Acta Histochemica xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Silymarin amplifies apoptosis in ectopic endometrial tissue in rats with endometriosis; implication on growth factor GDNF, ERK1/2 and Bcl-6b expression

Elaheh Nahari[a], Mazdak Razi[b,*]

[a] Department of Biology, Tabriz Branch, Islamic Azad University, Tabriz, Iran
[b] Department of Basic Sciences, Faculty of Veterinary Medicine, P.O. BOC: 1177, Urmia University, Urmia, Iran

ARTICLE INFO

Keywords:
Endometriosis
Silymarin
Angiogenesis
Apoptosis
Fibrosis

ABSTRACT

The present prospective study was done to evaluate the effect of silymarin (SMN) on endometriotic-like legions establishment and growth in experimentally-induced endometriosis. For this purpose, the experimental endometriosis was induced in 12 rats and then the animals subdivided into endometriosis-sole and SMN (50 mg kg^{-1}, orally) + endometriosis groups. Following 28 days, the legions establishment, size, Glial cell line-derived neurotrophic factor (GDNF), gfrα1, B Cell Lymphoma 6 (Bcl-6b), Bcl-2, extracellular regulator kinase (ERK1/2) expression ratios, angiogenesis, the apoptosis and fibrosis indices were investigated. The SMN significantly ($P < 0.05$) decreased the enometriotic-like legions establishment and size, decreased mRNA levels of GDNF, gfrα1, Bcl-6b and Bcl-2 and remarkably diminished GDNF, gfrα1, Bcl-6b and Bcl-2-positive cells distribution/mm^2 of tissue versus endometriosis-sole group. The SMN + endometriosis group exhibited a significant ($P < 0.05$) enhancement in ERK1/2 expression and represented diminished vascularized area and increased apoptosis and fibrosis indices, as well. In conclusion, the SMN by down-regulating GDNF and its receptor gfrα1 expression inhibits GDNF-gfrα1 complex generation and consequently suppresses Bcl-6b expression. Moreover, the SMN by enhancing the ERK1/2 expression and by suppressing the Bcl-2 expression promotes the apoptosis pathway. Finally, the SMN by down-regulating the angiogenesis ratio accelerates apoptosis and consequently induces severe fibrosis in endometriotic-like legions.

1. Introduction

Endometriosis is known as a disorder with a prevalence of 6%–10% of reproductive age group women. Indeed, the presence of endometriotic legions, consisting of functional endometrial glands as well as stroma outside the uterine cavity is named as endometriosis (Simoens et al., 2007). The roles of pro-inflammatory cytokines/transcription factors, such as interleukin-4,8,6 (IL-4,8,6), tumor necrosis factor-α "TNF-α", (Gonzalez-Ramos et al., 2010; Grund et al., 2008) and several growth factors including; macrophage migration inhibitory factor (MIF), vascular endothelial growth factor (VEGF) and nitric oxide (NO)-induced interactions have been reported as fundamental aspects in tissue development of ectopic endometrial tissue in women with endometriosis (Zhang et al., 2010). Lastly, the role of mitochondria-dependent apoptosis and the implications of proto-oncogenes B-cell lymphoma-2 (Bcl-2) and Bax have been shown in ectopic endometrial tissues (Mourtzikou et al., 2012). In line with this issue, Nezhat and co-workers showed the Bcl-2 overexpression in 23% of benign endometriotic cysts, 67% of endometrioid carcinomas, 73% of clear cell carcinomas, and 50% of papillary serous carcinomas (Nezhat et al., 2002). Further to Bcl-2-induced role in cell proliferation, the role of other extracellular regulator kinases (ERK), such as ERK2 and ERK1 (also known as p42 /p44MAPK, respectively, and officially named MAPK 1 and 3) have been implicated in ectopic endometrial cells proliferation (Lin et al., 2012; Murk et al., 2008; Wu et al., 2006). Actually, it has been shown that the ERK1/2 (depending on the duration) controls cell proliferation, migration, differentiation, and death in ectopic endometrial tissue (Murphy and Blenis, 2006; Ramos, 2008). Recent findings have illustrated the B Cell Lymphoma 6 (Bcl-6b) as a promising biomarker for acute diagnosis of endometriosis and associates with endometriotic cells proliferation (Evans-Hoeker et al., 2016; Yoo et al., 2017a,b). The Bcl-6b, also known as BAZF, ZNF62, and

Abbreviations: GDNF, Glial cell line-derived neurotrophic factor; Bcl-6b, B cell lymphoma 6; ERK1/2, extracellular regulator kinase; SMN, silymarin

* Corresponding author.
E-mail addresses: mazdak.razi@gmail.com, m.razi@urmia.ac.ir (M. Razi).

https://doi.org/10.1016/j.acthis.2018.08.003
Received 12 March 2018; Received in revised form 29 July 2018; Accepted 16 August 2018

Table 1
Antibodies, concentrations and details.

Name	Description	Species Reactivity	Concentration	Cat No:	Company
Anti-GDNF	Rabbit polyclonal to GDNF	Human, Rat	1:600	ab8026	Abcam, UK
Anti-gfrα1	Mouse polyclonal to gfrα1	Human, Rat, Mouse	1:500	ab10646	Gennova, Spain
Anti-Bcl-6b	Rabbit Polyclonal Antibody to Bcl-6b	Human, Rat, Mouse	1:600	sc-368	Santa Cruz, UK
Anti-Bcl-2	Rabbit Polyclonal Antibody to Bcl-2	Human, Rat, Mouse	1:200	ab59348	Abcam, UK
Anti-CD31	Rabbit Polyclonal Antibody to Bcl-2	Human, Rat	1:500	SKU: 347	Biocare, USA

ZBTB, belongs to the BCL6 gene family. Up to now, the roles of Bcl-6b in various physiologic interactions, including spermatogonial stem cells proliferation, (Oatley et al., 2006; Sakashita et al., 2002; Xu et al., 2012), expressions of p53 and p300 genes in carcinogenic condition (Phan and Dalla-Favera, 2004) and repression of cytokine-induced transcription in various cell systems (Arguni et al., 2006; Yoo et al., 2017a,b) have been reported.

The Glial cell line-derived neurotrophic factor (GDNF) family ligand (GFL) belongs to transforming growth factor b superfamily (Airaksinen and Saarma, 2002; Kang et al., 2009). Indeed, the GDNF interacts with its special receptor anchored co-receptor a (gfrα-1) and by this mechanism, it triggers self-renewal in proliferating cells (Glerup et al., 2013; Takahashi, 2001). More other studies have shown that, the GDNF amplifies the Sertoli (Yang and Han, 2010), mouse urogenital sinus (Park and Bolton, 2015) and murine retinal progenitor cells proliferation and differentiation (Wang et al., 2010). However, the role of GDNF and its restricted receptor gfrα1 has not been clarified clearly in ectopic endometriotic cells proliferation.

Silymarin (SMN) is a C25 containing flavonoid mixture, which is extracted from the Silybum marianum (milk thistle) plant (Comelli et al., 2007). The SMN contains high levels of flavonolignans (65% to 80%), including silybin A and silybin B, isosilybin A, isosilybin B, silychristin and silydianin. Moreover, the SMN contains flavonoids, 20% to 35% of fatty acids and polyphenolic compounds (Biedermann et al., 2014; Comelli et al., 2007). Several reports have illustrated the SMN-induced antioxidant (Asghar and Masood, 2008), anticancer (Chen et al., 2009; Ramasamy and Agarwal, 2008), immunosuppressant (Gharagozloo et al., 2013a), anti-inflammatory effects (Ashkavand et al., 2012; Hussain et al., 2009).

Indeed, the milk thistle interferes with the expression of the cell cycle regulator proteins, resulting in cell cycle arrest and/or even apoptosis. It has been reported that the SMN, in a dose- and time-dependent manner, is able to effectively suppress the A2780s and PA-1 cell growth/proliferation at G1/S phase. The same study has shown that the 50 and 100 μg/ml of SMN via up-regulating Bax, down-regulating Bcl-2 proteins expression and activation of caspase-9 and caspase-3 potentially promotes the intrinsic apoptosis pathway (Fan et al., 2014). In line with this issue, Lee et al., reported that the SMN attenuates the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and RSK2 in melanoma cells. In other words, the SMN suppresses the activation of these transcription factors and induces cell-cycle arrest at the G1 phase. Thus, according to these findings, the SMN can modulate the balance between cell survival and apoptosis (Lee et al., 2013). Gharagozloo et al., reported that, the SMN inhibits cell proliferation through the suppression of the PI3K/Akt/mTOR signaling pathway at the G1 phase in human activated T lymphocytes in vitro (Gharagozloo et al., 2013b).

The Present study was done in order to uncover the inhibitory effect of SMN (as an antioxidant, anti-inflammatory and cell cycle regulator) on cell proliferation and angiogenesis ratios in experimentally-induced endometriotic-like legions. For this purpose, the molecular and histological changes relating to GDNF, its specific receptor gfrα1 and Bcl-6b (as a target gene for GDNF-gfrα1 interaction) were analyzed. Moreover, in the second step, the inhibitory effects of SMN on Bcl-2 (as an anti-apoptotic proto-oncogene) and ERK1/2 (as an extracellular regulator kinase) expression and/or synthesis were investigated. Taking all the results together, the current study tried to find out the cross-link between these molecular alterations with angiogenesis and cellular apoptosis in endometriotic-like legions of rats with experimental endometriosis.

2. Methods and materials

2.1. Chemicals

The SMN (containing 80% silibin, Cat No: S0292 SIGMA) was obtained from, Sigma Chemical Co. (St. Louis, MO, USA). The primary antibodies of GDNF, gfrα1, Bcl-6b, Bcl-2 were purchased from Histoline laboratories (Istanbul, Turkey). The antibodies concentrations and details are presented in Table 1. Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories, (Vector Laboratories, USA). All other chemicals were commercial products of analytical grade.

2.2. Animals and experiment grouping

To follow-up current study, 12 mature female Wistar rats (200 ± 20 g) were obtained from the animal resource of the Faculty of Veterinary Medicine, Urmia University. The animals were acclimatized for a week. The diet and water were given ad libitum and all stress factors were reduced into minimum and standard condition (constant temperature and 12-h light). All the experimental protocols were approved by the ethical committee of Urmia University based on principles of laboratory animal care. The schematic flowchart for the methodology is presented in Fig. 1.

