca^{2+} into the cytoplasm and participate in regulating cell functions, which include cell pattern formation,

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differentiation, proliferation, migration, death and so on [10]. The L-type Ca^{2+} channel subunit α 1D, named Cav1.3, is highly expressed in prostate cancer, endometrial cancer and breast cancer, which participate tumorigenesis [11–13]. Also, Cav1.3 regulates calcium-dependent mitochondrial oxidant stress in Parkinson's disease [14]. Cav1.3 plays a well-known role in the occurrence and formation of many diseases, but its role in endometriosis is not clear.

In this study, we analyzed Cav1.3 expression in endometriosis patient using real-time PCR and immunohistochemistry assays; the relationship between Cav1.3 and PEG2 was investigated and the signaling pathway in primary endometrial stromal cell (hEM15A) was detected. Further, the effect of apoptosis by short hairpin (sh)RNA in hEM15A was assessed. The study might explore new insights into the treatment of endometriosis in clinical practice.

MATERIAL AND METHODS

Patients

In total, 30 ovarian endometriosis and 30 normal endometrial tissues were collected from patients undergoing first curative treatment resection in the Department of Obstetrics and Gynecology of the 1st Hospital of Lanzhou University (Lanzhou, China) between January 2017 and December 2017. None enrolled patients received hormonal therapy before surgery therapy. Moreover, 30 ovarian endometriosis samples diagnosed as endometriosis according to the American Society for Reproductive Medicine classification of endometriosis criteria [15]. The study was approved by the Ethics Committee of the 1st Hospital of Lanzhou University (No.LDYYLL2019-148) and each patient signed informed consent.

Immunohistochemistry

The paraffin sections of tissues were dewaxed as routine. 0.3% hydrogen peroxide methanol closed endogenous peroxidase for 30 minutes. After exposing 10% unimmunized goat serum to PBS for 10 minutes, the sections were treated with rabbit primary anti-cav1.3 antibody at 4°C overnight (1:200, Abcam, USA). Sections were incubated with biotinylated anti-rabbit immunoglobulin serum for 30 minutes, and then incubated with peroxidase-labeled avidin at room temperature for 20 minutes. Diaminobenzidine hydrochloride was used as chromogenic reagent. Finally, the sections were counter stained with hematoxylin. With percentage score (0~9) multiplied by the strength calculation expression of Cav1.3, final score < 4 patients for lower expression group, vice for low expression group.

Cell culture, treatment and reagents

hEM15A was purchased from China center for type culture collection and cultured as instruction [16]. Cell transfection involved use of lipofectamine 2000 (Invitrogen, USA) with Cav1.3 shRNA according to the manufacturer's protocol. The primer sequence for Cav1.3 shRNA was designed by Shanghai GenePharma Co. ,Ltd. The efficiency of knocking down the target proteins were detected by western blot assay.

Real-time PCR

Conventional Trizol method (TRI Reagent) is used to extract total RNA. The first strand of cDNA was synthesized using the Prime Script reverse transcriptase kit (TaKaRa, dalian, China) according to the manufacturer's instructions. Specific primers are as follows:β-actin: forward 5′-CACCATCCTGGCCTCGCTGT-3′, and reverse 5′-GCTACCTTCACCGTTCC-3′; Cav1.3: Forward: 5′- TCCAGGCAAAAACACTTCAAGG-3′ and Reverse:5′- GGAGGCTCT CACTGGCAAAT-3′.

Flow cytometry assay

Annexin-v /FITC kit (BD Biosciences, San Jose, CA, USA) was used to detect apoptosis as per the manufacturer's instructions, and flow cytometry was used for analysis following the series of treatments described previously [17].

Western blot assay

hEM15A was harvested and lysed. Total protein was extracted and quantified according to the manufacturer's instructions. Western blot assay was performed as described previously [18] anti — Cav1.3 polyclonal antibody (1:1000, Abcam, USA), cleaved caspase 3/caspase 3 (1:1000, Abcam, USA), cleaved PARP/PARP (1:1000, Abcam, USA) were used. Use ECL substrate to detect its expression. Intensity bands are detected by the Bio-rad imaging system (Hercules, CA, USA).

