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Saponin Extracts Induced Apoptosis of Endometrial Cells From Women With Endometriosis Through Modulation of miR-21-5p

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Abstract

Among the several components in Korean red ginseng, the saponin components are known to have various pharmacologic activities. The objective of this study was to evaluate therapeutic effects of saponin extracts from Korean red ginseng on endometriosis and to identify microRNAs (miRNAs) associated with saponin treatment. This is an in vitro study which used human endometrial stromal cells (HESCs) obtained from patients who underwent laparoscopic surgery for endometriosis and other benign conditions. Human endometrial stromal cells were treated with saponin extracts, and microarray profiling was performed. Human endometrial stromal cells were then transfected with miRNAs identified in the profiling. After the saponin extract treatment, the expression of caspase 3 was significantly increased in HESCs. Microarray profiling revealed several miRNAs that were differentially expressed, and miR-21-5p was further validated. Expression of miR-21-5p was significantly upregulated in the endometrium of patients with endometriosis, compared with controls. Transfection of a miR-21-5p inhibitor significantly increased caspase 3 expression in HESCs. The apoptotic potential of saponin extracts and the miR-21-5p inhibitor were further validated in HESCs using flow cytometry analysis. In conclusions, treatment with saponin extracts significantly decreased the expression of miR-21-5p in HESCs from patients with endometriosis. Inhibition of miR-21-5p effectively increased the apoptotic potential of HESCs. These findings suggest that saponin extract treatment may have therapeutic potential for endometriosis via modulation of specific miRNAs.

Keywords

apoptosis, endometriosis, microRNA, miR-21-5p, saponin extracts

Introduction

Endometriosis is the proliferation of endometrial tissue outside the uterine cavity. One of the most common gynecologic disorders, endometriosis affects approximately 10% of all reproductive-age women. In total, 20% to 50% of women with chronic pelvic pain or infertility, or both, have endometriosis.[1] The prevalence of the disease is relatively high in young reproductive-age women, but the optimal treatment approach has not been determined. Surgery may provide improvement of endometriosis-associated pain and infertility, but the long-term benefits of surgical treatment are unclear in terms of symptom management and clinical outcomes.[2] Medical treatment has an important role in the long-term management of endometriosis-related symptoms and prevention of recurrence. However, mainstream medical treatment involves reduction in ovarian-derived steroid hormone production. These medications often have limitations that include side effects, high costs, and patient compliance issues. Because many affected women desire to become pregnant, the development of new and effective nonhormonal medical treatment is needed.

Korean red ginseng is used frequently in the practice of traditional oriental medicine. It has various pharmacological activities (eg, anti-inflammatory, antioxidant, antitumor, antidiabetes, and antihepatotoxicity effects).[3] Among the various

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components in Korean red ginseng, the saponin components have therapeutic benefits when used in patients with thrombotic disease and inflammation. A rat model study revealed that saponins act on cyclooxygenase-1 (COX-1) and thromboxane A2 (TXA2) synthase to inhibit the production of TXA2[4] and increase cyclic adenosine monophosphate (cAMP) levels, which leads to reduction in [Ca+] mobilization during thrombin-induced platelet aggregation.[5] The saponins in Korean red ginseng have protective effects against myocardial infarction through the involvement of nuclear factor kappaB (NF-κB), Sirt1, and mitogen-activated protein kinase signaling pathways and inhibition of inflammation.[6] The results of a mouse model study suggested that saponin fractions might act as potential sources of therapeutic agents against inflammation via downregulation of pro-inflammatory mediators and provide protection against endotoxic shock.[7]

Current evidence indicates that endometriosis is likely associated with local and systemic inflammatory processes with increased cell proliferation and decreased apoptotic potentials. We hypothesized that apoptosis properties of saponin extracts from Korean red ginseng may have therapeutic potential for endometriosis treatment. A growing body of evidence suggests that aberrant miRNA expression is associated with the pathogenesis of endometriosis; modulation of these miRNAs has been suggested as a new approach for endometriosis treatment.[8-12] We also hypothesized that the therapeutic potential of saponin extracts may include modulation of specific miRNAs. In this study, we investigated the therapeutic effects of saponin extracts from Korean red ginseng in cells from patients with endometriosis and evaluated whether saponin extracts induced modulation of specific miRNAs involved in the pathogenesis of endometriosis.

Materials and Methods

Study Population and Sample Collection

Thirty-two women (range: 21-48 years of age) participated in this study after providing written informed consent. The study protocol was approved by the institutional review board of Gangnam Severance Hospital (Seoul, Korea). The participants were recruited from patients who underwent laparoscopy for various conditions, including pelvic masses, pelvic pain, endometriosis, infertility, and diagnostic evaluation of benign gynecologic disease, between June 2015 and July 2016. Postmenopausal patients or patients with previous hormone or gonadotropin-releasing hormone agonist use, adenomyosis, endometrial cancer or hyperplasia, endometrial polyps, infectious disease, chronic or acute inflammatory disease, malignancy, autoimmune disease, cardiovascular disease, or any combination of these conditions were excluded from the study.

All possible endometriotic lesions were excised during surgery and were sent for a pathology examination for confirmation of the diagnosis. A patient was assigned to the endometriosis group only after pathologic confirmation of the excised tissue. The extent of endometriosis was determined using the American Society for Reproductive Medicine revised classification guidelines.[13] Sixteen patients had histologically confirmed peritoneal or ovarian endometriosis, or both, with moderate-to-severe disease (stage III or IV). The control group consisted of 16 patients: 12 patients had dermoid cysts, 3 had serous cystadenoma, and 1 had a simple ovarian cyst. Endometrial biopsies were obtained from each patient using a Pipelle catheter (Cooper Surgical, Trumbull, Connecticut).

