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The expression of Bcl-2 in adenomyosis and its effect on proliferation, migration, and apoptosis of endometrial stromal cells

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ABSTRACT

Adenomyosis is a common gynecologic disease that severe impact on women. Previous studies have found that Bcl-2 abnormally expressed in adenomyosis. However, the exact mechanisms of Bcl-2 in the pathogenesis of adenomyosis are unclear. In this study, we are to explore the effect of Bcl-2 on proliferation, migration, and apoptosis of endometrial stromal cells. The expression of Bcl-2 were evaluated by Western blot and RT-qPCR. We used RNA interference to silence Bcl-2 gene of endometrial stromal cells, and then Cell Counting Kit(CCK-8), cell scratch repair test, and Annexin V-APC/propidium iodide (PI) staining were performed to detect the cell viability, migration ability and apoptotic rate. The results of the present study revealed that the expression of Bcl-2 was evidently higher than that in control group. After silencing the Bcl-2 gene, the cytoactive and migration ability of endometrial stromal cells of adenomyosis decreased, and the apoptotic rate increased. In conclusion, Bcl-2 is overexpressed in adenomyosis and participate in the pathogenesis of adenomyosis. Bcl-2 may be a potential novel target for adenomyosis treatment.

1. Introduction

Adenomyosis (AM) is defined as a benign gynecological disease which endometrial tissues (containing glandulars and mesenchyme) are located in the myometrium and grow diffusely, resulting in uterus diffuse enlargement [1]. The clinical manifestations of adenomyosis are characterised by menstrual changes (menorrhagia, prolonged menstruation, and ingravescent dysmenorrhea) and sterility [2]. The etiologies of adenomyosis are complex. Adenomyosis is a benign disease, but it also has some characteristics of malignant tumors, such as endometrial cells limitless growth and infiltration to the myometrium. Previous studies considered that the biological characteristics of the eutopic endometrium changed, such as the enhancement of the cell adhesion, invasiveness [3] and anti-apoptosis [4]. The conventional wisdom suggests that the abnormal down-growth and invagination of the endometrium into the myometrium result in adenomyosis [5]. Abnormal stromal cells invasion has been reported in the etiology of adenomyosis [6,7]. The stromal cells may have a primary pathogenetic role to accelerate epithelial downgrowth [5]. However, the exact mechanisms of adenomyosis are unclear. It is of great significance to explore the pathogenesis of adenomyosis and seek new conservative treatment methods.

Apoptosis is an important physiological mechanism for multicellular organisms to maintain the cell homeostasis. Apoptosis was controlled by multiple cell signals, including extracellular signals (such as toxins, hormones, growth factors, NO, cytokines, etc.) and intracellular signals (such as radiation, denutrition, virus infection, hypoxia, intracellular calcium concentration augment, etc.) [8]. Report demonstrated apoptosis impaired in adenomyosis [9]. The resistance to apoptosis of endometrium suggested the possible pathogenesis of adenomyosis. Bcl-2 is one of the apoptosis regulators. Studies have found that Bcl-2 abnormally expressed in adenomyosis, whether in proliferative or secretory phase [10]. However, the exact mechanisms of increased Bcl-2 in the pathogenesis of adenomyosis require further investigation.

In this study, we will use Western blot and quantitative real-time PCR (RT-qPCR) to detect the expression of Bcl-2 in the adenomyosis tissues and endometrial stromal cells. The RNA interference is carried out to decrease Bcl-2 in endometrial stromal cells of adenomyosis, then Cell Counting Kit(CCK-8), cell scratch repair test, and Annexin V-APC/propidium iodide (PI) staining are to detect cell viability, migration ability and apoptotic rate, respectively. These will explain the role of Bcl-2 in adenomyosis and provide a theoretical basis for effective treatment of adenomyosis.