Statistical Analysis

Data were expressed as mean ± standard deviation and analyzed by GraphPad Prism 5 software. Student's t-test was used for the comparison of the two groups, one-way analysis of variance was used for the comparison of the multiple groups, and Tukey's test was used for the comparison of the two groups. When the square difference between two groups is not even, non-parametric test is used. $p < 0.05$ was considered statistically significant.

RESULT

Cav1.3 high expressed in endometriosis tissues

Real-time PCR analysis was used to identify the different expression of Cav1.3 in endometriosis (EM) and normal endometrial (EN) tissues. Cav1.3 was significantly upregulated in EM tissues (n = 30) compared with in EN tissues (n = 30) ($p < 0.01$) (Fig. 1A). Moreover, the protein expression of Cav1.3 was detected by immunohistochemistry in the ovarian endometriosis. A total of 68.0% of ovarian endometriosis were positive for Cav1.3 staining in

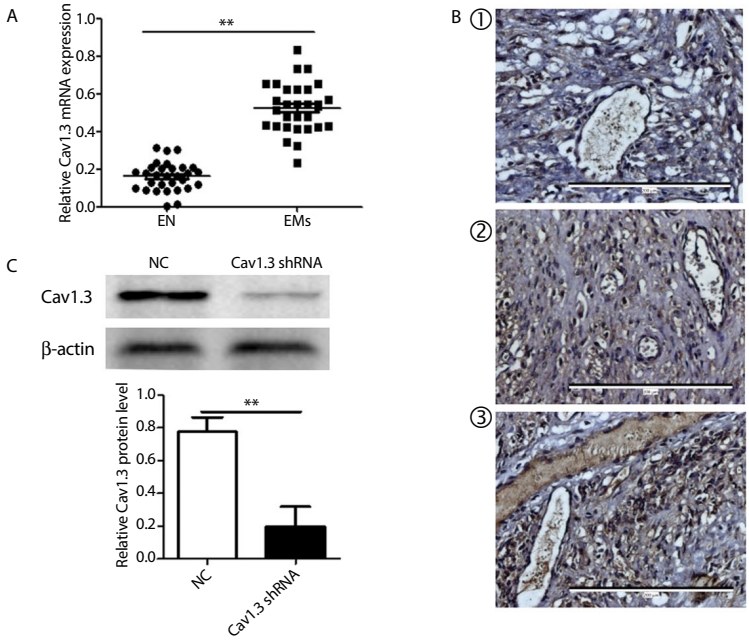

Figure 1. Expression of Cav1.3 in endometriosis tissues and hEM15A; **A.** Realtime PCR analysis of Cav1.3 expression levels in endometriosis (EM) and normal endometrial (EN) tissues (**p < 0.01); **B.** IHC staining of Cav1.3 expression in ovarian endometriosis (① — low expression; ② — moderate expression; ③ — high expression) × 400; **C.** The expression of the channel protein Cav1.3 after shRNA transfection was significantly reduced compared with that of NC cells (**p < 0.01)

the nucleus and cytoplasm (Fig. 1B). Also, the Cav1.3 was highly expressed in hEM15A. Therefore, Cav1.3 was knocked down by transfecting hEM15A with shRNA. The efficiency about knockdown Cav1.3 protein was detected by Western blot after 48 h The result showed that the protein of Cav1.3's expression was significantly reduced in shRNA transfection hEM15A compared with negative control cells ($p < 0.01$) (Fig. 1C).