Culture of Primary Endometrial Stromal Cells

Endometrial stromal cells were cultured as previously described.[12] In brief, endometrial tissue was finely minced, and the cells were dispersed using incubation in Hanks' balanced salt solution–containing HEPES (2 mmol/mL), 1% penicillin/streptomycin, and collagenase (1 mg/mL, 15 U/mg) for 60 minutes at 37°C with agitation and pipetting. The cells were pelleted, washed, suspended in Ham's F12: dulbecco's modified eagle's medium (DMEM) (1:1) mixture containing 10% fetal bovine serum and 1% penicillin/streptomycin, passed through a 40-μm cell strainer (Falcon, Corning, New York), and plated into 75-cm^2 Falcon tissue culture flasks (BD Biosciences, Bedford, Massachusetts). The cultured human endometrial stromal cells (HESCs) were used for analysis at 3 to 5 passages.

MicroRNA Microarray Analysis

For quality control, RNA purity and integrity were evaluated using the 260/280 optical density (OD) ratio as analyzed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Santa Clara, California). The Affymetrix GeneChip miRNA array (Affymetrix, Santa Clara, California) process was performed following the manufacturer's protocol. RNA samples (1 μg) were labeled using the FlashTag Biotin RNA Labeling Kit (Genisphere, Hatfield, Pennsylvania). The labeled RNA was quantified, fractionated, and hybridized to the miRNA microarray following the standard procedure provided by the manufacturer. The labeled RNA was heated to 99°C for 5 minutes and then to 45°C for 5 minutes. RNA-array hybridization was performed with agitation at 60 rotations per minute for 16 to 18 hours at 48°C on an Affymetrix 450 Fluidics Station. The chips were washed and stained using a Genechip Fluidics Station 450 (Affymetrix). The chips were then scanned using an Affymetrix GeneChip Scanner 3000. Signal values were computed using the Affymetrix GeneChip® Command Console® software (AGCC; version 1.3.1.187). The raw data were extracted automatically using an Affymetrix data extraction protocol and the software provided by Affymetrix GeneChip Command Console Software. The intensity data file import, miRNA level robust multichip average+detection above background-All analysis, and result export were performed using Affymetrix Expression Console™ Software (version 1.4.1.46). The array data were filtered using probes annotated by species. The comparative analysis between each test sample and control sample was performed using a fold-change approach. All statistical tests and visualization of differentially expressed genes were performed using R statistical language v. 2.15.0 software.

Cell Viability Assay

Cytotoxicity was measured using the $TACS_{TM}$ MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) cell proliferation assay kit (TREVIGEN, Gaithersburg, Maryland). The cells were seeded into 96-well plates and treated with indicated concentrations of saponin extracts for 48 hours. After this treatment, 10 μL MTT was added to each well, and the plates were incubated at 37°C. DMSO (100 μL) was then added to each well, and the dark blue formazan product was quantified using a microplate reader (570-nm absorbance wavelength; 690-nm reference filter wavelength; Molecular Device, Sunnyvale, California). The relative cell viability (%) result was expressed as a percentage value relative to the value for the untreated control cells.

Cell Transfection

Cells were cultured to 70% to 80% confluence after being seeded into 6-well plates. They were transfected with an homo sapiens-microRNA (hsa-miR)-negative inhibitor as a control or with an hsa-miR-21-5p inhibitor (Ambion, Inc, Life Technologies Corporation, Carlsbad, California). Lipofectamine 2000 (Invitrogen, Carlsbad, California) transfection reagent was used following the manufacturer's instructions to a final concentration of 50 nM. The transfected cells were harvested 48 hours after the reagent was added.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

To measure caspase 3 messenger RNA (mRNA) levels, total RNAs were isolated from cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA sample concentrations were analyzed using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Using 1 μg of total RNA, complementary RNA (cDNA) was synthesized with oligo-dT (Superscript III kit, Invitrogen) in a C1000 Thermal Cycler (Bio-Rad, Hercules, California). The resultant cDNA mixtures were stored at −20°C. Using a 2 μL synthesized cDNA template, quantitative real-time polymerase chain reaction (PCR) amplification was performed using the 7300 Real Time PCR System (Applied Biosystems, Foster City, California). The Power SYBR Green PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Woolston Warrington, UK) was used for nucleic acid quantitation during the real-time PCR. The reaction mixture included the cDNA template, forward and reverse primers, ribonuclease-free water, and the SYBR Green master mix, at a 20-μL final reaction volume. The thermal cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. The threshold cycle (Ct) and melting curves were estimated using the 7300's software program (Applied Biosystems). Each reaction was performed in triplicate. The mRNA level for each sample was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels.

To assess miRNA expression levels, RNA was extracted from human endometrial cells and cultured cells using the miRVana RNA Isolation Kit (Ambion Inc, Austin, Texas) per the manufacturer's instructions; 30 μL nuclease-free water was used to elute the RNA. A Nanodrop ND-2000 spectrophotometer was used to determine the RNA yield. A total of 10 ng isolated RNA and the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Baltics, Lithuania) were also used. Quantitative real-time PCR for the miRNAs was performed using a Taqman Universal Master Mix II, no uracil-N-glycosylase (UNG) (Applied Biosystems), with sets for miR-21-5p and U6 small nuclear RNA (U6 snRNA) (Applied Biosystems). All real-time PCR reactions were performed in triplicate using a 7300 Real Time PCR system; 40 amplification cycles were performed. Relative expression was calculated using the comparative Ct method, and the miRNA levels were normalized to the U6 levels.[14]

