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Asperosaponin VI promotes progesterone receptor expression in decidual cells via the notch signaling pathway

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ABSTRACT

Recurrent spontaneous abortion (RSA) is a common clinical condition, but its reasons remain unknown in 37–79% of the affected women. The steroid hormone progesterone (P4) is an integral mediator of early pregnancy events, exerting its effects via the progesterone receptor (PR). Dipsaci Radix (DR) has long been used for treating gynecological diseases in Chinese medicine, while its molecular mechanisms and active ingredients are still unclear. We report here the progesterone-like effects of the alcohol extraction and Asperosaponin VI from DR in primary decidual cells and HeLa cell line. We first determined the safe concentration of Asperosaponin VI in the cells with MTT assay and then found by using dual luciferase reporter and Western blotting that Asperosaponin VI significantly increased PR expression. Moreover, we explored the mechanisms of action of the DR extracts and Asperosaponin VI, and the results showed that they could activate Notch signaling, suggesting that they may function by promoting decidualization.

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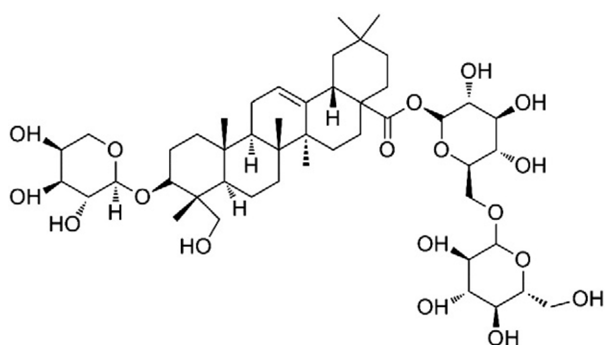

Chemical structure of Asperosaponin VI

1. Introduction

As traditional tonifying Chinese medicine to prevent miscarriage, DR has a complicated chemical composition. In modern pharmacological studies, components of DR have been isolated and identified. It has been found that saponin has the highest content, followed by alkaloid, volatile oil, iridoid, etc. Particularly, Asperosaponin VI, which is also called akebia saponin, is the most abundant content in DR. Most recent studies on the pharmacological properties of Asperosaponin VI were focused on neuroprotection, osteoporosis prevention, myocardial protection, anti-apoptosis and analgesia [1–3], while its progesterone-like effects have not been reported.

Progesterone (P) plays a critical role in pregnancy maintenance by activating PR. As a member of the steroid hormone nuclear receptor family, PR functions as a ligand-activated transcription factor. Increase in phosphorylation of PR, binding of the dimerized receptors with the specific hormone to the promoter region of the target gene, and interactions of the receptor complex with specific coactivator proteins or general transcription factors, promote PR to form an effective transcription initiation complex with the specific target gene promoter [4].

For women in 6–8 weeks of pregnancy, the progesterone secreted by corpus luteum declines gradually, and placenta begins to replace corpus luteum. Decline of the progesterone level in blood incurring decidua necrosis in early pregnancy is a reason that results in miscarriage [5].

Abbreviations: RSA, recurrent spontaneous abortion; DR, Dipsaci Radix; PR, progesterone receptor; ER, estrogen receptor; MTT, thiazolyl blue tetrazolium bromide; PRL, prolactin; HPLC, high performance liquid chromatography; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride.

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RU486, or mifepristone, was discovered in the early 1980's as the first known progesterone antagonist. Studies later determined that RU486 inhibits progesterone-mediated gene transcription, and if administered post implantation, it can ultimately lead to conceptus abortion [6]. The abortifacient properties of RU486 later lead to its use as an emergency contraceptive, demonstrating the critical role progesterone signaling in pregnancy [7].

In recent studies, researchers investigated the progesterone-like or progesterone antagonistic effects of extractions of traditional Chinese medicines for tonifying effects and preventing miscarriage by using progesterone receptor luciferase report assays, and the results indicated that DR extract showed strong progesterone-like effects [8].

In this paper, we report the dramatic progesterone-like effects of Asperosaponin VI, which is the most abundant content in DR, and its actions on PR expression and Notch signaling pathway.