Cav1.3 shRNA promoted apoptosis in hEM15A

Because Cav1.3 was upregulated in endometriosis and hEM15A , the biological role of Cav1.3 in hEM15A was examined. The Cav1.3 shRNA cells and Cav1.3-negative control cells were analyzed by flow cytometry to determine whether Cav1.3 inhibited cell death through decreasing apoptosis. The results showed that the apoptotic cells increased strikingly in Cav1.3 shRNA-transfected cells, as compared with controls ($p < 0.01$). Furthermore, after 50 μM PGE2 treated 24 h, PGE2 inhibited the apoptosis in Cav1.3-negative control cells. However, the apoptosis was unchanged in Cav1.3 shRNA-transfected cells after PGE2 stimulation 24 h ($p > 0.05$) (Fig 2).

PGE2 suppressed apoptosis by Cav1.3/PARP/caspase3 in hEM15A

To further investigate the mechanism of apoptosis in hEM15A , the expression of Cav1.3, cleaved caspase3 and poly-ADP-ribose polymerase (PARP) were detected by western blot in hEM15A. After 50μM PGE2 treated 24 h, the result revealed that Cav1.3 increased in Cav1.3-negative control cells ($p < 0.01$), but cleaved PARP and caspase 3 were decreased in Cav1.3-negative control cells ($p < 0.01$). Meanwhile, after 50 μM PGE2 treated 24 h in Cav1.3 shRNA-transfected cells, Cav1.3,cleaved PARP and caspase3 were unchanged ($p > 0.05$). The result revealed that PGE2 can upregulated the expression of Cav1.3, which inhibited hEM15A ' apoptosis by down-regulated cleaved PARP and caspase3 (Fig. 3).

DISCUSSION

Our results show that Cav1.3 was upregulated in endometriosis and hEM15A. In addition, upregulated Cav1.3 inhibited apoptosis in hEM15A. Moreover, PGE2 suppressed apoptosis by upregulated Cav1.3's expression in hEM15A. The results suggested that Cav1.3 expression plays a crucial apoptosis role in endometriosis progression. The mechanistic study showed that PGE2 suppressed apoptosis by upregulated Cav1.3 expression, which finally decreased cleaved PARP and caspase3 in hEM15A. This study proposes a novel mechanism that PARP/caspase3 pathway are the responsible factors for the high expression of Cav1.3 after PGE2 stimulation.

To our knowledge, the research about apoptosis role of Cav1.3 is little in endometriosis. Recent study show that increased expression of Cav1.3 in prostate cancer induces

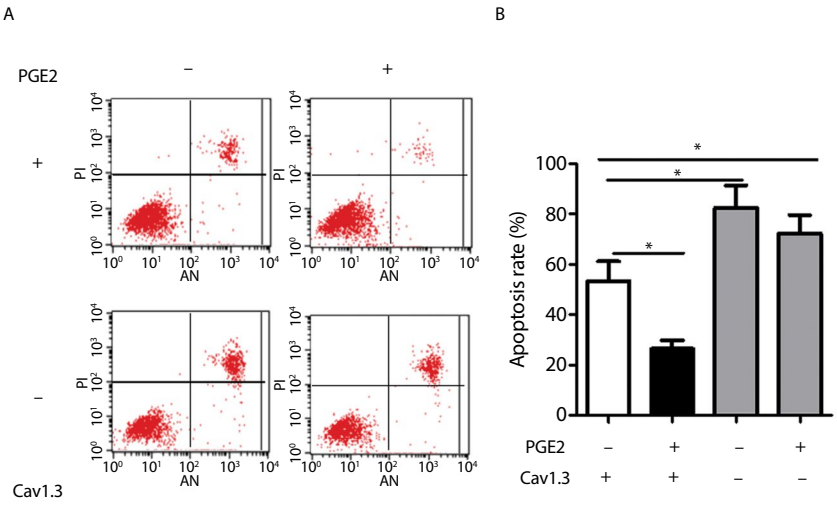

Figure 2. Cav1.3 shRNA promoted apoptosis in hEM15A; **A.** Cells transfected with shRNA Cav1.3 was stained with Annexin-V-FITC. Apoptosis analysis was performed by flow cytometry; **B.** The bar graph showed a significant increase in the apoptotic index (*p < 0.05)