The primers for *caspase 3*, *caspase 8*, *p53*, *ki-67*, matrix metalloproteinase-2 (*MMP2*), *MMP9*, and GAPDH were caspase 3 forward, 5′-GGAAGCGAATCAATGGACTCTGG-3′ and reverse, 5′-GCATCGACATCTGTACCAGACC-3′; caspase 8 forward, 5′-CCAGAGACTCCAGGAAAAGAGA-3′ and reverse, 5′- GATAGAGCATGACCCTGTAGGC-3′; p53 forward, 5′-GCCCAACAACACCAGCTCCT-3′ and reverse, 5′-CCTGG GCACCTTGAGTTCC-3′; ki-67 forward, 5′-GAAAGAGTGG CAACCTGCCTTC-3′ and reverse, 5′-GCACCAAGTTTTACTA CATCTGCC-3′; MMP2 forward, 5′-ACCGCGACAA-GAAGT ATGGC-3′ and reverse, 5′-CCACTTGCGGTCAT CATCGT-3′; MMP9 forward, 5′-CGATGACGAGTTGTG GTCCC-3′ and reverse, 5′-TCGTAGTTGGCCGTGGTACT-3′; GAPDH forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCAC CACCCTGTTGCTGTA-3′, respectively.

Apoptosis Assay

Apoptosis was determined using Hoechst 33342 nucleic acid stain (Sigma-Aldrich, St. Louis, Missouri). The cells were incubated with 10 μg/mL stain for 20 minutes at 37°C and were evaluated using fluorescence microscopy (EVOS All-in-One Digital Inverted Fluorescence Microscope, Thermo Fisher Scientific).

Protein Extraction and Western Blot Analysis

The protein extracts were prepared using RIPA lysis buffer (Thermo Scientific, Rockford, Illinois) containing freshly added protease and phosphatase inhibitor cocktail (Thermo Scientific). The concentrations of total cell lysates were measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). A total of 20 μg total protein was mixed with 5× sample buffer and heated at 95°C for 5 minutes. The samples were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred to a polyvinylidene

fluoride membrane (Millipore Corporation, Billerica, Massachusetts) using a Transblot apparatus (Bio-Rad). The membranes (Millipore Corporation) were blocked using 5% nonfat skim milk in Tris-buffered saline solution (10 mmol/L Tris-HCl [pH 7.4] and 0.5 mol/L NaCl) and adding Tween-20 (0.1% vol/vol). The blots were probed using primary antibodies: caspase 3 (1:500; Cell Signaling Technology, Danvers, Massachusetts) and GAPDH (1:1000, Abcam, Cambridge, UK), followed by horseradish peroxidase–conjugated secondary anti-mouse or anti-rabbit antibody (1:2000; Thermo Scientific). The proteins were detected using enhanced chemiluminescence (Santa Cruz Biotechnology, Dallas, Texas). The experiment was repeated 3 times for analysis.

Flow Cytometry Analysis of Apoptosis

Cells were seeded onto 6-well plates, incubated with indicated concentrations of chemical for 24 or 48 hours, and transfected for 48 hours. Phosphate-buffered saline was used to wash the cells twice; 400 μL binding buffer was used to suspend the cells. A total of 5 μL Annexin fluorescein isothiocyanate (V-FITC) and 4 μL propidium iodide (PI) were then added, and the samples were incubated for reaction at room temperature for 10 minutes (fluorescein isothiocyanate [FITC] Annexin V Apoptosis Detection Kit; BD Biosciences, San Diego, California). Flow cytometry (Canto II, BD Sciences, Bedford, Massachusetts) was used to quantify the samples, and the data were analyzed using DIVA software (version 8.0; BD Biosciences, Heidelberg, Germany).

Statistical Analysis

The results were presented as mean ± standard error of the mean (SEM) values. The data were checked to determine whether they met the requirements for a normal distribution using the Kolmogorov-Smirnov test or the Shapiro-Wilk test. Continuous variables were compared using the Student *t* test or the Mann-Whitney *U* test, where appropriate. SPSS 16.0 software (SPSS Inc, Chicago, Illinois) was used for the statistical analysis. A *P* value <.05 was considered to indicate a statistically significant result.

Results

Clinical Characteristics

The clinical characteristics of the participants are presented in Table 1. There was no significant difference in age between the endometriosis group and the control group. Compared with the control group patients, the endometriosis group patients had significantly lower gravidity, parity, and body mass index. Serum CA-125 levels were significantly greater in the endometriosis, compared with the control, group patients (85.83 ± 28.73 vs 22.40 ± 4.34, respectively, $P = .049$). All endometriosis patients had moderate-to-severe disease. The mean ± SEM revised American Fertility Society (rAFS) score was 40.13 ± 4.97.

Table 1. Clinical Characteristics of Study Participants With and Without Endometriosis.[a]

Characteristic	Endometriosis (n = 16)	Control (n = 16)	*P* Value
Age, years	34.75 ± 1.66	38.31 ± 1.68	.143
Gravidity	1.00 ± 0.32	2.37 ± 0.44	.019
Parity	0.37 ± 0.20	1.50 ± 0.24	.001
BMI (kg/m^2)	20.90 ± 0.39	22.49 ± 0.61	.039
CA-125 (IU/mL)	85.83 ± 28.73	22.40 ± 4.34	.049
rAFS scores	40.13 ± 4.97	NA	

Abbreviations: BMI, body mass index; rAFS, revised American Society for Reproductive Medicine guideline.
[a]Data are expressed as mean ± standard error mean (SEM).