2. Materials and methods

2.1. Cells, reagents and antibodies

Hela cells were obtained from Cell Bank of Chinese Academy of Sciences, and primary decidua cells were isolated from decidua tissues in our lab. DR was purchased from Kangmei Pharmaceutical co., Ltd. (Guangdong, China). Asperosaponin VI was obtained from Guangdong Food and Drug Administration, China. Both progesterone and Mifepristone (RU486) were obtained from Sigma Aldrich. DMEM/F12 With and without phenol red, FBS, 0.25% trypsin, Charcoal dextran-treated FBS and Lipofectamine™ 2000 were all from Gibco. Progesterone receptor luciferase reporter plasmid was obtained from YeaSen Co., Ltd. (Shanghai, China) and dual luciferase kit was purchased from Promega. Rabbit monoclonal anti-Vimentin antibody [EPR3776], mouse monoclonal anti-Cytokeratin7 antibody [RCK105], rabbit monoclonal anti-Prolactin antibody [PRL2], and anti-Progesterone Receptor antibody [YR85] were products of Abcam. Mouse monoclonal anti-GAPDH antibody was purchased from Beyotime (Jiangsu, China). Anti-Rabbit IgG (H + L), HRP Conjugate and Anti-Mouse IgG (H + L), HRP Conjugate and Renilla Luciferase Assay System were purchased from Promega. Alexa Fluor 488- and 594-conjugated secondary antibodies were from Molecular Probes.

2.2. DR preparation and quality control

DR was ground into powder, 10 g of which were soaked in 95% alcohol for 72 h. The extraction was filtered under vacuum, dried in a rotary evaporator, and stored in −2 °C until use. Asperosaponin VI, a typical bioactive triterpenoid saponin in DR, was used as the reference standard for quality control of the DR extracts. High performance liquid chromatography (HPLC) analysis was performed according to reference [9]. Briefly, the HPLC system (Agilent 1100 Series) including a quaternary pump, C_{18} gravity column (Alltima C_{18}, 4.6 nm × 250 nm, 35 °C) and a DAD detector was used with the following parameters: mobile phase, acetonitrile/water = 30/70; flow rate, 1.0 ml/min; injection volume, 20 μl, detector wavelength, 212 nm.

2.3. Primary decidual cell isolation and HeLa cell culture

Decidua samples were obtained at 6–9 week of gestation from singleton pregnancies of mothers requesting termination of normal pregnancy or who were undergoing evacuation of retained products of conception following spontaneous pregnancy failure. All patients gave their written informed consent according to Declaration of Helsinki, and we obtained the permission of Ethics Committee of The First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine.

Fresh decidua tissues were obtained in sterile condition and washed with PBS to remove blood, cut into pieces and digested with trypsin-EDTA (0.25%) for 5–10 min, DMEM/F12 medium with 10% FBS was then added to stop digestion.

Cell clusters in the final digestate were dissociated by aspiration with a 23-gauge needle, filtered through a 200-mesh sieve, and then centrifuged at 2000 rpm for 5 min, The pellet was resuspended with DMEM/F12 medium with 10% FBS and cultured in flask at 5×10^5 cells/ml. After 30 min, non-adherent cells were removed, and the medium was changed after 48 h.

HeLa cells were cultured in DMEM supplemented with 4.5 g/l glucose, 10% FBS, L-glutamine, nonessential amino acids, pyruvate and antibiotics (penicillin/streptomycin).

2.4. Cell viability test

Cell viability was determined by assessing the degree of MTT reduction. in brief, the decidual cells were harvested with 0.05% trypsin and 0.53 mM EDTA·4Na and resuspended in 10% FBS in DMEM/F12. The cells were seeded in 96-well plates at 2000 cells/well and cultured for 48 h. To avoid steroids in the culture media, the seeding media (10% FBS in DMEM/F12) was replaced with phenol red-free DMEM/F12 supplemented with 10% Charcoal dextran-treated FBS (CDFBS). After treated with the alcohol extraction of Dipsaci Radix or Asperosaponin VI, cells were incubated at 37 °C in 1 mg/ml MTT solution for 4 h. After the removal of the MTT solution, 150 μl of dimethyl sulfoxide were added, and the absorbance at the wavelength of 490 nm was recorded with a microtiterplate reader. Cell viability was expressed as a percentage of the control culture.