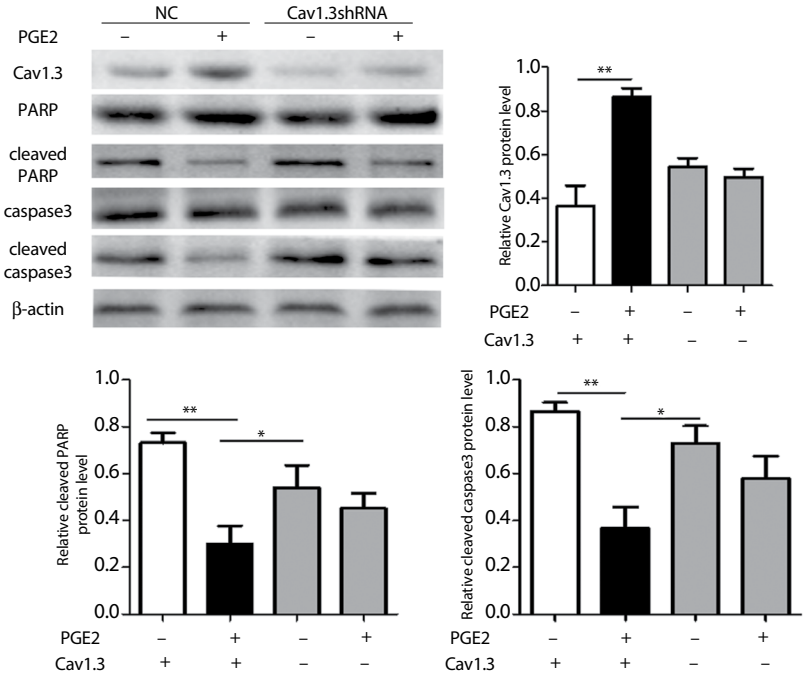

Figure 3. PGE2 suppressed apoptosis through Cav1.3/PARP/caspase3 in hEM15A. Cav1.3 increased in Cav1.3-negative control cells after 50μM PGE2 treated 24 h (**p < 0.01), meanwhile, cleaved PARP and caspase3 were decreased after 50μM PGE2 treated 24h in Cav1.3-negative control cells (**p < 0.01). After 50μM PGE2 treated 24h in Cav1.3 shRNA-transfected cells, Cav1.3,cleaved PARP and caspase3 were unchanged (p > 0.05). Cleaved PARP and caspase3 were activated after knocking down Cav1.3 in hEM15A (*p < 0.05)

androgen mediated cell growth [11]. In endometrial cancer cells, estrogen upregulates Cav1.3's expression by GPR30, which activates the erk1/2/CREB pathway, initiates calcium influx through Cav1.3, and promotes cell proliferation and migration [12]. In breast cancer, Cav1.3 is high expressed and activated Ca^{2+} influx promoting cell proliferation [13]. Our study showed that Cav1.3 was significantly upregulated in endometriosis tissues and hEM15A. Reducing Cav1.3 significantly promoted apoptosis in hEM15A. PGE2 upregulated the expression of Cav1.3 and inhibited apoptosis by down-regulated the protein of cleaved caspase3 and PARP. These data support the suggestion that Cav1.3 may act an important role in endometriosis progression through regulate cell apoptosis.

Apoptosis refers to the cell regulated by genes in order to maintain the stability of the internal environment, which

is not a phenomenon of self-injury under pathological conditions, but a death process actively fought for to better adapt to the living environment [19]. The cytomorphological features of apoptosis include: cells are rounded, chromatin condensed and fragmented, and the cytoplasm is shrunken. After that, the whole cell forms some spherical processes by means of germination and foaming, and breaks off at its base to form apoptotic corpuscles containing cytoplasm, organelles and nuclear fragments of different sizes, which are then devoured by surrounding cells. Also there are other biological features which include controlled by genes, not caused by inflammation, plasma membrane does not break and so on [20]. Abnormal apoptosis relates with the incidence of tumors, autoimmune diseases, neurodegenerative diseases.