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2. Materials and methods

2.1. Tissue collection

Tissues were collected from patients who underwent a laparoscopic or transabdominal hysterectomy at the gynecological ward of The Second Affiliated Hospital, Zhejiang University School of Medicine. The study group was 36 women ranged 40–50 years with adenomyosis. The control group consisted of 21 patients with uterine leiomyoma who were between 39 and 50 years old. According to the literature, there was no significant difference in the expression of Bcl-2 between proliferative phase and secretory phase, therefore women in the proliferative and secretory phase were all included in this study. Tissue collection was approved by the ethics committee and patients. The diagnosis of adenomyosis or uterine leiomyoma(without endometrial lesion) had been done with a histological examination performed by pathologists. Tissue samples were placed in DMEM/F-12 medium containing 10%FBS(purchased by Gibco and Sigma, respectively) immediately and shipped to the laboratory within 40 min on an ice bath. Endometrium partly was used to extract stromal cells for follow-up experiments, and the rest was stored in a −80 ˚C freezer for tissue total protein and RNA detection.

2.2. Primary cell culture

We cut endometrium into 0.5-1 mm^3, adding 2–3 volumes of cell dissociation buffer (DMEM/F-12 diluted 0.25% trypsin to 1.25 mg/ml, purchesed by Gibco and Solarbio, respectively) to the cultrue dish, then put it in an incubator at 37 ˚C and 5%CO_2 to digest for 70–80 min. Afterward, the cell suspension was filtered by nylon mesh(140 um and 37 um successively) and centrifugated at 800 r/min for 5 min. And the cells were resuspended in culture medium supplemented with 10% FBS (commercially availabled from Sigma), and cultrued in an incubator at 37℃ and 5% CO_2. The purity was above 90% through verification. Logarithmic growth phase cells were used for subsequent experiments.

2.3. RNA interference

Three pairs of specific siRNA(GenePharma, Shanghai, China) sequences were designed and synthesized to ensure Bcl-2 silenced specifically (Table 1). According to the manufacturer's protocol, 3–5 × 10^5 cells/well were seeded in 6-well plates and cell density reached 60% when transfection. Mixing 5ul/well of Lipofectamine 2000(Invitrogen) diluted with 100ul Opti-MEM(Gibco) and 5ul FAM-siRNA diluted with 100ul Opti-MEM, and added it to 6-well plate. Then transfected cells were cultured in an incubator at 37℃ and 5% CO_2 for 4–6 h. Changing the medium to complete medium and examining the transfection efficiency under a fluorescence microscope roughly. Carrying out RT-PCR, Western blot and subsequent experiments 48 h later.

2.4. Western blot

Proteins acquired from cells or frozen uterine tissue grinding with a mortar in liquid nitrogen were separated by SDS-PAGE (consisted of 3% concentration gel and 12% separation gel). Then the proteins were electrotransferred onto the nitrocellulose membrane, and incubated with primary antibody (anti-beclin 1, 1:1000, sc-48381, Santa Cruz; anti-Bcl-2, 1:10000, Proteintech Group; anti-GAPDH, 1:10000, Wuhan Google Biotechnology) overnight at 4 ˚C followed by 2 h incubation with secondary antibody (1:5000,Wuhan Google Biotechnology). GAPDH as an internal reference, the immunoreactive bands were analysed with Enhanced Chemiluminescence(Gibco). The experiment was repeated three times.

Table 1
siRNA gene sequence.

Gene name	Gene sequence (5´to3´)
Bcl-2-homo-528	sence GGGAGAUAGUGAUGAAGUATT antisence UACUUCAUCACUAUCUCCCTT
Bcl-2-homo-928	sence GAGGAUUGUGGCCUUCUUUTT antisence AAAGAAGGCCACAAUCCUCTT
Bcl-2-homo-1014	sence CCCUGUGGAUGACUGAGUATT antisence UACUCAGUCAUCCACAGGGTT
Negative control	sence UUCUCCGAACGUGUCACGUTT antisence ACGUGACACGUUCGGAGAATT