To induce experimental endometriosis, the uterine horns were transplanted to the bowel mesentery, as previously described (Bilotas et al., 2010). In brief, the animals were anesthetized with an

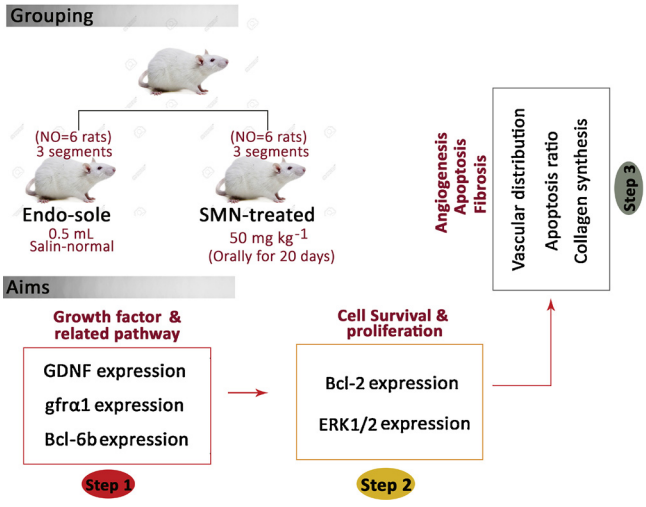

Fig. 1. Schematic flowchart for used methodology for illustrating the effect of SMN on a- growth factor GDNF and its related genes expression as first aim of trial, b- endometrial cells survival and proliferation in endometriotic-like legion and c- angiogenesis, apoptosis and fibrosis ratios.

intraperitoneal injection of 5% ketamine (100 mg kg^{-1},Alfasan, Woerden, The Netherland), 40 mg/kg, and 2% xylazine (40 mg kg^{-1}, Trittau, Germany). Following laparotomy, the midventral incision was performed to expose the uterus and intestine. The right uterine horns of the animals were removed by standard hysterectomy and cut into approximately 4 mm^2 square pieces. Then, 3 equal tissue segments were sutured onto the serosal layer using a single 6-0 nylon suture (Supralon; Ethicon). The transplanted endometrial tissues were faced to the serosa. Finally, the incision of the abdomen was closed using 3-0 silk suture (Supa medical devices, Iran). Next, the animals were assigned into two endometriosis-sole and SMN-treated endometriosis-induced (SMN + endometriosis) groups. The six rats with three implanted segments were considered for each group (NO = 18 samples for each group). Considering previous publications regarding the SMN-induced therapeutic properties (Amin and Arbid, 2015; Muriel et al., 2005; Pradeep et al., 2007) 50 mg kg^{-1} of SMN (orally) was administrated daily, starting postoperative day 1, and continued for 28 days. The 28 days treatment with SMN was selected based on standardized necessary time for evaluating the ameliorative effect of different chemicals against endometriotic-like legions establishment and development using animal models (Jin et al., 2015; Olivares et al., 2011; Quereda et al., 1996). The animals in endometriosis-sole group received the same volume (0.5 mL) of salin-normal (used as the solvent for SMN).

2.3. Evaluation of endometriotic-like legion

Following test termination (28 days), the animals were euthanized by CO_2 gas using a special device (Adaco, Urmia). The abdominal region was opened by midline incision. The 3 implantation sites were identified by the presence of endometriotic-like lesions. The lesions were counted and their volume was determined using the following formula:

V = (4/3) π2R (where V is volume, and r and R are the radiuses, r < R), which was standardized previously (Brodie et al., 2003; Olivares et al., 2011). Following tissue dissection, the lesions were fixed in Bouin's fixative solution, and following 72 h embedded in paraffin. Thereafter, the specimens were cut into 5 µm sections using the rotary microtome (Leitz Wetzlar, Germany). Ten serial sections from each sample were stained with hematoxylin and eosin and examined microscopically for the presence of the histologic hallmarks (glands and stroma) of endometriosis.

2.4. Assessment of fibrosis by collagen specific staining

To assess the fibrosis in endometriotic-like legions tissue, the Masson's trichrome staining technique was used. In brief; the Bouin's solution was added on sections and then the sections were microwaved for 1 min and allowed to incubate with Bouin's solution for 15 min. Next, the slides were washed with tap water (5 min) to remove picric acid. The slides, thereafter were stained with Weigert's working hematoxylin solution for 10 min. Then the slides were washed in running tap water and consequently incubated with Biebrich scarlet solution for 5 min. Next, the slides were washed with distilled water and incubated with Phosphotungstic/phosphomolybdic acid for 10 min and directly were transferred into Aniline blue for 5 min. Next, the slides were rinsed in distilled water and incubated with 1% acetic acid for 1 min. Finally, the slides were dehydrated and cleared and cloversliped. The collagen bundles were represented in blue.

2.5. Immunohistochemical (IHC) staining for GDNF, gfrα1, Bcl-6b, Bcl-2, CD31 and ERK1/2

For IHC staining, the tissue sections were de-paraffinized in xylene (2 changes) and rehydrated using descending degrees of ethanol (90%, 80%, 70%, 50%). Following rehydration, the antigen retrieval process was performed in 10 mM sodium citrate buffer (pH: 7.2) and the endogenous peroxidase was blocked in a peroxidase blocking solution (0.03% hydrogen peroxide containing sodium acid) for 5 min. Thereafter, the sections were incubated overnight with the primary antibodies (at 4 °C). The sections were incubated with a sufficient amount of streptavidin–HRP (streptavidin conjugated to horseradish PBS containing an anti-microbial agent) for 20 min. The positive-reacted sites for target proteins were visualized by incubating the sections with diaminobenzidine (DAB). The hematoxylin staining dye was used to counterstain the slides before permanent mounting.

Table 2
Neuclotid sequences, for primers used in RT-PCR.

Target Gene	Primer sequence 5'-3'	Ref
GDNF	FWD: ATGAAGTTATGGGATGTCGTGGCT REV: GGTCAGATACATCCACACCG	Takashima et al., 2015
gfrα1	FWD: GCACAGCTACGGGATGCTCTTCGG REV: GTAGTTGGGAGTCATGACTGTGCCAATC	Fouchecourt et al., 2006
Bcl-6b	FWD: CCCGGGCTCAAGAGACTTC REV: TTCCTGGGCGGTGGATTAGC	Takashima et al., 2015
Bcl-2	FWD: CTCGTCGCTACCGTCGTGACTTCG REV: CAGATGCCGGTTCAGGTACTCAGTC	Ortega et al., 2003
ERK1/2	FWD: GCTGACCCTGAGCACGACCA REV: CTGGTTCATCTGTCGGATCA	Zhang et al., 2006
GAPDH	FWD: AGCTGAGAGGGAAATCGTGCG REV:CCAGCAAGCTTGCAACCTTAACCA	Zhang et al., 2006

2.6. Assessment of apoptosis using TUNEL staining

The apoptosis ratio was evaluated by using TUNEL (terminal deoxynucleotidyl transferase enzyme mediated dUTP nick end labeling) assay kit (Roche, Germany). In brief, the sections (5 µm) were deparaffinized with xylene (3 changes, each change 5 min) and rehydrated in graded alcohol (each 2 min). Next, the sections were incubated with 1 µl proteinase K (for 20 min) and washed 3 times with PBS. Then, the sections were incubated with 5 µl TUNEL solution (for 40 min), and washed 3 times with PBS and incubated with 10 µl POD-convertor (for 30 min). The slides were washed 3 times in PBS, incubated with 10 µl DAB substrate (for 60 min) and washed with distilled water. Finally, the sections were counter stained with Hematoxylin and then dehydrated using ascending alcohol. The clear and dark brown cells were considered as apoptotic cells.

2.7. Evaluating angiogenesis ratio

To investigate theangiogenesis ratio, the vessels distribution per mm^2 of tissue was assessed using both Hematoxiline-eosin and Immunohistochemically CD31 staining techniques.

3. RNA isolation, cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR)

Previously collected and stored (−70 °C) endometriotic-like tissues were used for RNA extraction. The RNA extraction was performed based on the standard TRIZOL method (Adibnia et al., 2016). For this purpose, 20–30 mg of implanted endometrial tissues from each group was homogenized in 1 ml of TRIZOL. The colorless aqueous phase collected with extra care in order to avoid genomic DNA contamination. The RNA amount was determined using nanodrop spectrophotometer (260 nm and A260/280 = 1.8–2.0), and the samples were then stored at −70 °C. For RT-PCR, the cDNA was synthesized in a 20 µl reaction mixture containing 1 µg RNA, oligo (dT) primer (1 µl), 5 × reaction buffer (4 µl), RNAse inhibitor (1 µl), 10 mM dNTP mix (2 µl), M-MuLV Reverse Transcriptase (1 µl) according to the manufacturer's protocol (Fermentas, GmbH, Germany). The cycling protocol for 20 µl reaction mixtures was 5 min at 65 °C, followed by 60 min at 47 °C, and 5 min at

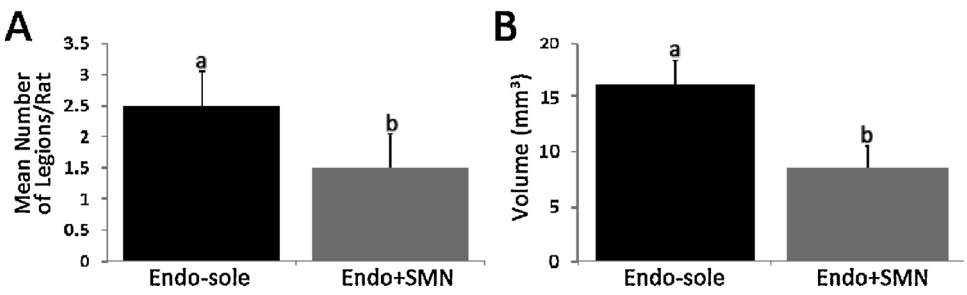

Fig. 2. (A) SMN statistically significantly (NO = 6 rats in each group, a Vs b: $P < 0.01$) decreased the endometriotic-like legions development after 28 days administration, (B) SMN statistically significantly (NO = 6 rats in each group, a Vs b: $P < 0.001$) diminished endometriotic-like legions size versus endometriosis-sole group. All data are presented in Mean ± SD, **Endo-sole:** endometriosis-sole and **Endo + SMN:** SMN + endometriosis.