PARP is a molecular cell receptor that can effectively monitor DNA damage in cells, repair damage and induce apoptosis. When a large amount of DNA damage is not effectively repaired, the cell protection function of PARP changes. The damaged cells bypass the DNA damage regulation electricity and clear from the organism through a predetermined self-destruction process, which is apoptosis. Once apoptosis is activated, PARP will participate in the process of apoptosis in the form of substrate. The proteolysis of PARP by caspase3 is an early event or prerequisite of apoptosis. Caspase3 is a key protease in apoptosis, which is at the core of apoptosis cascade reaction pathway and is considered as a death protease. A variety of apoptosis-stimulating factors initiate different protease cutting of prokaryogen caspase3 and activate caspase3, and the activated caspase3 further cuts different substrates, leading to the amplification of protease cascade reaction cutting and finally leading to apoptosis of cells. Caspase3 is a necessary pathway for the cascade of apoptotic proteins [21]. In this study, our results demonstrated that the cleaved caspase3 and PARP were activated through up-regulating of Cav1.3 after PGE2 stimulation. However, the mechanism underlying how Cav1.3 activated cleaved caspase3 and PARP need further investigation.

In conclusion, the available evidence suggests that Cav1.3 is required for PGE2 induction anti-apoptosis, contributes broadly to the pathophysiology of endometriosis. Interference with the calcium channel Cav1.3 may offer a new therapeutic window for endometriosis treatment.

Funding

The study was supported by the Foundation of Gansu natural science grant 18JR3RA340 and Lanzhou chengguan district science and technology bureau grant 2018SHFZ0022.