2.5. RT-qPCR

Total RNA was isolated from tissues or cells with TRIzol® reagent (TaKaRa). Then mRNA(1 μg) was converted to cDNA via reverse transcription with a TaKaRa kit(TaKaRa). Specifc primers along with cDNA and PCR reagents were placed into a fast real-time PCR system(7500 Fsat DX, KITOOLI). The melt curve analysis was used to confirm the amplified product. GAPDH mRNA was used as an internal control. Each PCR reaction was performed in triplicate wells. The samples underwent reverse transcription at 37℃ for 15 min and 85℃ for 5 s, followed by denaturation at 95 ˚C for 30 s, annealing at 60 ˚C for 30 s, Melt Curve. The primers were designed in Genepharma of Shanghai as follows: Bcl-2, forward GAGGATTGTGGCCTTCTTTG, reverse GCCGGTTCAGGTAC TCAGTC. GAPDH, forward ATCCCATCACCATCTTCCAG, reverse GAG TCCTTCCACGATACCAA. (Table 2). Finally, we used the $2^{-\Delta\Delta Ct}$ method for relative quantifcation of the gene. The experiment was repeated three times.

2.6. Scratch wound repair experiment

Cells were seeded in a 6-well culture plate at the density of 5 × 10^5cells/well, and incubated to a density of 90%. Scratch wounds were made on the monolayer cells using a 10 ul pipette. Suspended cells were washed and the cells in 6-well culture were cultured in serum-free medium. The repairing process of the scratch wound was observed under an inverted microscope. The cells were photographed at 0 h, 24 h and 48 h in the same position to compare migration rate. Relative migration distance = (0 h scratch width - 24 h or 48 h scratch width)/0 h scratch width × 100%.

2.7. Flow cytometry assay

Annexin V-APC/PI apoptosis detection kit (keyGEN Biotech, Jiangsu, China) was used to detect cell apoptosis ratio. Briefly, Cells were centrifuged at 1000 rpm for 5 min and resuspended in PBS for washing. Cells were resuspended in 500 μl Binding Buffer, with 5 μl Annexin V-APC and 5 μl propidium iodide (PI), and incubated in the dark for 15 min at room temperature. Applying flow cytometry detected apoptotic rate.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 20.0 software. The Kolmogorov-Smimov method found all data was approximate normal distribution. The Levene test method was to test homoscedasticity. All

Table 2
RT-qPCR primer sequence.

Gene name	Primer sequence
Bcl-2	forward GAGGATTGTGGCCTTCTTTG reverse GCCGGTTCAGGTACTCAGTC
GAPDH	forward ATCCCATCACCATCTTCCAG reverse GAGTCCTTCCACGATACCAA

Table 3
General clinical data.

group	number of examples	age	menstrual cycle		number of pregnancies	number of labor
			proliferative phase	secretory phase		
experimental group	36	45.70 ± 3.02	21	15	3.90 ± 2.42	1.60 ± 1.07
control group	21	44.85 ± 4.22	12	9	2.67 ± 1.86	1.33 ± 0.5

data was expressed as mean ± standard deviation (M ± SD). The two groups were compared using the independent-sample *t* test. Comparison between variables was analysed with one-way ANOVA: multiple comparisons were performed using the LSD method. Dunnett's method was used for comparison between multiple experimental groups and the control group(e.g. the comparison of Bcl-2-siRNA group, siNC group to blank control group). Repeated measures were used for relative migration distance. Data was considered statistically significant at a value of $P < 0.05$.

3. Results

Patients characteristics are shown in Table 3. There was no significant difference in age, gravidity and parity between the two groups.

3.1. Increased expression of Bcl-2 in the eutopic endometrium of adenomyosis

Western blotting was performed to analyze the expression of Bcl-2. The expression of Bcl-2 in the eutopic endometrium of adenomyosis were significantly higher than the control group ($P < 0.05$). Fig. 1a. RT-qPCR demenstrated the expression of Bcl-2 mRNA in the adenomyosis group were significantly higher than the control (1.33 ± 0.03 vs.1, $P < 0.05$). Fig. 1b. The above indicates the Bcl-2 is overexpressed in the eutopic endometrium of adenomyosis.