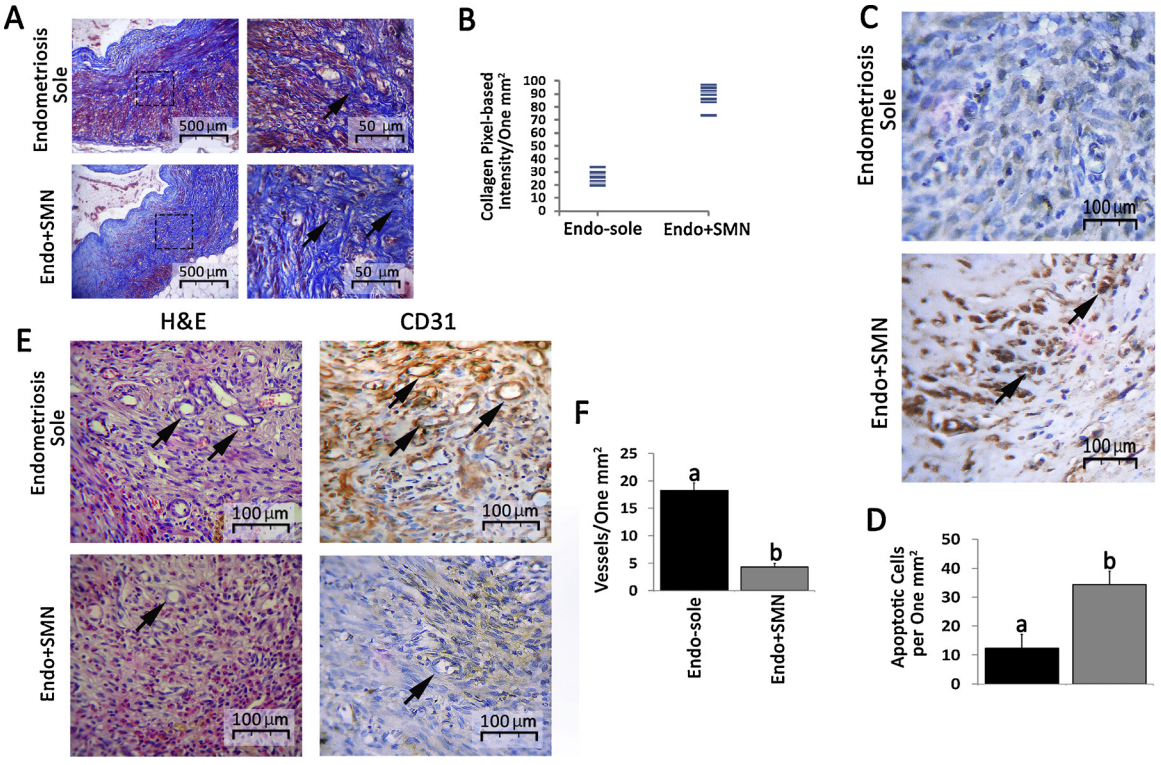

Fig. 3. (A) Photomicrograph of Masson's trichrome staining, representing collagen bundles and bonds in endometriotic-like legions from endometriosis-sole (Endo-sole) and SMN + endometriosis groups (Endo + SMN), (B) Software analysis for pixel-based collagen intensity in 50,000 × 50,000 µm of tissue, (C) Photomicrograph od TUNEL staining, (D) Mean ± SD of apoptotic cells/mm^2 of tissue; SMN statistically significantly (NO = 10, a Vs b: $P < 0.001$) increased apoptotic cells number/mm^2 of tissue, (E) Photomicrograph of Hematoxylin-Eosin (H&E) and IHC staining for CD31, representing vessels (arrows) in both groups and (F) Note decreased number of vessels/mm^2 of tissue in SMN + endometriosis group (NO = 10, a Vs b: $P < 0.001$). All data are presented in Mean ± SD. **Endo-sole:** endometriosis-sole **and Endo + SMN:** SMN + endometriosis.

70 °C to terminate the reaction. The PCR reaction was carried out in a total volume of 27 µl containing PCR master mix (13 µl), FWD and REV specific primers (each 1 µl), and cDNA as a template (1.5 µl) and nuclease free water (10.5 µl). PCR conditions were run as follows: general denaturation at 95 °C for 4 min, 1 cycle, followed by 30–35 cycles of 95 °C for 20 s; annealing temperature (58 °C for GDNF, 62 °C for gfrα1, 52 °C for ERK1/2, 60 °C for Bcl-6b, 60 °C for Bcl-2 and finally 52 °C for GAPDH) for 60 s; elongation: 72 °C for 1 min and 72 °C for 5 min. Specific primers were designed and manufactured by Gen Fanavaran (Cinna-Gen Co. Tehran, Iran). Primers pair's sequences, for each individual gene are presented in Table 2.

3.1. Statistical analyses

For the measured parameters, mean and standard deviations were calculated. Results were analyzed using SPSS (version 16.00, California, USA). The comparisons between groups were made by analysis of variance (One-way ANOVA) followed by Bonferroni post-hoc test. A value $p < 0.05$ was considered significant. The photomicrographs were taken by using SONY onboard camera (Zeiss, Cyber-Shot, Japan). Image pro-insight software (version 9.00) was used for evaluating the pixel-based distribution of GDNF, gfrα1, Bcl-6b, Bcl-2, CD31 and ERK1/2 positive cells as well as pixel-based intensity of collagen per 50000 × 50000 µm.

3.2. Image cytometry

The photomicrographs were taken by using SONY onboard camera (Zeiss, Cyber-Shot, Japan). In order to analyze the histological parameters, 10 sections from each sample (NO = 18 samples for each group) were prepared and 10 microscopic fields from each section, which represented positive reaction for target proteins, were considered to analyze the IHC alterations and apoptotic cells distribution. The GDNF+, gfrα1+, Bcl-6b+, Bcl-2+, CD31+ and ERK1/2+ and apoptotic cells as well as vessel numbers *per* mm^2 of tissue were evaluated by using a standard light microscope (Olympus, CH27, Japan) and compared between groups. The image pro-insight software (version 9.00) was used for evaluating the pixel-based immunoreactivity of

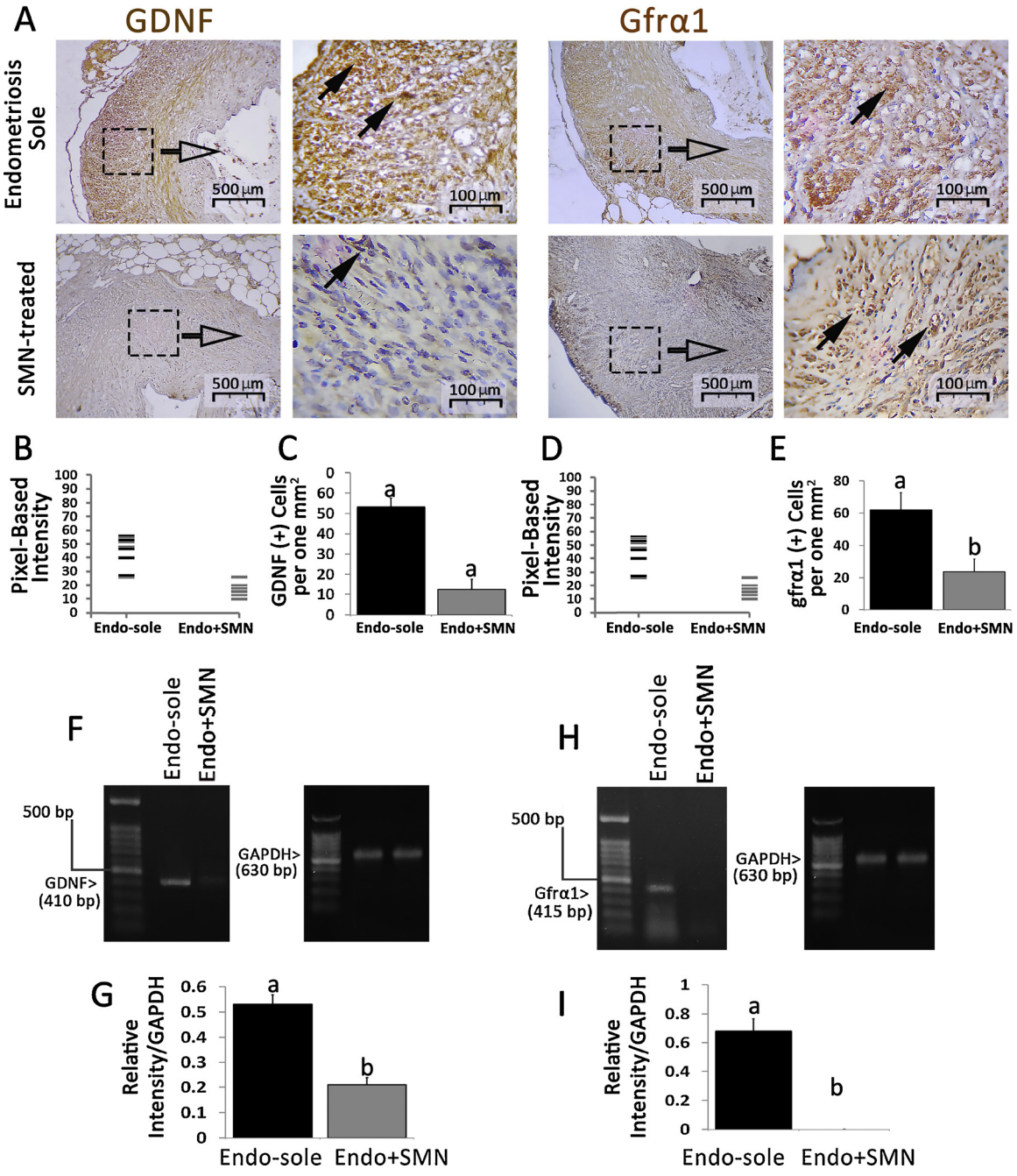

Fig. 4. (A) Photomicrograph representing immunohistochemical staining techniques of GDNF and gfrα1, note decreased expression of GDNF and gfrα1 (arrows) in SMN + endometriosis group (B) Software analyses for pixel-based intensity of GDNF in 50,000 × 50,000 µm of tissue, (C) Mean ± SD of GNDF-positive cells/mm^2 of tissue, (NO = 10, a Vs b: P < 0.01), (D) Software analyses for pixel-based intensity of gfrα1 in 50,000 × 50,000 µm of tissue, (E) Mean ± SD of gfrα1-positive cells/mm^2 of tissue, (NO = 10, a Vs b: P < 0.03), (F) Electrophoresis photomicrograph of GDNF, (G) The mean ± SD density of GDNF mRNA levels that were measured by densitometry and normalized to GAPDH mRNA expression level, (NO = 6, a Vs b: P < 0.001), (H) Electrophoresis photomicrograph of gfrα1, (I) The mean ± SD density of gfrα1 mRNA levels that were measured by densitometry and normalized to GAPDH mRNA expression level. **Endo-sole:** endometriosis-sole **and Endo + SMN:** SMN + endometriosis.