REFERENCES

1. Klemmt PAB, Starzinski-Powitz A. Molecular and Cellular Pathogenesis of Endometriosis. Curr Womens Health Rev. 2018; 14(2): 106–116, doi: 10.2174/1573404813666170306163448, indexed in Pubmed: 29861704.
2. Prescott J, Farland LV, Tobias DK, et al. A prospective cohort study of endometriosis and subsequent risk of infertility. Hum Reprod. 2016; 31(7): 1475–1482, doi: 10.1093/humrep/dew085, indexed in Pubmed: 27141041.
3. Tanbo T, Fedorcsak P. Endometriosis-associated infertility: aspects of pathophysiological mechanisms and treatment options. Acta Obstet Gynecol Scand. 2017; 96(6): 659–667, doi: 10.1111/aogs.13082, indexed in Pubmed: 27998009.
4. Hurt KJ. Pocket Obstetrics and Gynecology. Wolters Kluwer Health, Philadelphia, PA 2015.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5): 646–674, doi: 10.1016/j.cell.2011.02.013, indexed in Pubmed: 21376230.
6. Vanaja SK, Rathinam VAK, Fitzgerald KA. Mechanisms of inflammasome activation: recent advances and novel insights. Trends Cell Biol. 2015; 25(5): 308–315, doi: 10.1016/j.tcb.2014.12.009, indexed in Pubmed: 25639489.
7. Chuang PC, Lin YJ, Wu MH, et al. Inhibition of CD36-dependent phagocytosis by prostaglandin E2 contributes to the development of endometriosis. Am J Pathol. 2010; 176(2): 850–860, doi: 10.2353/ajpath.2010.090551, indexed in Pubmed: 20035060.
8. Banu SK, Lee J, Speights VO, et al. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 induces apoptosis of human endometriotic cells through suppression of ERK1/2, AKT, NFkappaB, and beta-catenin pathways and activation of intrinsic apoptotic mechanisms. Mol Endocrinol. 2009; 23(8): 1291–1305, doi: 10.1210/me.2009-0017, indexed in Pubmed: 19407222.
9. Hsu CC, Lu CW, Huang BM, et al. Cyclic adenosine 3',5'-monophosphate response element-binding protein and CCAAT/enhancer-binding protein mediate prostaglandin E2-induced steroidogenic acute regulatory protein expression in endometriotic stromal cells. Am J Pathol. 2008; 173(2): 433–441, doi: 10.2353/ajpath.2008.080199, indexed in Pubmed: 18583320.
10. Bulayeva NN, Wozniak AL, Lash LL, et al. Mechanisms of membrane estrogen receptor-alpha-mediated rapid stimulation of Ca2+ levels and prolactin release in a pituitary cell line. Am J Physiol Endocrinol Metab. 2005; 288(2): E388–E397, doi: 10.1152/ajpendo.00349.2004, indexed in Pubmed: 15494610.
11. Chen R, Zeng X, Zhang R, et al. Cav1.3 channel α1D protein is overexpressed and modulates androgen receptor transactivation in prostate cancers. Urol Oncol. 2014; 32(5): 524–536, doi: 10.1016/j.urolonc.2013.05.011, indexed in Pubmed: 24054868.
12. Hao J, Bao X, Jin Bo, et al. Ca2+ channel subunit α 1D promotes proliferation and migration of endometrial cancer cells mediated by 17β-estradiol via the G protein-coupled estrogen receptor. FASEB J. 2015; 29(7): 2883–2893, doi: 10.1096/fj.14-265603, indexed in Pubmed: 25805831.
13. Ji Y, Han Z, Shao L, et al. Ultrasound-targeted microbubble destruction of calcium channel subunit α 1D siRNA inhibits breast cancer via G protein-coupled receptor 30. Oncol Rep. 2016; 36(4): 1886–1892, doi: 10.3892/or.2016.5031, indexed in Pubmed: 27572936.
14. Guzman JN, Ilijic E, Yang B, et al. Systemic isradipine treatment diminishes calcium-dependent mitochondrial oxidant stress. J Clin Invest. 2018; 128(6): 2266–2280, doi: 10.1172/JCI95898, indexed in Pubmed: 29708514.
15. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril. 1997; 67(5): 817–821, doi: 10.1016/s0015-0282(97)81391-x, indexed in Pubmed: 9130884.
16. Cho S, Mutlu L, Zhou Y, et al. Aromatase inhibitor regulates let-7 expression and let-7f-induced cell migration in endometrial cells from women with endometriosis. Fertil Steril. 2016; 106(3): 673–680, doi: 10.1016/j.fertnstert.2016.05.020, indexed in Pubmed: 27320036.
17. Sun Y, Guo W, Ren T, et al. Gli1 inhibition suppressed cell growth and cell cycle progression and induced apoptosis as well as autophagy depending on ERK1/2 activity in human chondrosarcoma cells. Cell Death Dis. 2014; 5: e979, doi: 10.1038/cddis.2013.497, indexed in Pubmed: 24384722.
18. Yang Y, Zhou J, Li X, et al. Gefitinib enhances sensitivity of endometrial cancer cells to progestin therapy via dual-specificity phosphatase 1.

Oncotarget. 2017; 8(70): 115360–115369, doi: 10.18632/oncotarget.23264, indexed in Pubmed: 29383165.
19. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972; 26(4): 239–257, doi: 10.1038/bjc.1972.33, indexed in Pubmed: 4561027.
20. Gruber HE, Hoelscher GL, Bethea S, et al. Mitochondrial Membrane Potential and Nuclear and Gene Expression Changes During Human Disc Cell Apoptosis: In Vitro and In Vivo Annulus Findings. Spine (Phila Pa 1976). 2015; 40(12): 876–882, doi: 10.1097/BRS.0000000000000936, indexed in Pubmed: 25909354.
21. Xu P, Cai X, Zhang W, et al. Flavonoids of Rosa roxburghii Tratt exhibit radioprotection and anti-apoptosis properties via the Bcl-2(Ca(2+))/Caspase-3/PARP-1 pathway. Apoptosis. 2016; 21(10): 1125–1143, doi: 10.1007/s10495-016-1270-1, indexed in Pubmed: 27401922.