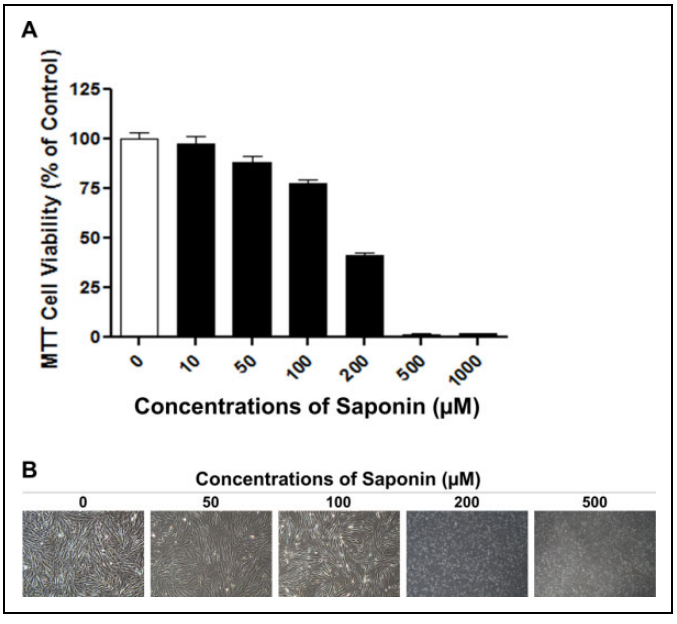

Figure 1. Cells were treated with indicated concentrations of saponin extracts for 48 hours and assessed using (A) MTT assay of endometrial stromal cells. B, Representative changed cell morphology. Results are presented as mean ± standard error of mean (SEM) values (triplicate; n = 4).

Cell Death Induced by Saponin Extracts in Endometrial Stromal Cells from Patients With Endometriosis

Saponin extract treatment significantly decreased cell viability in a dose-dependent (0-1000 μM) manner in HESCs, as determined using the MTT assay (Figure 1A). To characterize the cell death response to saponin extract treatment in HESCs from patients with endometriosis, cells were treated with various concentrations of saponin extracts (0-200 μM). Cell death was determined after 24 and 48 hours using the Hoechst assay (Figure 2). Saponin extract treatment caused a time- and dose-dependent increase in cell death compared with the control level, as indicated by decreases in cell nuclear fluorescence numbers of saponin extract–treated cells. Based on these results, a saponin extract concentration of 100 μM was selected for further in vitro experiments.

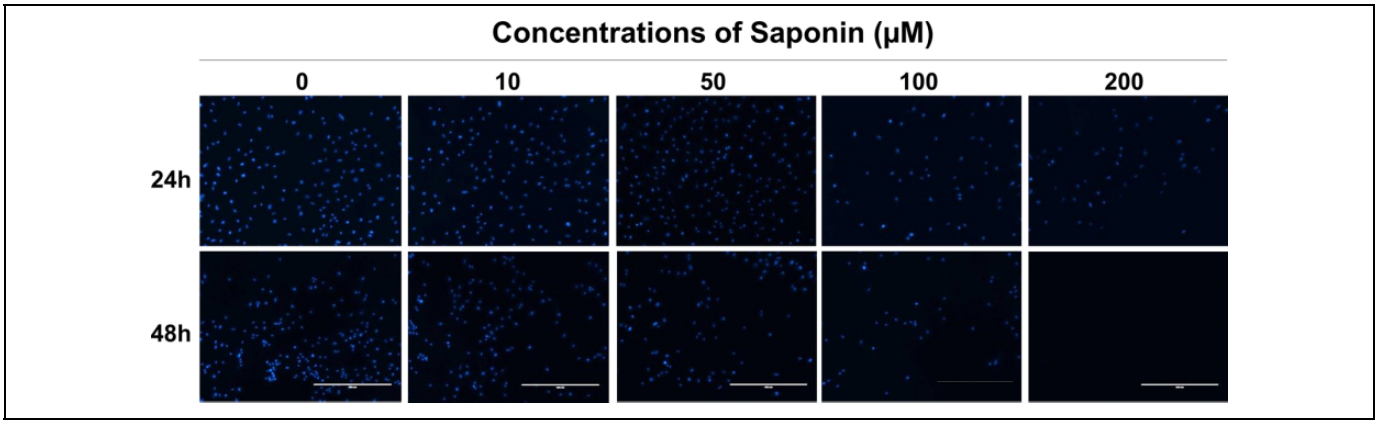

Figure 2. Saponin extract–induced cell death of endometrial stromal cells. A, Representative fluorescence micrographs of cells treated with saponin extracts (0-200 μM) for 24 hours or 48 hours; scale bar, 400 μm).