2.5. Immunofluorescence staining

After the treatment, decidual cells on coverslips were fixed with Immunol Staining Fix Solution (Beyotime, China), and blocked for 30 min with 10% normal goat serum in PBS containing 0.1% Triton X-100. Cells were then incubated with primary antibodies for 1 h at RT, followed by incubation with appropriate Alexa Fluor-labeled secondary antibodies (1:1000, in blocking buffer). The coverslips were mounted with Prolong Gold anti-fade reagent and DAPI, and the cells were observed under a confocal microscope (Zeiss 710) [10].

2.6. Plasmid transection and luciferase reporter gene assays

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum without antibiotics. The cells at 80–90% confluent were transfected with the Progesterone receptor luciferase reporter plasmid using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 12 h of transfection, cells were treated with the alcohol extract of Dipsaci Radix or Asperosaponin VI. After 12 h of incubation, the cells were lysed with 100 μl of passive lysis buffer in a dual luciferase kit according to the manufacturer's protocol. The luciferase activity was normalized with pRL-Tk control vector expression [11].

2.7. Western blotting

Decidual cells or HeLa cells were cultured in 6-well plates, treated with progesterone, the alcohol extract of Dipsaci Radix or Asperosaponin VI with or without RU486. After the treatment, cells were harvested and lysed with ice-cold RIPA buffer (Beyotime, China) supplemented with an EDTA-free protease inhibitor cocktail. Samples of lysate (20 μg) were separated by 10% SDS–PAGE and transferred to NC membrane (Merk Millipore). After blocking with 5% non-fat milk in TBS containing 0.1% Tween 20 for 1 h at room temperature (RT), membranes were incubated with appropriately diluted primary antibodies at 4 °C overnight and then probed with HRP-conjugated secondary antibodies for 1 h at RT. Immunoreactive bands were detected by enhanced chemiluminescence and visualized with the Las4000 (GE Healthcare). The intensities of the signals were quantified by densitometry using Quantity One software according to the manufacturer's instructions as previously described [12].

2.8. Quantitative RT-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed (RT) into cDNA according to the manufacturer's instructions. Quantitative real time PCR (qPCR) was performed using SYBR Green II Master Mix in PikoReal Real-Time PCR System (Thermo Scientific). The profile of thermal cycling consisted of initial denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. All primers used for qPCR analysis were synthesized by Invitrogen. The specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis. GAPDH was used as an internal control. Sequences of primers (5′-to-3′): for PR, forward: CTATCTCAACTACCTGAGGCCG; reverse: CAGCTCCCACAGGTAAGGAC; and for GAPDH, forward: CTCTGCTCCTCCTGTTCGAC, reverse: GCGCCCAATACGACCAAATC.

2.9. Statistical analysis

Data were analyzed and plotted by using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and presented in the form of mean ± S.D., then. Statistical analyses were performed with Student's *t*-test and ANOVA using SPSS 13.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Verification of primary decidual cells

After being cultured for 3 generations, decidual cells were verified by immune-staining of the decidual cell marker Vimentin and the trophoblast marker Cytokeratin 7. The results showed that the cells were strongly stained with Vimentin, while almost negative Cytokeratin 7 staining was observe, indicating that >99% of the cells were decidual cells. Besides, we also stained the cells with prolactin antibody and obtained a positive result (Fig. 1).

3.2. Herbal evaluation and effects of Asperosaponin VI on cell viability

HPLC analysis showed that the average percentage of Asperosaponin VI contained in DR extract was 30.36%, which fulfilled the requirement of chemical content of DR specified by the Commission of Chinese Pharmacopoeia (2010). A typical HPLC chromatogram is shown (Fig. 2A and B).

The dose-response relationship of Asperosaponin VI on the viability of decidual cells was measured after 24 h of treatment. As shown in Fig. 2C, Asperosaponin VI did not significantly change the cell viability at the dosages of 0.5–20 μg/ml, suggesting that it is non-toxic to the cells.