GDNF, gfrα1, Bcl-6b, Bcl-2, CD31, and ERK1/2 as well as collagen bundles *per* 50000 × 50000 µm. All histological analyses were performed by using 100 and 400 × objective magnification lens.

4. Results

4.1. SMN decreased endometriotic-like legions establishment and size

Observations revealed that the SMN significantly (P < 0.01) decreased the legions establishment *per* animal versus endometriosis-sole group (Fig. 2A). Moreover, the SMN remarkably (P < 0.001) diminished legion development compared to endometriosis-sole group (Fig. 2B).

4.2. SMN enhanced fibrosis in endometriotic-like legions and enhanced apoptosis ratio

Histological observations showed intensive collagen bundles generation in endometriotic-like legions of animals from SMN + endometriosis group (Fig. 3A, B). Accordingly, the software

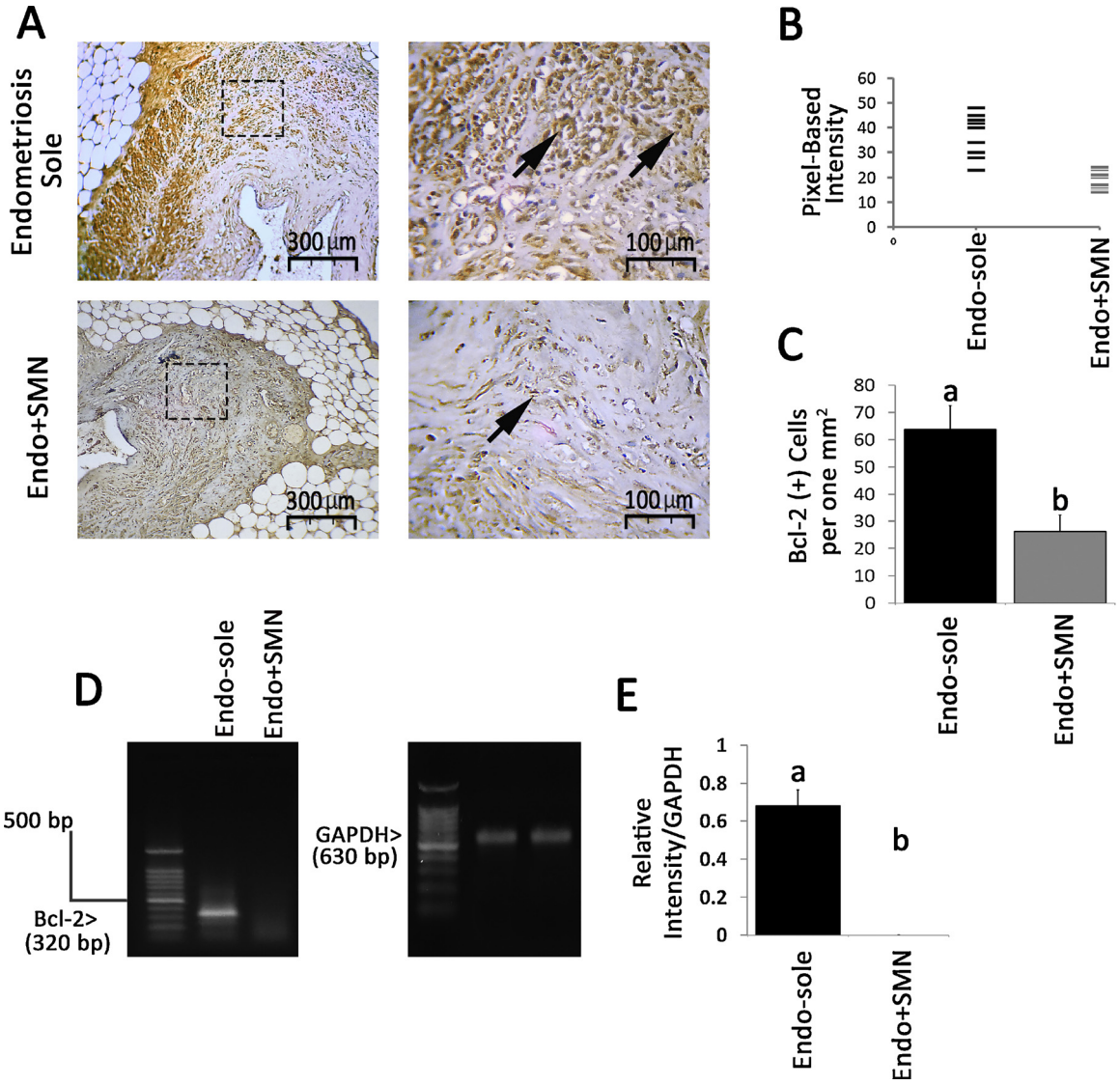

Fig. 5. (A) photomicrograph of immunohistochemical staining of Bcl-2, Note decreased expression of Bcl-2 (arrows) in SMN + endometriosis group versus endometriosis-sole group. (B) Software analyses for pixel-based intensity of Bcl-2 in 50,000 × 50,000 µm of tissue, (C) Mean ± SD of Bcl-2-positive cells/mm^2 of tissue, (NO = 10, a Vs b: $P < 0.01$), (D) Electrophoresis photomicrograph of Bcl-2, (E) The mean ± SD density of Bcl-2 mRNA levels that were measured by densitometry and normalized to GAPDH mRNA expression level (NO = 6). **Endo-sole:** endometriosis-sole **and Endo + SMN:** SMN + endometriosis.

analyses showed the higher pixel-based intensity of the blue color (collagen) in SMN + endometriosis group versus endometriosis–sole group. In order to show the apoptosis ratio, the apoptotic cells number *per* mm^2 of tissue was evaluated. The animals in SMN + endometriosis group showed statistically significant ($P < 0.001$) enhancement in numbers of apoptotic cells *per* mm^2 of tissue (Fig. 3C, 3D)

4.3. SMN decreased the angiogenesis ratio

Light microscopic analyses of H&E and IHC staining techniques showed that, the SMN significantly ($P < 0.001$) decreased the vascular distribution/mm^2 of tissue compared to endometriosis-sole group (Fig. 3E, 3 F).

4.4. SMN decreased GDNF and gfrα1 expression

In order to estimate the effect of SMN on GDNF and gfrα1 expression, IHC staining and semi-quantitative RT-PCR analyses were performed. The IHC staining exhibited that the SMN significantly decreased the GDNF and gfrα1 expression versus endometriosis-sole group. Accordingly, the animals in SMN + endometriosis group showed a decreased number of GDNF$^+$ ($P < 0.01$) and gfrα1$^+$ cells ($P < 0.03$) distribution/mm^2 of tissue compared to the endometriosis-sole group. Moreover, the software analyses showed decreased pixel-based intensity for GDNF and gfrα1 reacted sited in SMN + endometriosis group (Fig. 4A–E). The results obtained from RT-PCR corroborated the IHC results. Accordingly, the RT-PCR analyses exhibited decreased mRNA level of GDNF ($P < 0.001$) and represented no detectable mRNA of gfrα1 in SMN + endometriosis group (Fig. 4F–I).

4.5. SMN decreased Bcl-2 and Bcl-6b expression

The IHC and semi-quantitative RT-PCR ($P < 0.001$) analyses showed that the SMN significantly diminished the Bcl-2 expression in endometriotic-like legion compared to the endometriosis-sole group. Accordingly, the animals in SMN + endometriosis group exhibited a remarkable ($P < 0.01$) reduction in a number of Bcl-2$^+$ cells/mm^2 of tissue versus endometriosis-sole group. Moreover, the software analyses exhibited decreased pixel-based intensity for Bcl-2 intensity in