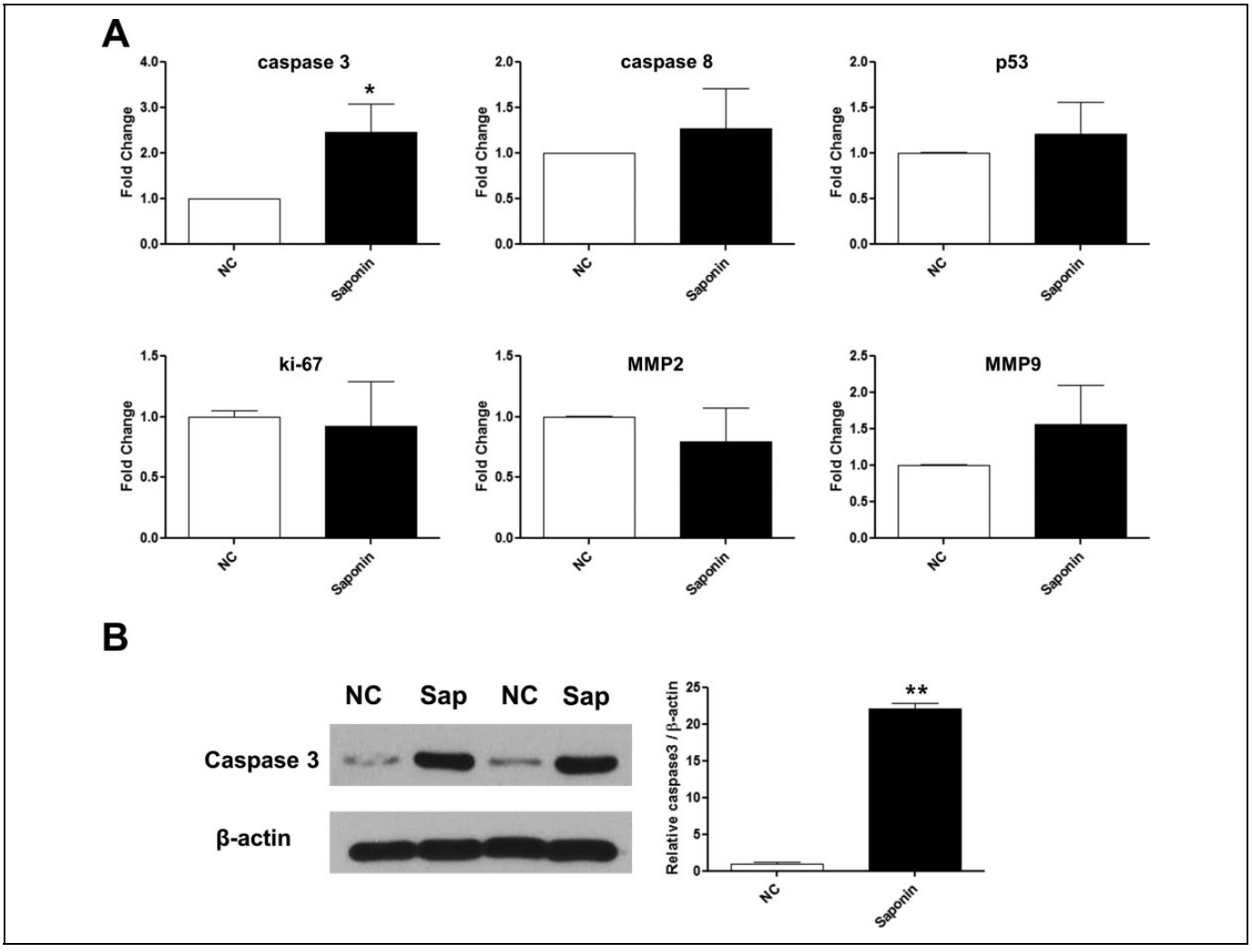

Figure 3. Cellular characteristics after saponin extract treatment for 48 hours. The concentration of saponin extracts was 100 μM. A, Expression of caspase 3, caspase 8, p53, ki-67; and MMP2 and MMP9 (triplicate; n = 9). B, Western blot analysis of caspase 3 expression. *$P < .05$, **$P < .01$. Results are presented as mean ± SEM values. NC indicates negative control; Sap, saponin.

Table 2. MicroRNA Profiles After Saponin Extract Treatment of Endometrial Stromal Cells From Patients With Endometriosis.

Upregulated			Downregulated		
miRNA	FC	*P* Value	miRNA	FC	*P* Value
hsa-miR-24-2-5p	1.69	.043	hsa-miR-21-5p	−1.56	.022
hsa-miR-132-3p	2.19	.044	hsa-miR-181b-5p	−1.56	.034
hsa-miR-138-1-3p	1.95	.049	hsa-miR-503-5p	−2.51	.010
hsa-miR-324-5p	1.67	.037	hsa-miR-642a-3p	−1.53	.047
hsa-miR-324-3p	1.80	.044	hsa-miR-3180-3p	−1.94	.038
hsa-miR-574-5p	1.72	.040	hsa-miR-3180	−2.22	.046
hsa-miR-320b	1.65	.049	hsa-miR-3937	−2.97	.018
hsa-miR-320c	1.58	.030	hsa-miR-4498	−2.10	.033
hsa-miR-3195	1.96	.040	hsa-miR-4690-5p	−1.60	.035
hsa-miR-4521	1.79	.046	hsa-miR-6075	−2.85	.027
hsa-miR-4739	1.96	.002	hsa-miR-6080	−2.53	.023
hsa-miR-8071	9.93	.048	hsa-miR-6802-5p	−1.86	.023
			hsa-miR-6820-5p	−2.54	.013
			hsa-miR-7110-5p	−3.38	.008

Abbreviations: FC, fold change; miRNA, microRNA.

Cellular Characteristics of HESCs From Patients With Endometriosis, After Saponin Extract Treatment

After 48 hours of saponin extract treatment, the expression of caspase 3, caspase 8, p53, Ki-67, MMP2, and MMP9 in HESCs from the patients with endometriosis were evaluated using quantitative real time polymerase chain reaction (qRT-PCR) (Figure 3A). Expression of caspase 3 was significantly increased after saponin extract treatment (2.47-fold change, $P = .015$). There were no significant differences in caspase 8 or p53 expression. The results for ki-67, which is a cell proliferation marker, indicated a trend toward downregulation ($P = .073$), but there were no significant changes in cell invasion markers (MMP2 and MMP9) after the saponin extract treatment. Expression of caspase 3 after saponin extract treatment was further validated using Western blot analysis (Figure 3B). After 48 hours of saponin extract treatment, caspase 3 expression showed an approximately 22-fold increase in HESCs from the patients with endometriosis; that increase was statistically significant ($P < .001$).