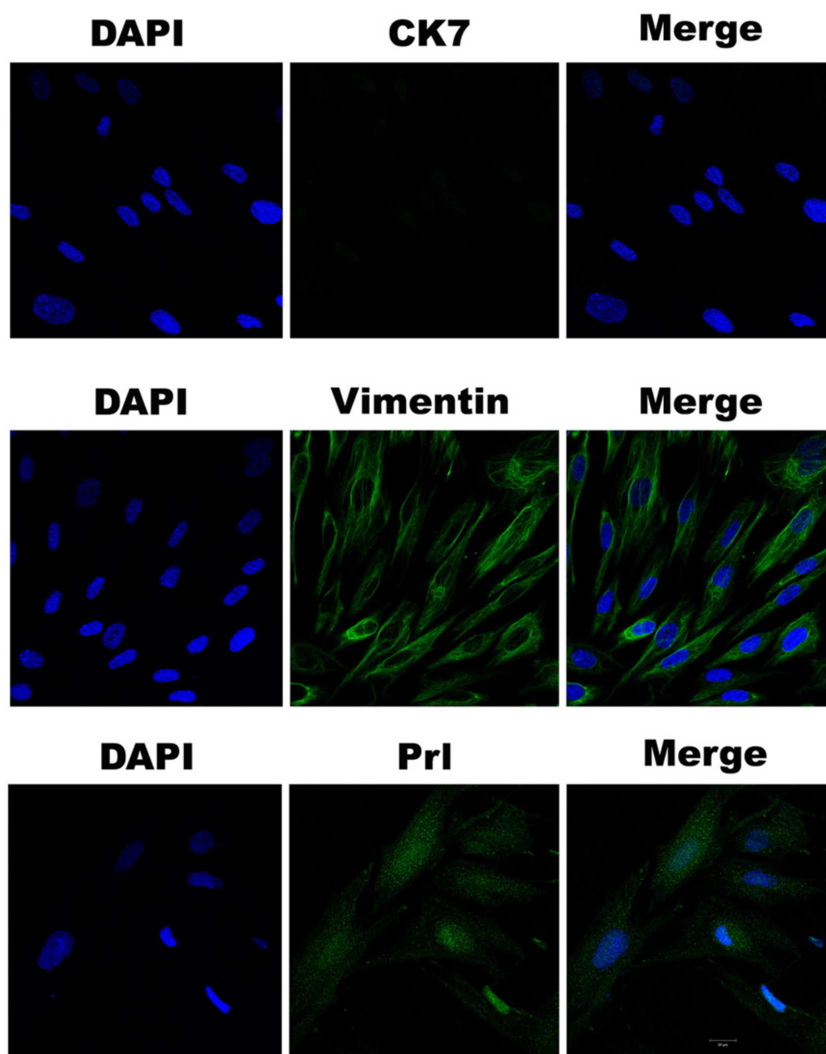

Fig. 1. Verification of decidual cells by immuno-staining. Primary decidual cells were cultured on coverslips in 24-well plates and then fixed with formaldehyde solution for immuno-staining with Vimentin, Cytokeratin7 and Prolactin antibodies. The immunofluorescence signals were imaged under a confocal microscope. Bar, 20 μm.