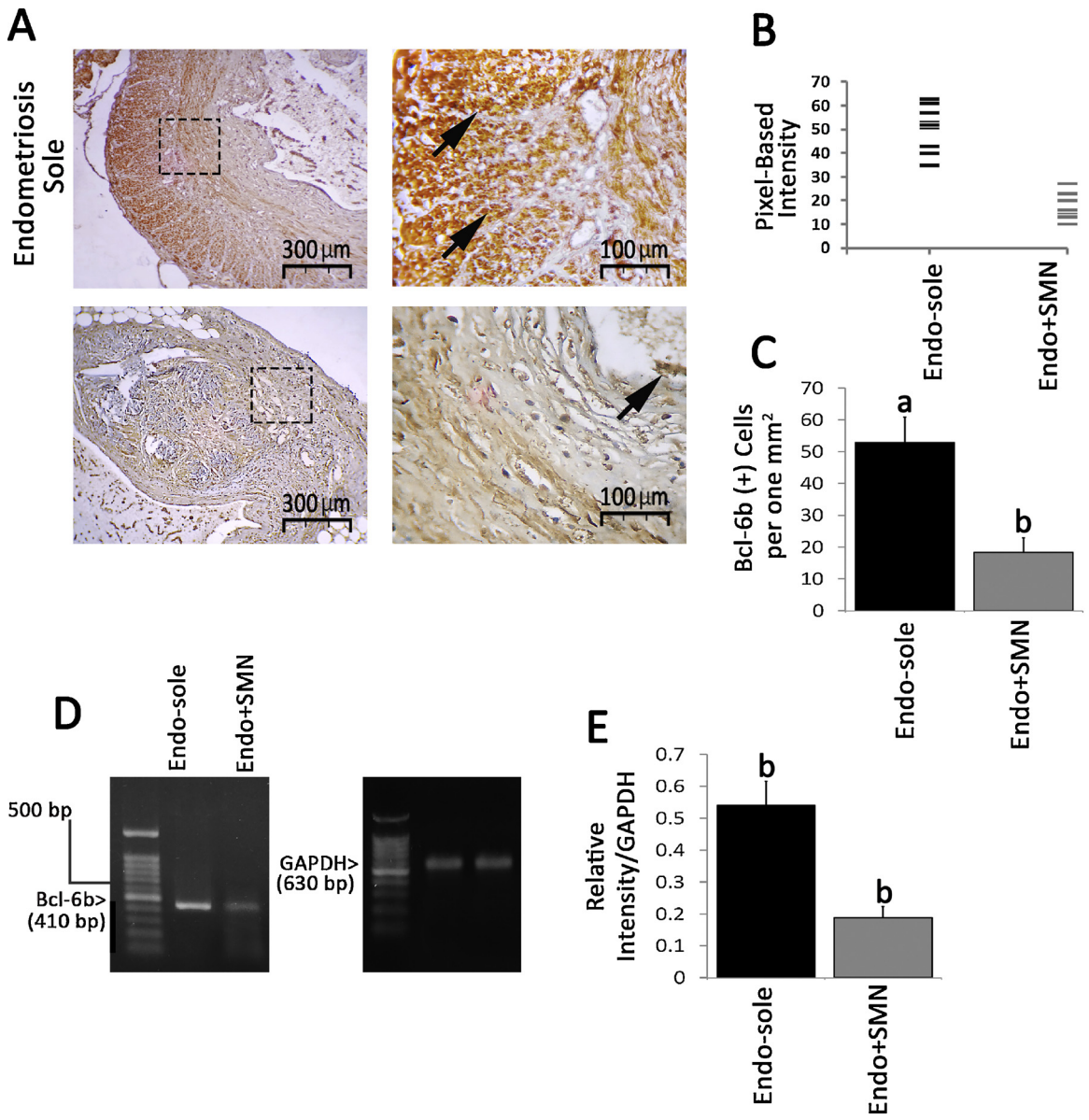

Fig. 6. (A) photomicrograph of immunohistochemical staining of Bcl-6b, Note decreased expression of Bcl-6b (arrows) in SMN + endometriosis group versus endometriosis-sole group. (B) Software analyses for pixel-based intensity of Bcl-6b in 50,000 × 50,000 µm of tissue, (C) Mean ± SD of Bcl-6b -positive cells/mm^2 of tissue, (NO = 10, a Vs b: P < 0.02), (D) Electrophoresis photomicrograph of Bcl-6b, (E) The mean ± SD density of Bcl-6b mRNA levels that were measured by densitometry and normalized to GAPDH mRNA expression level (NO = 6, a Vs b: P < 0.001). **Endo-sole:** endometriosis-sole **and Endo + SMN:** SMN + endometriosis.

SMN + endometriosis group (Fig. 5A-E). More analyses showed that the mRNA level (P < 0.001) of Bcl-6b as well as the number of Bcl-6b$^+$ cells (P < 0.02) were decreased in SMN + endometriosis group in comparison to endometriosis-sole animals. Similar to Bcl-2, the software analyses exhibited a decreased pixel-based intensity of Bcl-6b in SMN + endometriosis animals versus endometriosis-sole group (Fig. 6A-E).

4.6. SMN enhanced ERK1/2 expression in endometriotic-like legion

The ERK1/2$^+$ cells number *per* mm^2 of tissue was compared between groups. Observations showed that the SMN significantly (P < 0.01) increased the ERK1/2$^+$ cells number *per* mm^2 of tissue versus endometriosis-sole group. Moreover, the software analyses exhibited increased pixel-based intensity for ERK1/2 intensity in SMN + endometriosis group compared to the endometriosis-sole group. The semi-quantitative RT-PCR analyses showed that SMN statistically significantly (P < 0.03) increased the ERK1/2 mRNA levels versus endometriosis-sole group. (Fig. 7A-E).

5. Discussion

The current study showed that administrating SMN significantly decreased the endometriotic-like legions establishment and size. More molecular and histopathological analyses revealed that the SMN significantly diminished the growth factor GDNF expression/synthesis, diminished the GDNF-restricted receptor gfrα1 and target gene Bcl-6b expression/synthesis. Moreover the SMN remarkably decreased the proto-oncogene Bcl-2 expression/synthesis. Finally, the SMN significantly up-regulated the ERK1/2 expression in endometriotic-like legions, diminishedthe angiogenesis ratio and consequently resulted in severe apoptosis and fibrosis (Fig. 8).

Despite the role of various growth factors, including VEGF (Zhang et al., 2010), epidermal growth factor family of growth factors and

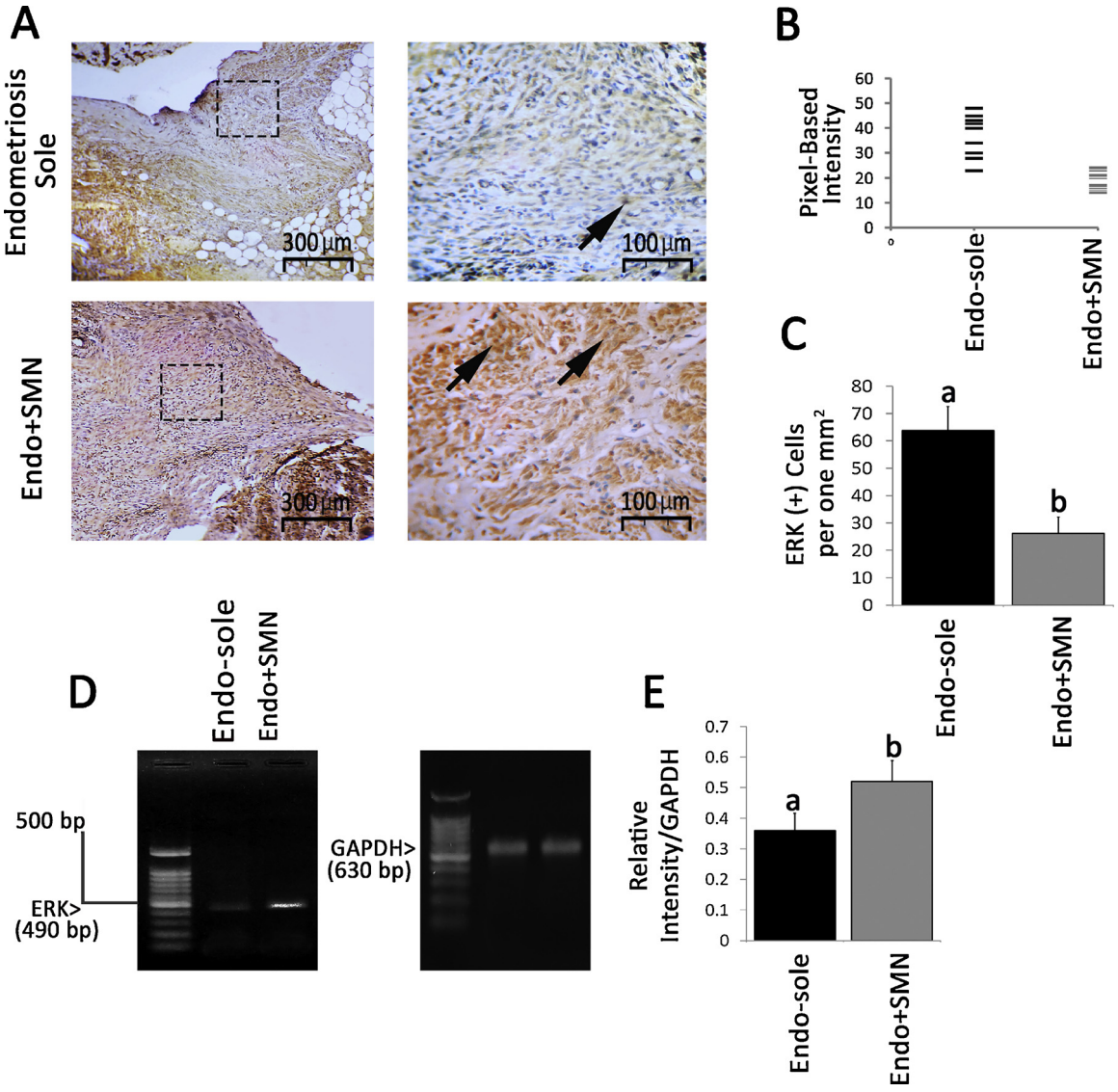

Fig. 7. (A) photomicrograph of immunohistochemical staining of ERK1/2, Note increased expression of ERK1/2 (arrows) in SMN + endometriosis group versus endometriosis-sole group. (B) Software analyses for pixel-based intensity of ERK1/2 in 50,000 × 50,000 µm of tissue, (C) Mean ± SD of ERK1/2-positive cells/mm^2 of tissue, (NO = 10, a Vs b: P < 0.01), (D) Electrophoresis photomicrograph of ERK1/2, (E) The mean ± SD density of ERK1/2 mRNA levels that were measured by densitometry and normalized to GAPDH mRNA expression level (NO = 6, a Vs b: P < 0.03). **Endo-sole:** endometriosis-sole **and Endo + SMN:** SMN + endometriosis.

receptors (Large et al., 2014), fibroblast growth factor "FGFs",(Mihalich et al., 2003), insulin like growth factor "IGFs", (Mu et al., 2015) and insulin-like growth factor-binding proteins, transforming growth factor-ß "TGF-β", (Omwandho et al., 2010), activin A and related proteins (Zheng et al., 2016), the role of GDNF, a novel growth factor, and its specific receptor gfrα1, as well as the inhibitory effect of SMN (as anti-proliferative agent), are not analyzed in endometriotic tissue.The GDNF and its ligand-binding subunit GFRα1 receptor complex initiate intracellular signaling cascades [for example MAPK kinase (MEK1/2) and MAPK (ERK1/2) pathway (MEK-ERK)], leading to cell survival and proliferation (Fisher et al., 2001; Hasegawa et al., 2013). Our results showed that the SMN significantly suppressed the GDNF and gfrα1 expression versus endometriosis-sole group. On the other hand, it should be noted that the SMN inhibits the kinase activity of MEK1/2 and through this mechanism; the SMN is able to suppress the cell proliferation machinery (Lee et al., 2013). Although the MEK 1/2 expression is not assessed in the current study, it could be concluded that the SMN may inhibited the cell proliferation by suppressing both the promoter GDNF and activator MEK1/2 expressions.