3.2. Functional analysis of the siRNA-treated endometrial stromal cells of adenomyosis

The expression of Bcl-2 protein in the eutopic endometrial stromal cells of adenomyosis was higher than that of the control group. Fig. 2a. The Bcl-2 mRNA was examined by means of RT-qPCR. The expression of Bcl-2 mRNA in the adenomyosis group was (1.29 ± 0.06), which was higher against the control group ($P < 0.05$). Fig. 2b.

To further examine the effects of Bcl-2 on cell proliferation, migration and apoptotic rate, siRNA was transiently transfected into endometrial stromal cells of adenomyosis for 48 h. RT-qPCR and Western blot showed that the mRNA and protein expression of Bcl-2-homo-528 group (recorded as Bcl-2-siRNA group) was the most inhibited obviously. Therefore, this group of siRNAs was selected for subsequent experiments.

The expression of Bcl-2 mRNA in the Bcl-2-siRNA group and the negative control siNC group were 0.43 ± 0.12 and 0.93 ± 0.22 relative to the blank control group. Western blot analysis showed the expression of Bcl-2 protein in Bcl-2-siRNA group, negative control siNC group and blank control group were 0.58 ± 0.10, 0.99 ± 0.16 and 1.05 ± 0.23, respectively. The expression of Bcl-2 mRNA and protein in Bcl-2-siRNA group were significantly lower than those in the negative control group ($P < 0.05$). The negative control siNC group were not different from the blank control group ($P = 0.17$ in mRNA, $P = 0.26$ in protein). The above indicated that the expression of Bcl-2 in stromal cells was significantly inhibited in Bcl-2-siRNA group. Fig. 3a.

After Bcl-2 gene was interfered for 48 h, the cell viability was detected by Cell counting kit-8. The blank control group was as a reference. The results showed that the survival rate of Bcl-2-siRNA group was 0.74 ± 0.09, and the negative control group was 0.92 ± 0.13.

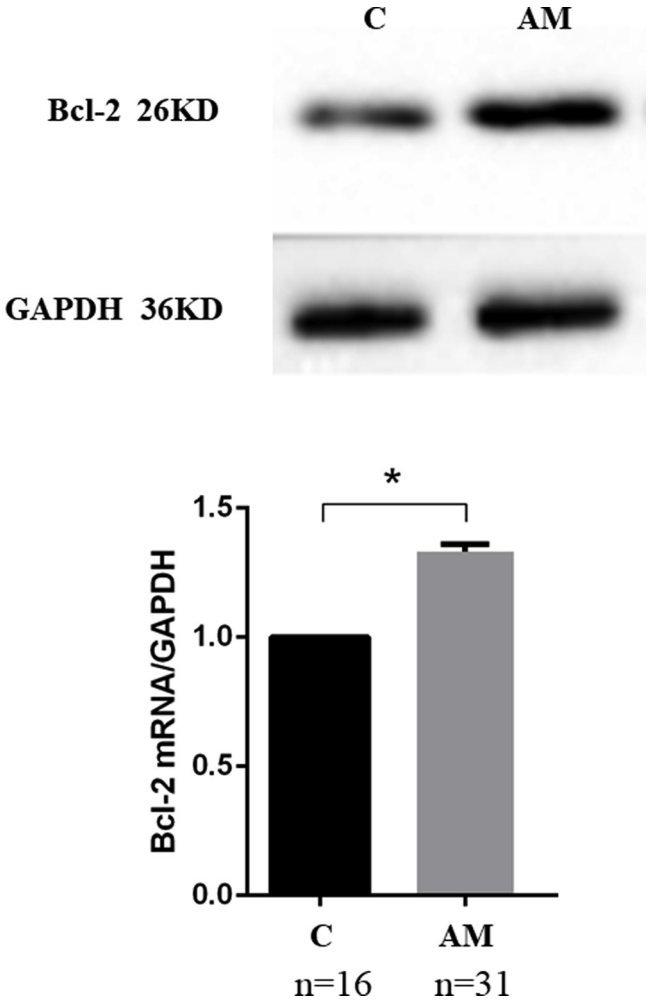

Fig. 1. The Bcl-2 in the eutopic endometrium of adenomyosis was overexpressed. Western blot assay(Fig.1a) and RT-PCR(Fig.1b) for the expression of Bcl-2. The GAPDH served as reference. The molecular weight markers are shown on the left. C: endometrium of control group. AM: eutopic endometrium of adenomyosis. * P < 0.05.