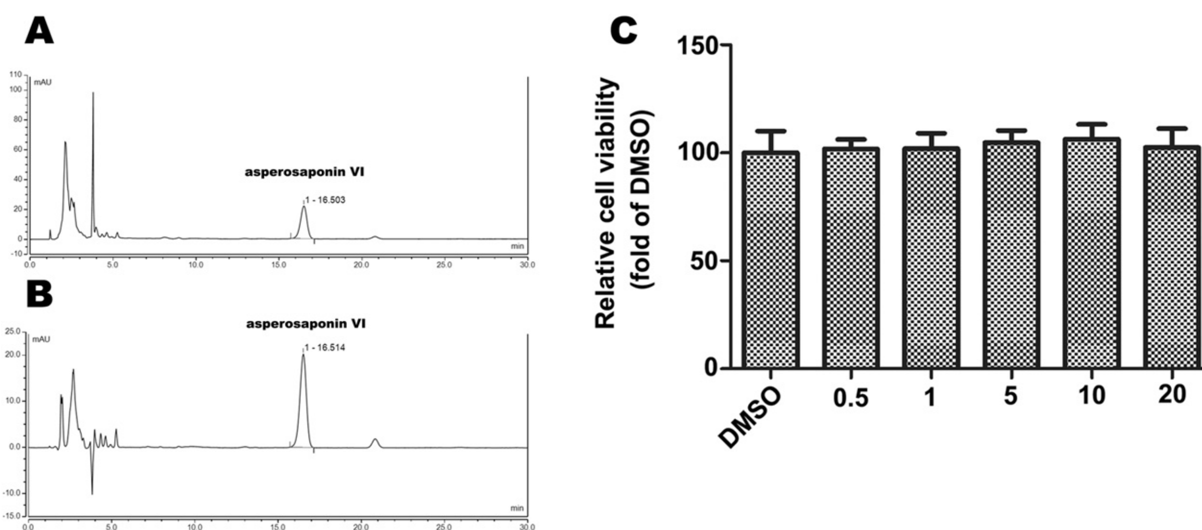

Fig. 2. HPLC chromatographs showing the presence of Asperosaponin VI in the DR alcohol extract (A) and the reference sample (B) and MTT assay results (C). (A, B) No significant difference was found between the retention time of VI in the reference solution and DR alcohol extract under the same HPLC conditions. (C) The MTT assay showed no decrease of cell viability after treatment with Asperosaponin VI at the concentrations of 0.5, 1, 5, 10, 20 μg/ml.

3.3. Asperosaponin VI targets PR

Although Dipsaci Radix is widely used in traditional medicine in China for tocolysis, its mechanisms of actions remain unclear. We explored the potential effects of one of its components, Asperosaponin VI, and its alcohol extract on PR expression. By using dual-luciferase reporter gene assay, we found that both Asperosaponin VI and the alcohol extract could upregulate the luciferase activity, although their effects were slightly weaker than the positive control progesterone, and that their effects were abolished by mifepristone (RU486) (Fig. 3A). Furthermore, we found that both Asperosaponin VI and the alcohol extract could increase PR expression in primary decidual cells, and that their effects were diminished by RU486. These data suggest that Asperosaponin VI targets PR.

3.4. Asperosaponin VI up-regulates the Notch signaling pathway

To investigate the mechanism of action of Asperosaponin VI in promoting PR expression in decidual cells, we explored the function of Asperosaponin VI and the alcohol extract on Notch expression. As shown in Fig. 4, progesterone, Asperosaponin VI and the alcohol extraction could increase the expression of both Notch1 and Notch1, albeit with weaker activities than progesterone, and the effects were interfered by RU486.

4. Discussion

In order to make successful pregnancy, the embryo must attach to the luminal epithelial cells and parasitic on the stroma. Then, surrounding stromal cells need to undergo decidualization in order to establish the musculature necessary for the embryo survival [13]. These events in early pregnancy are intimately regulated by two hormones, estrogen and progesterone, through their corresponding receptors, the estrogen receptor (ER) and the progesterone receptor (PR), respectively. PR has been shown to be pivotal for uterine receptivity and embryo implantation [14,15].

Progesterone receptors can be defined as cellular proteins with a high affinity for progesterone that, upon binding progesterone, become functionally transformed to perform specific intracellular actions, and hence the cells show response to progesterone [16]. The PR exists as two isoforms, PR-A and PR-B, which are transcribed from two different initiation sites in the same gene [17,18]. Multiple PRs have been identified. These include classic nuclear PRs (nPRs) and another one that appears to reside on the cell surface known as membrane PRs (mPRs) [19]. Progesterone-PR interaction can affect cellular functions via genomic pathways and non-genomic pathways. In non-genomic pathways, progesterone may affect cell functions by its interactions with mPRs, PR membrane component-1 and -2 (PGRMC-1 and PGRMC-2), and other cell surface receptors that are directly linked with intracellular signaling cascades, and by activation of intracellular signal molecules by ligand-activated nPRs via non-transcriptional effects of the extranuclear pathways [20].