MicroRNA Profiles After Saponin Extract Treatment of Endometrial Stromal Cells From Patients With Endometriosis

MicroRNA profiling of endometrial stromal cells from the patients with endometriosis revealed numerous miRNAs that were differentially expressed after saponin extract treatment (n = 4). Twenty-six miRNAs were found to have differential expression with a significant fold change (Table 2). Among these miRNAs, 12 were significantly upregulated and 14 were significantly downregulated; miR-21-5p had a statistically significant fold change of −1.56335 ($P = .021$). After a database search and literature review, miR-21-5p was selected for further validation.

Expression of miR-21-5p in Endometrial cells of Patients With and Without Endometriosis and the Effects of Saponin Extract Treatment on miR-21-5p Expression

The relative expression of miR-21-5p in endometrial cells of patients with and without endometriosis was assessed using qRT-PCR (Figure 4A). The expression level of miR-21-5p was significantly greater in patients with endometriosis, compared with the control group (21-fold difference, $P < .001$). To evaluate the association between saponin extracts and the expression of miR-21-5p, HESCs from patients with endometriosis were treated with 100 μM saponin extracts for 48 hours. The results indicated that there was significant downregulation of miR-21-5p expression (2-fold decrease; $P = .005$, Figure 4B).

miR-21-5p Inhibitor Transfection Increased Caspase 3 Expression in HESCs From Patients With Endometriosis

To evaluate transfection efficacy, miR-21-5p expression was measured in HESCs 48 hours after transfection with an hsa-miR-negative control or has-miR-21-5p inhibitor. Treatment with the hsa-miR-21-5p inhibitor resulted in an approximately 400-fold decrease in miR-21-5p expression, compared with hsa-miR-negative control treatment (data not shown). miR-21 targets the genes associated with apoptotic factors such as caspase 3,[15] and saponin extract treatment induced downregulation of miR-21 expressions in HESCs. Therefore, we evaluated caspase 3 expression in HESCs from patients with endometriosis after hsa-miR-21-5p inhibitor transfection. After a 48-hour miR-21-5p inhibitor treatment, caspase 3 mRNA and protein expression were significantly increased in the HESCs (1.4-fold increase, $P = .005$, and 3.8-fold increase, $P < .001$, respectively; Figure 4C).

Saponin Extracts and miR-21-5p Increase Cell Apoptotic Cell Death in Endometrial Stromal Cells

To determine the effects of saponin extracts and miR-21-5p on cell apoptosis during endometriosis, HESCs from patients with endometriosis were treated with saponin extracts or transfected with the miR-21-5p inhibitor. Cell apoptosis was evaluated using flow cytometry analysis. The proportion of Annexin V-FITC(+) cells undergoing apoptosis during the early and late phases increased in these HESCs. After saponin extract treatment for 24 or 48 hours, 8.86% and 32.36%, respectively, of the cells were apoptotic (Figure 5A). Moreover, we found an increase in the proportion of cells undergoing apoptosis in HESCs when they were transfected with miR-21-5p inhibitor. Compared with the miR-negative control result of 1.94%, the proportion of Annexin V-FITC(+) cells undergoing apoptosis was much higher (6.72%) after miR-21-5p inhibitor transfection (Figure 5B). Caspase 3 is the ultimate enforcer of apoptotic activity, and its cleavage is a conventional hallmark of the activation of apoptotic cell death.[16] Taken together, our results indicated that apoptosis was modulated by the saponin extracts