The GDNF initiates other intracytoplasmic signaling cascades, which finally ends with expression/synthesis of Bcl-6b, Ets variant 5 (Erm), and LIM homeobox 1 (Lhx1). All these target genes in turn directly involve in cells self-renewal and proliferation (Oatley et al., 2007, 2006). In line with this issue, lastly, the effective role of Bcl-6b has been discovered in the ectopic endometrial tissue of women with endometriosis (Evans-Hoeker et al., 2016; Yoo et al., 2017a, b). Our observations revealed that the Bcl-6b mRNA level and Bcl-6b$^+$ cells distribution *per* mm^2 of tissue were decreased in endometriosis + SMN group. Thus, we can come close to this fact that the SMN inhibited the cell proliferation not only by suppressing initiators GDNF and gfrα1 expression but also by down-regulating the finisher gene Bcl-6b expression. In addition to our findings, previous researches exhibited other mechanisms, by which the SMN negatively affects the cell proliferation of different cell types at the G1 phase of the cell cycle. For instance, the SMN through PP2 A pathway (Switzer et al., 2009) and via suppressing PI3K/Akt/mTOR signaling pathway (Gharagozloo et al., 2013b) is able to potentially induce cell cycle arrest at the G1 stage. (Kondoh and Nishida, 2007; Owens and Keyse, 2007). The proto-

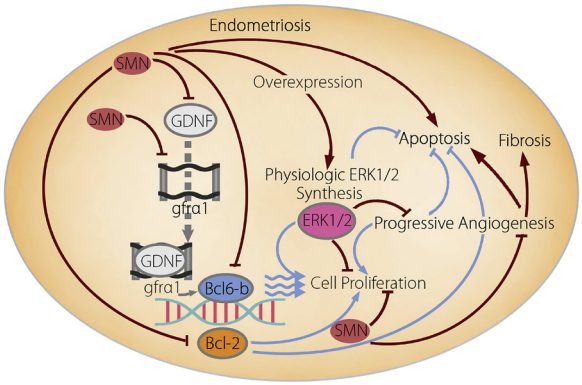

Fig. 8. Schematic summary representing effect of SMN on endometriotic-like legion establishment, development and cellularity; in endometriosis-sole condition, the GDNF interacts with gfrα1 and then the GDNF-gfrα1 complex activate the Bcl-6b expression/synthesis, which consequently ends with cell proliferation. On the other hand, high Bcl-2 expression and physiologic expression/synthesis of ERK1/2 accomplished by high GDNF expression/synthesis accelerated/promotes angiogenesis and cellularity in endometriotic-like legions. However, SMN by a- suppressing GDNF, gfrα1, Bcl-6b, Bcl-2 expression and via b- stimulating ERK1/2 overexpression/synthesis inhibits angiogenesis, promotes apoptosis and finally results in severe fibrosis in endometriotic-like legions.

oncogene Bcl-2 and other genes, including Bax, PUMA and Bak exert key regulating roles in cell survival/proliferation and/or apoptosis in ectopic endometrial tissue (Huang et al., 2003; Meresman et al., 2000; Nezhat et al., 2002; Subramaniam and Unsicker, 2010). Of these proteins, the Bcl-2 has been reported to exert essential role in cellular proliferation through inducing anti-apoptotic impact (Meresman et al., 2000; Mourtzikou et al., 2012). In corroboration with these reports, our data showed decreased expression of Bcl-2 in endometriotic-like legions of endometriosis + SMN group, suggesting the pro-apoptotic effect of SMN. More other studies have shown that the SMN induces apoptosis through the p53-dependent pathway, which is mediated via the Bcl-2/Bax/caspase-3 proteins involvement in JB6 C141 cells (Katiyar et al., 2005). The concentration of SMN is shown therein to be correlated with p53 concentrations, suggesting that silymarin acts via increasing the p53 levels. Considering these findings, we can come close to these facts that, aside the SMN-induced impact on Bcl-6b pathway, the SMN by down-regulating the Bcl-2 expression up-regulated the apoptosis ratio, and consequently decreased the cell proliferation. The increased number of apoptotic cells *per* mm^2 of tissue in SMN + endometriosis group confirms this theory. As it has been shown previously, the physiologic expression and/or phosphorylation of ERK1/2 promotes the cell survival and/or proliferation and consequently accelerates angiogenesis ratio (Andradas et al., 2011; Cai et al., 2010). However depending on the cell type and the nature of the injury, the overexpression and/or activation of ERk1/2 down-regulates the Bcl-2 expression, resulting in impaired mitochondrial respiration (Wang et al., 2013; Yeh et al., 2004), membrane potential (Nowak, 2002; Rasola et al., 2010), and finally ends with mitochondrial-dependent apoptosis (Wu et al., 2005; Yang et al., 2008). In fact, the active ERK1/2 has been found on mitochondrial membranes (Zhuang et al., 2007). In line, here in the current study, we found that the SMN significantly increased ERK1/2 mRNA level and ERK1/2$^+$ cells number *per* mm^2 of tissue. Considering diminished Bcl-2 expression simultaneous with ERK1/2 overexpression and elevated apoptosis ratio in SMN + endometriosis group, we can conclude that the up-regulated ERK1/2 expression suppressed the Bcl-2 expression, which in turn resulted in intensive apoptosis (may be through the intrinsic apoptosis pathway).

As all tissues need angiogenesis to develop, the ectopic endometriotic legions need the angiogenesis, as well. It has been illustrated that, administrating anti-angiogenic chemicals significantly inhibit endometriotic (Khan et al., 2010; Novella-Maestre et al., 2010), as well as endometriotic-like legions (Soysal et al., 2014; Wan et al., 2013), development. Our observations demonstrated a remarkable reduction in the angiogenesis ratio by representing decreased vessel numbers/mm^2 of tissue in SMN + endometriosis group. This finding suggests the anti-angiogenic effect of SMN. On the other hand, various in-vitro and in-vivo studies have shown that, in association with VEGF, the GDNF promotes angiogenesis (Abe et al., 1997; Klopp et al., 2012; Kloth and Suter-Crazzolara, 2000). In addition, it has been well-established that the GDNF represents a cross talk with VEGF:VEGFR and NGF:TrkA pathways, and stimulates the VEGF-independent angiogenesis (Tufro et al., 2007; Zhong et al., 2016), promotes the angiogenesis via up-regulating the interleukin-8 (Iwahashi et al., 2002), and finally, it acts as a key regulator of the endothelial cells network and proliferation (Zhong et al., 2016). In line with this issue, the anti-angiogenic properties of SMN has been reported by previous experimental studies. Accordingly, the SMN, and its major pure componenet silibinin significantly reduces the endothelial cells (EA.hy 926) proliferation, when they are cocultured with colon cancer (LoVo) cell lines (Yang et al., 2003). The silibinin induces the endothelial cells apoptosis by modulation of NF-kappaB, Bcl-2 family and caspases expressions (Yoo et al., 2004). Thus, considering the mentioned findings, it is logic to conclude that the SMN exerted anti-angiogenic properties by suppressing the GDNF expression and/or by inducing apoptosis in endothelial cells.

6. Conclusion

Our data showed that, the SMN as an anti-inflammatory and anti-oxidant agent inhibits the endometriotic-like legions development by diminishing the growth factor GDNF, its restricted receptor gfrα1 expression. Moreover, we showed that, the SMN inhibits cell survival and/or proliferation via reducing the Bcl-2 (as core gene involving in cell survival) and the Bcl-6b (as main gene involving in cell proliferation) expressions. On the other hand, our findings represented that, the enhanced ERK1/2 expression in SMN-treated group amplifies apoptosis pathway. Finally, we showed that, the SMN by down-regulating angiogenesis ratio results in intensive apoptosis and fibrosis in endometriotic-like legions. Nevertheless, more studies are necessary to show, whether the SMN therapy is appropriate for the treatment of endometriosis.

7. Study limitations

There are several limitations of the current study, including possible bias due to small sample number. Although gfrα1 was considered as an especial receptor for GDNF, investigating the molecular changes for Rearranged during Transfection (c-RET) receptor confirms the findings of this research. Additionally, analyzing complete pathway of Ras/RAF1/MEK1/2/ERK1/2 following SMN administration promotes/completes the findings of the current prospective study. Moreover, further studies are needed to illustrate the ameliorative effects of SMN against endometriotic legions development and to uncover the effect of other molecular changes in women with endometriosis.

Author statement

The authors contributions are as below; Dr. Mazdak Razi (corresponding author for research work): Conception and design of the study, analysis and interpretation of data, final approval of the version to be submitted; Mrs. Elaheh Nahari: Study design, drafting the article or revising it critically for important intellectual content.

Acknowledgments

The authors wish to thank AYANDEH lab. Co, for laboratory helps.

Moreover, authors wish to thank the Departments of Basic Sciences, Faculty of Veterinary medicine, Urmia University and Biology, Islamic AZAD university for supportive and laboratory helps. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Abe, K., Hayashi, T., Itoyama, Y., 1997. Amelioration of brain edema by topical application of glial cell line-derived neurotrophic factor in reperfused rat brain. Neurosci. Lett. 231 (1), 37–40.

Adibnia, E., Razi, M., Malekinejad, H., 2016. Zearalenone and 17 beta-estradiol induced damages in male rats reproduction potential; evidence for ERalpha and ERbeta receptors expression and steroidogenesis. Toxicon 120, 133–146.

Airaksinen, M.S., Saarma, M., 2002. The GDNF family: signalling, biological functions and therapeutic value. Nat. Rev. Neurosci. 3 (5), 383–394.

Amin, M.M., Arbid, M.S., 2015. Estimation of the novel antipyretic, anti-inflammatory, antinociceptive and antihyperlipidemic effects of silymarin in Albino rats and mice. Asian Pac. J. Trop. Biomed. 5 (8), 619–623.

Andradas, C., Caffarel, M.M., Perez-Gomez, E., Salazar, M., Lorente, M., Velasco, G., Guzman, M., Sanchez, C., 2011. The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. Oncogene 30 (2), 245–252.

Arguni, E., Arima, M., Tsuruoka, N., Sakamoto, A., Hatano, M., Tokuhisa, T., 2006. JunD/AP-1 and STAT3 are the major enhancer molecules for high Bcl6 expression in germinal center B cells. Int. Immunol. 18 (7), 1079–1089.

Asghar, Z., Masood, Z., 2008. Evaluation of antioxidant properties of silymarin and its potential to inhibit peroxyl radicals in vitro. Pak. J. Pharm. Sci. 21 (3), 249–254.