Progestogen activation of PR involves dimerization, phosphorylation, and subsequent binding to the cis-acting progestogen response elements on DNA that modulate activity of target gene promoters in target tissues [21]. Both PR-A and PR-B show the same binding affinity for progestogen. Data from co-transfection with different reporter constructs in cell lines not normally expressing PR indicated that PR-B displays hormone-dependent transactivation, whereas PR-A displays cell-specific and reporter-specific activities that may be similar to PR-B, or may be inactive, or may behave as a trans-dominant PR-B inhibitor [22].

Herbal medicines have been widely used by oriental medical doctors to treat recurrent spontaneous abortion for thousands of years, and Dipsaci Radix is one of such herbs. The root of Dipsacus asper Wall.ex Henry is a well-known herbal medicine named Dipsaci Radix (DR) for the clinical treatment of menoxenia, leucorrhea disorder, puerperal disorder, breast nodule, and other female diseases [9]. Particularly, DR was ranked as the first selective herb reported in clinical studies to prevent and treat miscarriages in Chinese women [23], and its effects on uterine bleeding and tocolysis during pregnancy were highlighted in traditional experiences cumulated for thousands of years. However, its effects on PR are not clear. In this study, we found that the alcohol extraction of DR or Asperosaponin VI showed strong effects on PR expression, comparable to though not better than the positive control progesterone, indicating that Asperosaponin VI may play a progesterone-like function if used during pregnancy.

Notch signaling plays a critical role in decidualization [24,25], spiral artery remodeling [26] and placental development during pregnancy [27]. Notch proteins are ligand-dependent transmembrane