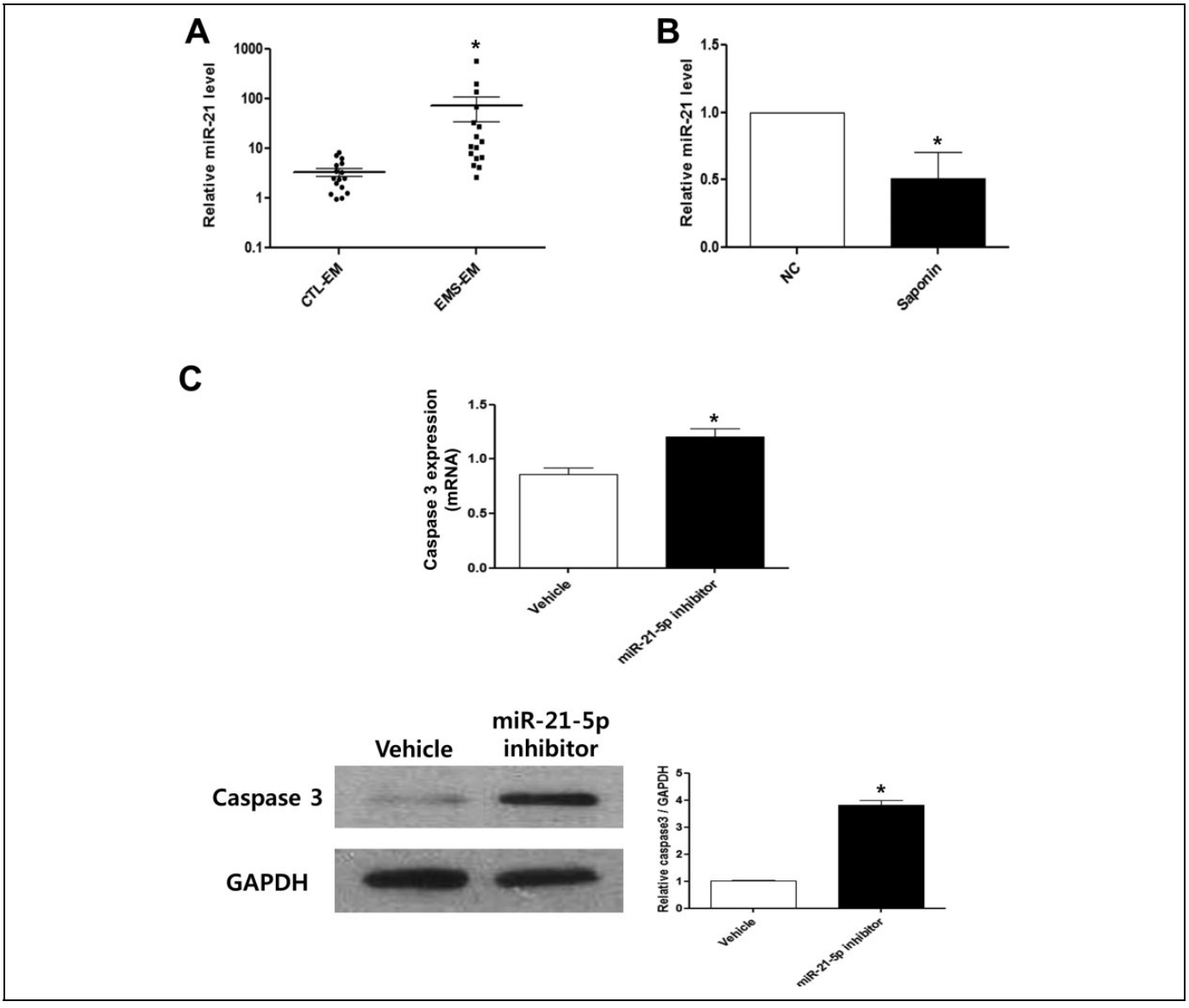

Figure 4. miR-21-5p and caspase 3 expression. A, miR-21-5p expression in endometrial cells from patients with and without endometriosis (triplicate; n = 16). B, miR-21-5p expression in HESCs from patients with endometriosis, after saponin extract treatment (n = 9). C, caspase 3 mRNA expression and Western blot analysis after miR-negative control (Vehicle) or miR-21-5p inhibitor transfection of HESCs from patients with endometriosis (n = 9 and n = 5, respectively). CTL-EM, eutopic endometrium from the patients without endometriosis; EMS-EM, eutopic endometrium from the patients with endometriosis; Vehicle, miR-negative control. Results are presented as mean ± standard error mean (SEM) values. *$P < .01$.

and miR-21-5p inhibitor in HESCs from the patients with endometriosis, via enhancement of caspase 3.

Discussion

We found that saponin extracts enhanced the apoptotic potential of HESCs from patients with endometriosis via caspase 3 activation. We used miRNA profiling and identified several miRNAs that were differentially expressed after saponin extract treatment. Among these miRNAs, we found that miR-21-5p expression in endometrial cells from patients with endometriosis was significantly greater than in those without the disease. Saponin extract treatment effectively reduced the levels of miR-21-5p expression. With transfection of miR-21-5p inhibitor, caspase 3 expression and apoptotic potential increased in HESCs. These results indicated that saponin extracts may enhance the apoptotic potential of HESCs from patients with endometriosis via modulation of miR-21-5p.

To our knowledge, this is the first study to evaluate the effects of saponin extracts when they are used for endometriosis. Our results indicated that saponin extracts increased the apoptosis of endometriotic cells via caspase-3 activation. These findings are consistent with previous study findings indicating