Ashkavand, Z., Malekinejad, H., Amniattalab, A., Rezaei-Golmisheh, A., Vishwanath, B.S., 2012. Silymarin potentiates the anti-inflammatory effects of Celecoxib on chemically induced osteoarthritis in rats. Phytomedicine 19 (13), 1200–1205.

Biedermann, D., Vavrikova, E., Cvak, L., Kren, V., 2014. Chemistry of silybin. Nat. Prod. Rep. 31 (9), 1138–1157.

Bilotas, M., Meresman, G., Stella, I., Sueldo, C., Baranao, R.I., 2010. Effect of aromatase inhibitors on ectopic endometrial growth and peritoneal environment in a mouse model of endometriosis. Fertil. Steril. 93 (8), 2513–2518.

Brodie, A., Jelovac, D., Long, B.J., 2003. Predictions from a preclinical model: studies of aromatase inhibitors and antiestrogens. Clin. Cancer Res. 9 (1 Pt 2) 455s-9s.

Cai, W.J., Wang, M.J., Ju, L.H., Wang, C., Zhu, Y.C., 2010. Hydrogen sulfide induces human colon cancer cell proliferation: role of Akt, ERK and p21. Cell Biol. Int. 34 (6), 565–572.

Chen, C.H., Huang, T.S., Wong, C.H., Hong, C.L., Tsai, Y.H., Liang, C.C., Lu, F.J., Chang, W.H., 2009. Synergistic anti-cancer effect of baicalein and silymarin on human hepatoma HepG2 Cells. Food Chem. Toxicol. 47 (3), 638–644.

Comelli, M.C., Mengs, U., Schneider, C., Prosdocimi, M., 2007. Toward the definition of the mechanism of action of silymarin: activities related to cellular protection from toxic damage induced by chemotherapy. Integr. Cancer Ther. 6 (2), 120–129.

Evans-Hoeker, E., Lessey, B.A., Jeong, J.W., Savaris, R.F., Palomino, W.A., Yuan, L., Schammel, D.P., Young, S.L., 2016. Endometrial BCL6 overexpression in eutopic endometrium of women with endometriosis. Reprod. Sci. 23 (9), 1234–1241.

Fan, L., Ma, Y., Liu, Y., Zheng, D., Huang, G., 2014. Silymarin induces cell cycle arrest and apoptosis in ovarian cancer cells. Eur. J. Pharmacol. 743, 79–88.

Fisher, C.E., Michael, L., Barnett, M.W., Davies, J.A., 2001. Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney. Development 128 (21), 4329–4338.

Fouchecourt, S., Godet, M., Sabido, O., Durand, P., 2006. Glial cell-line-derived neurotropic factor and its receptors are expressed by germinal and somatic cells of the rat testis. J. Endocrinol. 190 (1), 59–71.

Gharagozloo, M., Jafari, S., Esmaeil, N., Javid, E.N., Bagherpour, B., Rezaei, A., 2013a. Immunosuppressive effect of silymarin on mitogen-activated protein kinase signalling pathway: the impact on T cell proliferation and cytokine production. Basic Clin. Pharmacol. Toxicol. 113 (3), 209–214.

Gharagozloo, M., Javid, E.N., Rezaei, A., Mousavizadeh, K., 2013b. Silymarin inhibits cell cycle progression and mTOR activity in activated human T cells: therapeutic implications for autoimmune diseases. Basic Clin. Pharmacol. Toxicol. 112 (4), 251–256.

Glerup, S., Lume, M., Olsen, D., Nyengaard, J.R., Vaegter, C.B., Gustafsen, C., Christensen, E.I., Kjolby, M., Hay-Schmidt, A., Bender, D., Madsen, P., Saarma, M., Nykjaer, A., Petersen, C.M., 2013. SorLA controls neurotrophic activity by sorting of GDNF and its receptors GFRalpha1 and RET. Cell Rep. 3 (1), 186–199.

Gonzalez-Ramos, R., Van Langendonckt, A., Defrere, S., Lousse, J.C., Colette, S., Devoto, L., Donnez, J., 2010. Involvement of the nuclear factor-kappaB pathway in the pathogenesis of endometriosis. Fertil. Steril. 94 (6), 1985–1994.

Grund, E.M., Kagan, D., Tran, C.A., Zeitvogel, A., Starzinski-Powitz, A., Nataraja, S., Palmer, S.S., 2008. Tumor necrosis factor-alpha regulates inflammatory and mesenchymal responses via mitogen-activated protein kinase kinase, p38, and nuclear factor kappaB in human endometriotic epithelial cells. Mol. Pharmacol. 73 (5), 1394–1404.

Hasegawa, K., Namekawa, S.H., Saga, Y., 2013. MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. Stem Cells 31 (11), 2517–2527.

Huang, F.Y., Lin, Q.H., Fang, X.L., 2003. Expression of Bcl-2 and Bax protein in endometriosis. Hunan Yi Ke Da Xue Xue Bao 28 (2), 102–106.

Hussain, S.A., Jassim, N.A., Numan, I.T., Al, K., Abdullah II, T.A., 2009. Anti-inflammatory activity of silymarin in patients with knee osteoarthritis. A comparative study with piroxicam and meloxicam. Saudi Med. J. 30 (1), 98–103.

Iwahashi, N., Murakami, H., Nimura, Y., Takahashi, M., 2002. Activation of RET tyrosine kinase regulates interleukin-8 production by multiple signaling pathways. Biochem. Biophys. Res. Commun. 294 (3), 642–649.

Jin, Z., Wang, L., Zhu, Z., 2015. Effect of GuiXiong Xiaoyi Wan in treatment of endometriosis on rats. Evid. Complement. Alternat. Med. 208514.

Kang, J., Perry, J.K., Pandey, V., Fielder, G.C., Mei, B., Qian, P.X., Wu, Z.S., Zhu, T., Liu, D.X., Lobie, P.E., 2009. Artemin is oncogenic for human mammary carcinoma cells. Oncogene 28 (19), 2034–2045.

Katiyar, S.K., Roy, A.M., Baliga, M.S., 2005. Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome c release, and caspase activation. Mol. Cancer Ther. 4 (2), 207–216.

Khan, K.N., Kitajima, M., Hiraki, K., Fujishita, A., Sekine, I., Ishimaru, T., Masuzaki, H., 2010. Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy. Hum. Reprod. 25 (3), 642–653.

Klopp, A.H., Zhang, Y., Solley, T., Amaya-Manzanares, F., Marini, F., Andreeff, M., Debeb, B., Woodward, W., Schmandt, R., Broaddus, R., Lu, K., Kolonin, M.G., 2012. Omental adipose tissue-derived stromal cells promote vascularization and growth of endometrial tumors. Clin. Cancer Res. 18 (3), 771–782.

Kloth, S., Suter-Crazzolara, C., 2000. Modulation of renal blood vessel formation by glial cell line-derived neurotrophic factor. Microvasc. Res. 59 (1), 190–194.

Kondoh, K., Nishida, E., 2007. Regulation of MAP kinases by MAP kinase phosphatases. Biochim. Biophys. Acta 1773 (8), 1227–1237.

Large, M.J., Wetendorf, M., Lanz, R.B., Hartig, S.M., Creighton, C.J., Mancini, M.A., Kovanci, E., Lee, K.F., Threadgill, D.W., Lydon, J.P., Jeong, J.W., DeMayo, F.J., 2014. The epidermal growth factor receptor critically regulates endometrial function during early pregnancy. PLoS Genet. 10 (6), e1004451.

Lee, M.H., Huang, Z., Kim, D.J., Kim, S.H., Kim, M.O., Lee, S.Y., Xie, H., Park, S.J., Kim, J.Y., Kundu, J.K., Bode, A.M., Surh, Y.J., Dong, Z., 2013. Direct targeting of MEK1/2 and RSK2 by silybin induces cell-cycle arrest and inhibits melanoma cell growth. Cancer Prev. Res. Phila. (Phila) 6 (5), 455–465.

Lin, S.C., Wang, C.C., Wu, M.H., Yang, S.H., Li, Y.H., Tsai, S.J., 2012. Hypoxia-induced microRNA-20a expression increases ERK phosphorylation and angiogenic gene expression in endometriotic stromal cells. J. Clin. Endocrinol. Metab. 97 (8), E1515–23.

Meresman, G.F., Vighi, S., Buquet, R.A., Contreras-Ortiz, O., Tesone, M., Rumi, L.S., 2000. Apoptosis and expression of Bcl-2 and Bax in eutopic endometrium from women with endometriosis. Fertil. Steril. 74 (4), 760–766.

Mihalich, A., Reina, M., Mangioni, S., Ponti, E., Alberti, L., Vigano, P., Vignali, M., Di Blasio, A.M., 2003. Different basic fibroblast growth factor and fibroblast growth factor-antisense expression in eutopic endometrial stromal cells derived from women with and without endometriosis. J. Clin. Endocrinol. Metab. 88 (6), 2853–2859.

Mourtzikou, A., Kosmas, K., Marouga, A., Stamouli, M., Pouliakis, A., Karakitsos, P., 2012. The use of an immunocytochemical double-labeling staining can display the distribution of Bcl-2/Ki-67 cells in endometrial adenocarcinomas as well as in normal endometrium. Clin. Lab. 58 (1-2), 133–144.

Mu, F., Hankinson, S.E., Schernhammer, E., Pollak, M.N., Missmer, S.A., 2015. A prospective study of insulin-like growth factor 1, its binding protein 3, and risk of endometriosis. Am. J. Epidemiol. 182 (2), 148–156.

Muriel, P., Moreno, M.G., Hernandez Mdel, C., Chavez, E., Alcantar, L.K., 2005. Resolution of liver fibrosis in chronic CCl4 administration in the rat after discontinuation of treatment: effect of silymarin, silibinin, colchicine and trimethylcolchicinic acid. Basic Clin. Pharmacol. Toxicol. 96 (5), 375–380.

Murk, W., Atabekoglu, C.S., Cakmak, H., Heper, A., Ensari, A., Kayisli, U.A., Arici, A., 2008. Extracellularly signal-regulated kinase activity in the human endometrium: possible roles in the pathogenesis of endometriosis. J. Clin. Endocrinol. Metab. 93 (9), 3532–3540.

Murphy, L.O., Blenis, J., 2006. MAPK signal specificity: the right place at the right time. Trends Biochem. Sci. 31 (5), 268–275.