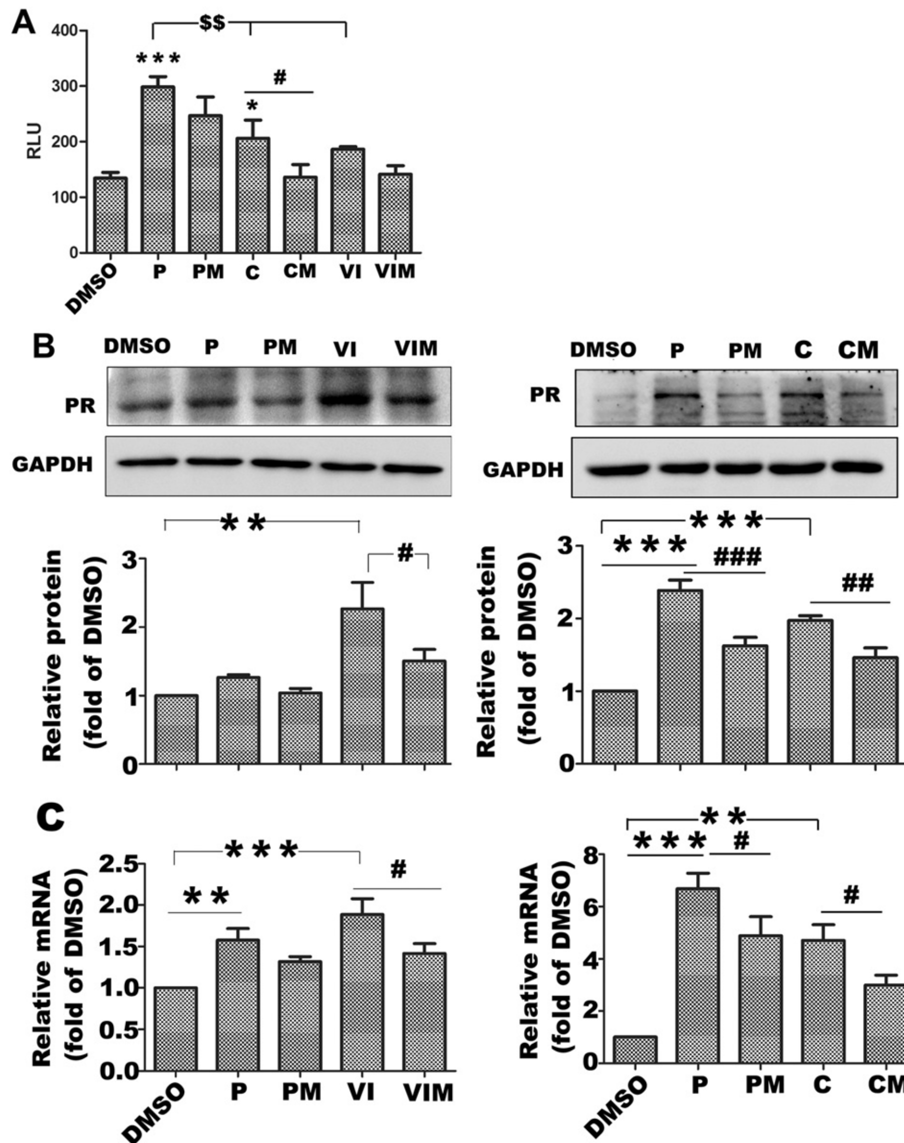

Fig. 3. Asperosaponin VI promoted PR expression. HeLa cells were transfected with PR luciferase reporter plasmid incubated with progesterone (P), Asperosaponin VI (VI) or the DR alcohol extract (C) with or without mifepristone (M). (A) The fluorescence intensities were analyzed by the dual luciferase kit. (B, C) Effects on PR protein and mRNA expression were evaluated by Western blotting and RT-qPCR. The bar graph represents mean ± SEM, from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared to DMSO; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to mifepristone-free group; $p < 0.05 compared to progesterone treatment.

receptors that transduce extracellular signals responsible for cell destiny and differentiation through development and across species. Notch ligands DLL-4, Jagged 1 and 2 are expressed in decidual and trophoblast cells and are involved in angiogenesis during placental vascularization via the secretion of growth factors such as VEGF [28,29]. The expression of DLL-1 ligand and Notch receptor increase in the uterus and placenta during inflammation-induced preterm labor. Inhibition of Notch signaling suppresses inflammatory responses in decidual and placental cells [30].

In mice, Notch signaling may be necessary for ER and PR-induced uterine function. Notch, ER, and PR signaling have been implicated in a few cancers, which, similarly to decidualization, involve a multiplicity of cellular signaling pathways and processes [31]. Our results show that the alcohol extract, Asperosaponin VI and the positive control progesterone could activate the PR promoter in both HeLa cells and primary decidual cells, increase PR expression, activate the downstream Notch signaling pathway, and induce decidualization for a better implantation, thereby helping to maintain pregnancy.

5. Conclusion

In summary, as a traditional medicine for RSA treatment, DR shows excellent treatment effectiveness, and our results revealed progesterone-like effects of the alcohol extraction of DR and Asperosaponin VI, by activating PR expression and Notch signaling pathway, promoting decidualization for a more successful pregnancy.

Conflicts of interest

The authors have declared that no conflict of interest exists.

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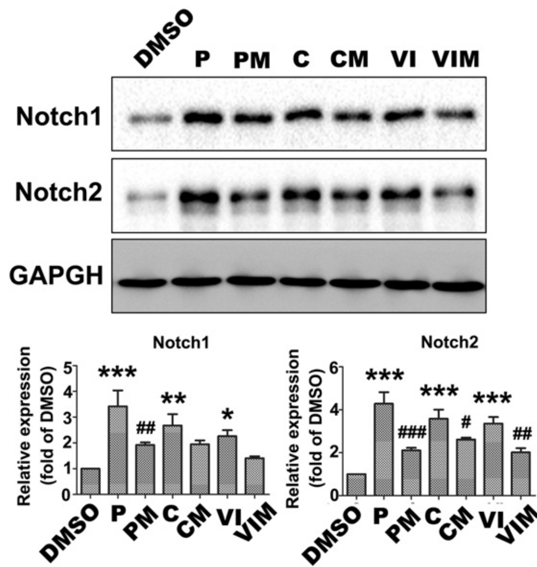

Fig. 4. Asperosaponin VI activated the Notch signaling pathway in decidual cells. Decidual cells were treated with progesterone (P), Asperosaponin VI (VI) or the DR alcohol extract (C) respectively, with or without mifepristone (M) for 24 h. Notch1 and Notch2 protein expression was detected by Western blotting. The bar graph represents mean ± SEM, from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to DMSO; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to mifepristone-free group.

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