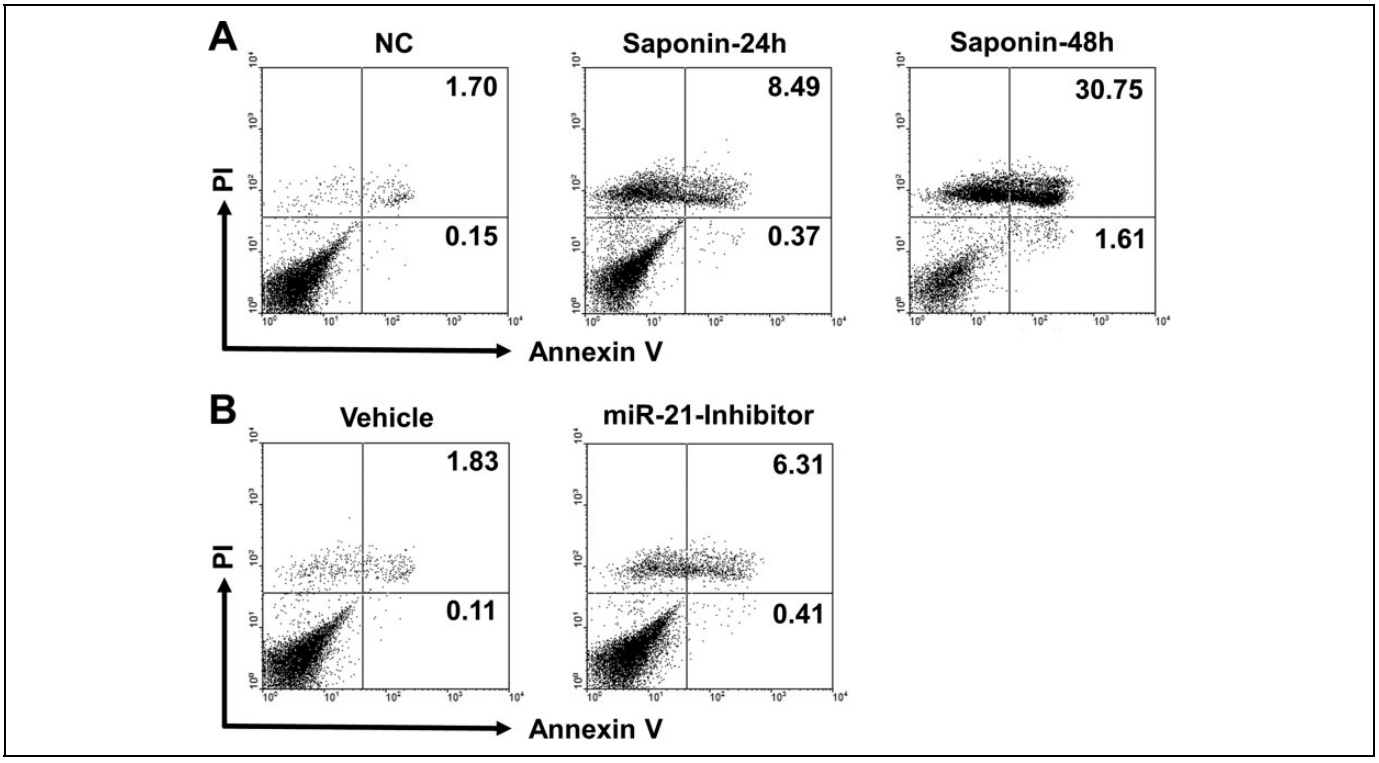

Figure 5. Apoptosis-inducing effects of saponin extract and miR-21-5p inhibitor in HESCs from patients with endometriosis. The percentage of apoptotic cells following Annexin V-FITC and PI binding. A, Human endometrial stromal cells were treated with saponin extracts for 24 or 48 hours (100 μM). B, Human endometrial stromal cells transfected with miR-negative control (Vehicle) or miR-21-5p inhibitor.

a possible link between saponin and apoptosis. Saponin induces apoptosis via inhibition of Bcl-2 and activation of apoptotic elements (eg, Bax and caspases).[17] Specifically, saponin activates caspase-3 and caspase-9 as apoptotic executioner and initiator, respectively, in human ovarian cancer cells.[18] Saponin induces changes in mitochondrial membrane potential in human lung cancer cell lines.[19] Because reduced apoptotic potential of endometriotic cells is one of the distinctive cellular characteristics involved in the pathogenesis of endometriosis,[20] enhancement of apoptotic potential using saponin extracts may suggest possibilities for new nonhormonal treatments for this enigmatic disease.

Although the results of this study suggested that there was an association between saponin extract and apoptosis of endometriotic cells, the one or more relevant specific apoptotic mechanisms remain to be fully understood for the endometrium and for endometriosis, and it has been shown that several miRNAs are involved in the apoptosis of endometrial cells. miR-183 may have a role in the induction of apoptosis in endometrial stromal cells.[21] miR-191 inhibits TNF-α-induced apoptosis in tissue samples from endometriosis and patients with endometrioid cancer, and miR-28c exerts its effects by suppressing cell proliferation and invasion and by stimulating apoptosis.[22,23] Consistent with the results of previous studies, our results also suggested that saponin extract–induced apoptosis involved alteration of specific miRNAs. Dysregulation of miRNAs has a key role in various therapeutic responses,[24] and growing evidence indicates possible involvement of miRNAs in the pathogenesis of endometriosis.[10,25] Therefore, we hypothesized that saponin extract treatment may alter miRNAs expression in endometriosis. Using microRNA profiling, we found several miRNAs that were expressed differently after saponin extract treatment of HESCs from patients with endometriosis. Our results indicated that miR-21-5p was one of the miRNAs that was involved in the apoptotic mechanism of the saponin extracts.

We found that miR-21-5p expression was more elevated in endometrial cells from patients with endometriosis than in those without the disease. Saponin extract treatment effectively reduced miR-21-5p expression in HESCs from the patients with endometriosis. miR-21-5p expression occurs at statistically significantly lower levels after endometriotic peritoneal fluid treatment of primary cell cultures from eutopic endometrial tissue obtained from patients.[26] miR-21 is one of the earliest identified cancer-promoting "oncomiRs," targeting numerous tumor suppressor genes associated with proliferation, apoptosis, and invasion.[27] miR-21 is also expressed in hematopoietic cells of the immune system (eg, B/T cells, monocytes, macrophages, and dendritic cells). A high miR-21 level is, therefore, considered to be a marker of immune cell activation.[28] During pathological necrosis, miR-21 enhances cellular necrosis by negatively regulating tumor suppressor genes associated with the death receptor–mediated intrinsic apoptotic pathway.[29] Experimental results have indicated that in numerous cell types, miR-21 functions as an anti-apoptotic and prosurvival factor.[15,30] Our study revealed

similar effects for miR-21 in endometrial cells from the patients with endometriosis. High expression levels of miR-21 may reflect characteristics of endometriosis, representing common features of pathological cell growth or proliferation; miR-21 inhibition effectively led to an increased apoptotic potential of endometrial cells. Although the specific underlying mechanisms are unclear, our results indicated that miR-21-5p inhibition induced apoptosis via caspase 3 activation in the endometrial cells from the patients with endometriosis. These results were consistent with the results of a previous study that indicated that miR-21 inhibitors significantly increase caspase 3/7 activation in melanoma cell lines.[31]

This study had several limitations. The results were based on an in vitro evaluation. To elucidate the effects of saponin extracts in endometriosis patients, in vivo study and evaluation of effects on ectopic endometrial tissues are needed. Although we did perform MTT assay to determine the effective dose of saponin extracts, further evaluation of the safety and efficacy of saponin extracts using human and case–control studies is needed before clinical application of this approach.

In conclusion, this study revealed that saponin extract treatment significantly decreased the expression of miR-21-5p and increased the expression of caspase3 in HESCs from patients with endometriosis. Inhibition of miR-21-5p effectively activated the apoptotic potential of HESCs. These results suggested that regulation of miRNA expression could be one of the molecular mechanisms involved in the therapeutic effects of saponin extracts used for endometriosis treatment. Regulation of miRNAs involved in the pathogenesis of endometriosis may provide new therapeutic targets for treatment of this condition.

Authors' Note

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