Nezhat, F., Cohen, C., Rahaman, J., Gretz, H., Cole, P., Kalir, T., 2002. Comparative immunohistochemical studies of bcl-2 and p53 proteins in benign and malignant ovarian endometriotic cysts. Cancer 94 (11), 2935–2940.

Novella-Maestre, E., Carda, C., Ruiz-Sauri, A., Garcia-Velasco, J.A., Simon, C., Pellicer, A., 2010. Identification and quantification of dopamine receptor 2 in human eutopic and ectopic endometrium: a novel molecular target for endometriosis therapy. Biol. Reprod. 83 (5), 866–873.

Nowak, G., 2002. Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells. J. Biol. Chem. 277 (45), 43377–43388.

Oatley, J.M., Avarbock, M.R., Telaranta, A.I., Fearon, D.T., Brinster, R.L., 2006. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc. Natl. Acad. Sci. U. S. A. 103 (25), 9524–9529.

Oatley, J.M., Avarbock, M.R., Brinster, R.L., 2007. Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J. Biol. Chem. 282 (35), 25842–25851.

Olivares, C., Ricci, A., Bilotas, M., Baranao, R.I., Meresman, G., 2011. The inhibitory effect of celecoxib and rosiglitazone on experimental endometriosis. Fertil. Steril. 96 (2), 428–433.

Omwandho, C.O., Konrad, L., Halis, G., Oehmke, F., Tinneberg, H.R., 2010. Role of TGF-betas in normal human endometrium and endometriosis. Hum. Reprod. 25 (1), 101–109.

Ortega, A., Ferrer, P., Carretero, J., Obrador, E., Asensi, M., Pellicer, J.A., Estrela, J.M., 2003. Down-regulation of glutathione and Bcl-2 synthesis in mouse B16 melanoma cells avoids their survival during interaction with the vascular endothelium. J. Biol.

Chem. 278 (41), 39591–39599.
Owens, D.M., Keyse, S.M., 2007. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene 26 (22), 3203–3213.
Park, H.J., Bolton, E.C., 2015. Glial cell line-derived neurotrophic factor induces cell proliferation in the mouse urogenital sinus. Mol. Endocrinol. 29 (2), 289–306.
Phan, R.T., Dalla-Favera, R., 2004. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature 432 (7017), 635–639.
Pradeep, K., Victor Raj Mohan, C., Gobianand, K., Karthikeyan, S., 2007. Silymarin: an effective hepatoprotective agent against diethylnitrosamine-induced hepatotoxicity in rats. Pharm. Biol. 45 (9), 707–714.
Quereda, F., Barroso, J., Acien, P., 1996. Individual and combined effects of triptoreline and gestrinone on experimental endometriosis in rats. Eur. J. Obstet. Gynecol. Reprod. Biol. 67 (1), 35–40.
Ramasamy, K., Agarwal, R., 2008. Multitargeted therapy of cancer by silymarin. Cancer Lett. 269 (2), 352–362.
Ramos, J.W., 2008. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int. J. Biochem. Cell Biol. 40 (12), 2707–2719.
Rasola, A., Sciacovelli, M., Chiara, F., Pantic, B., Brusilow, W.S., Bernardi, P., 2010. Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition. Proc. Natl. Acad. Sci. U. S. A. 107 (2), 726–731.
Sakashita, C., Fukuda, T., Okabe, S., Kobayashi, H., Hirosawa, S., Tokuhisa, T., Miyasaka, N., Miura, O., Miki, T., 2002. Cloning and characterization of the human BAZF gene, a homologue of the BCL6 oncogene. Biochem. Biophys. Res. Commun. 291 (3), 567–573.
Simoens, S., Hummelshoj, L., D'Hooghe, T., 2007. Endometriosis: cost estimates and methodological perspective. Hum. Reprod. Update 13 (4), 395–404.
Soysal, D., Kizildag, S., Saatli, B., Posaci, C., Soysal, S., Koyuncuoglu, M., Dogan, O., 2014. A novel angiogenesis inhibitor bevacizumab induces apoptosis in the rat endometriosis model. Balkan J. Med. Genet. 17 (2), 73–80.
Subramaniam, S., Unsicker, K., 2010. ERK and cell death: ERK1/2 in neuronal death. FEBS J. 277 (1), 22–29.
Switzer, C.H., Ridnour, L.A., Cheng, R.Y., Sparatore, A., Del Soldato, P., Moody, T.W., Vitek, M.P., Roberts, D.D., Wink, D.A., 2009. Dithiolethione compounds inhibit Akt signaling in human breast and lung cancer cells by increasing PP2A activity. Oncogene 28 (43), 3837–3846.
Takahashi, M., 2001. The GDNF/RET signaling pathway and human diseases. Cytokine Growth Factor Rev. 12 (4), 361–373.
Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Morimoto, H., Inoue, K., Ogonuki, N., Jijiwa, M., Takahashi, M., Ogura, A., Shinohara, T., 2015. Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep. 4 (3), 489–502.
Tufro, A., Teichman, J., Banu, N., Villegas, G., 2007. Crosstalk between VEGF-A/VEGFR2 and GDNF/RET signaling pathways. Biochem. Biophys. Res. Commun. 358 (2), 410–416.
Wan, Y.Y., Tian, G.Y., Guo, H.S., Kang, Y.M., Yao, Z.H., Li, X.L., Liu, Q.H., Lin, D.J., 2013. Endostatin, an angiogenesis inhibitor, ameliorates bleomycin-induced pulmonary fibrosis in rats. Respir. Res. 14 (1), 56.
Wang, J., Yang, J., Gu, P., Klassen, H., 2010. Effects of glial cell line-derived neurotrophic factor on cultured murine retinal progenitor cells. Mol. Vis. 16, 2850–2866.
Wang, X., Bai, H., Zhang, X., Liu, J., Cao, P., Liao, N., Zhang, W., Wang, Z., Hai, C., 2013. Inhibitory effect of oleanolic acid on hepatocellular carcinoma via ERK-p53-mediated cell cycle arrest and mitochondrial-dependent apoptosis. Carcinogenesis 34 (6), 1323–1330.
Wu, Z., Wu, L.J., Tashiro, S., Onodera, S., Ikejima, T., 2005. Phosphorylated extracellular signal-regulated kinase up-regulated p53 expression in shikonin-induced HeLa cell apoptosis. Chin. Med. J. (Engl.) 118 (8), 671–677.
Wu, Y., Kajdacsy-Balla, A., Strawn, E., Basir, Z., Halverson, G., Jailwala, P., Wang, Y., Wang, X., Ghosh, S., Guo, S.W., 2006. Transcriptional characterizations of differences between eutopic and ectopic endometrium. Endocrinology 147 (1), 232–246.
Xu, L., Li, X., Chu, E.S., Zhao, G., Go, M.Y., Tao, Q., Jin, H., Zeng, Z., Sung, J.J., Yu, J., 2012. Epigenetic inactivation of BCL6B, a novel functional tumour suppressor for gastric cancer, is associated with poor survival. Gut 61 (7), 977–985.
Yang, Y., Han, C., 2010. GDNF stimulates the proliferation of cultured mouse immature Sertoli cells via its receptor subunit NCAM and ERK1/2 signaling pathway. BMC Cell Biol. 11, 78.
Yang, S.H., Lin, J.K., Chen, W.S., Chiu, J.H., 2003. Anti-angiogenic effect of silymarin on colon cancer LoVo cell line. J. Surg. Res. 113 (1), 133–138.
Yang, R., Piperdi, S., Gorlick, R., 2008. Activation of the RAF/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway mediates apoptosis induced by chelerythrine in osteosarcoma. Clin. Cancer Res. 14 (20), 6396–6404.
Yeh, P.Y., Chuang, S.E., Yeh, K.H., Song, Y.C., Chang, L.L., Cheng, A.L., 2004. Phosphorylation of p53 on Thr55 by ERK2 is necessary for doxorubicin-induced p53 activation and cell death. Oncogene 23 (20), 3580–3588.
Yoo, H.G., Jung, S.N., Hwang, Y.S., Park, J.S., Kim, M.H., Jeong, M., Ahn, S.J., Ahn, B.W., Shin, B.A., Park, R.K., Jung, Y.D., 2004. Involvement of NF-kappaB and caspases in silibinin-induced apoptosis of endothelial cells. Int. J. Mol. Med. 13 (1), 81–86.
Yoo, J.-Y., Ahn, J.I., Kim, T.H., Yu, S., Ahn, J.Y., Lim, J.M., Jeong, J.-W., 2017a. G-protein coupled receptor 64 is required for decidualization of endometrial stromal cells. Sci. Rep. 7, 5021.
Yoo, J.Y., Kim, T.H., Fazleabas, A.T., Palomino, W.A., Ahn, S.H., Tayade, C., Schammel, D.P., Young, S.L., Jeong, J.W., Lessey, B.A., 2017b. KRAS activation and over-expression of SIRT1/BCL6 contributes to the pathogenesis of endometriosis and progesterone resistance. Sci. Rep. 7 (1), 6765.
Zhang, X.L., Liu, J.M., Yang, C.C., Zheng, Y.L., Liu, L., Wang, Z.K., Jiang, H.Q., 2006. Dynamic expression of extracellular signal-regulated kinase in rat liver tissue during hepatic fibrogenesis. World J. Gastroen. 12 (39), 6376–6381.
Zhang, J.J., Xu, Z.M., Dai, H.Y., Ji, X.Q., Duan, Y.Y., Zhang, C.M., Qin, D.Y., 2010. Application of the nuclear factor-kappaB inhibitor pyrrolidine dithiocarbamate for the treatment of endometriosis: an in vitro study. Fertil. Steril. 94 (7), 2942–2944.
Zheng, J., Qu, J., Lu, P., Hou, Z., Cui, Y., Mao, Y., Qi, X., Ji, H., Liu, J., 2016. Activin a stimulates aromatase via the ALK4-Smad pathway in endometriosis. Biomed Res. Int. 2016, 5791510.
Zhong, Z., Gu, H., Peng, J., Wang, W., Johnstone, B.H., March, K.L., Farlow, M.R., Du, Y., 2016. GDNF secreted from adipose-derived stem cells stimulates VEGF-independent angiogenesis. Oncotarget 7 (24), 36829–36841.
Zhuang, S., Yan, Y., Daubert, R.A., Han, J., Schnellmann, R.G., 2007. ERK promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of Akt in renal epithelial cells. Am. J. Physiol. Renal. Physiol. 292 (1), F440–447.