The difference was statistically significant ($P < 0.05$). The cell survival rate of Bcl-2-siRNA group was significantly lower than that of the negative control group. Fig. 3b.

The cell scratch repair experiment was used to detect the relative migration distance after silencing the Bcl-2 gene of endometrial stromal cells. At 24 h, the Bcl-2-siRNA group (0.12 ± 0.01) was shorter than the negative control group (0.14 ± 0.06) and the blank control group (0.17 ± 0.01). The difference was statistically significant ($P < 0.05$). At 48h, the Bcl-2-siRNA group was also shorter than the negative control group and the blank control group (0.24 ± 0.01 vs. 0.30 ± 0.04 vs. 0.31 ± 0.03, $P < 0.05$). But there was no significant difference between the latter two groups at 24h and 48h($P = 0.33$, $P = 0.82$). Fig. 3c.

The apoptotic rates were measured by flow cytometry. The sum of

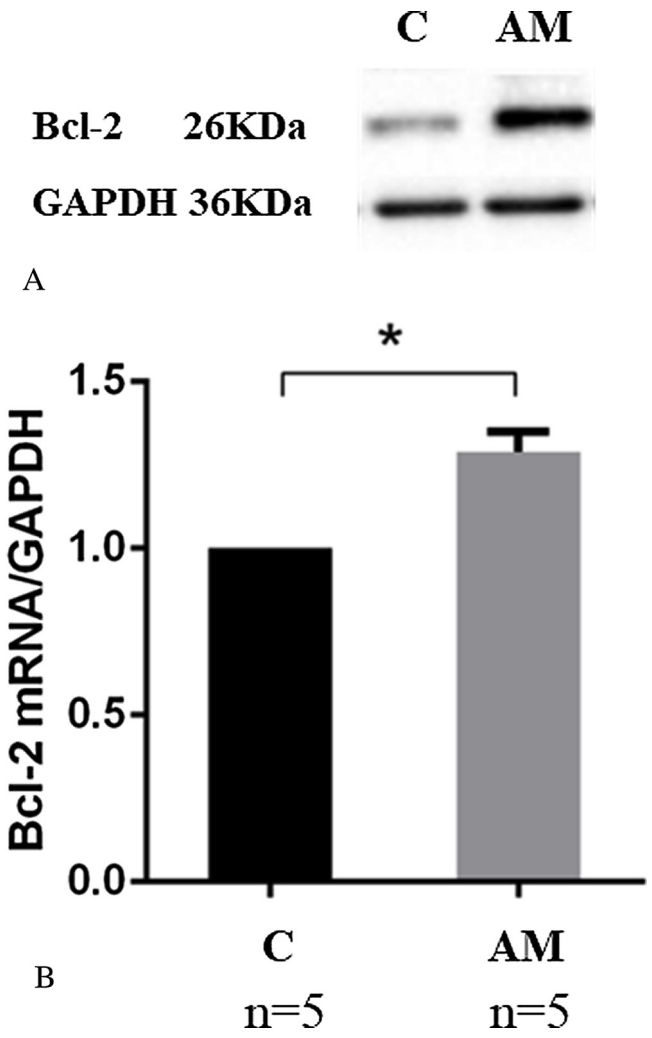

Fig. 2. The expression of Bcl-2 protein and mRNA in the eutopic endometrial stromal cells of adenomyosis were higher than that of the control group. Western blot assay(Fig.2a) and RT-PCR(Fig.2b) for the expression of Bcl-2. The GAPDH served as reference. The molecular weight markers are shown on the left. C: endometrial stromal cells of control group; AM: eutopic endometrial stromal cells of adenomyosis. n = 5, * $P < 0.05$.

Q2 and Q4 represented the apoptotic rate. The apoptotic rates of Bcl-2-siRNA group, negative control siNC group and blank control group were (26.49 ± 5.42)%, (18.10 ± 4.18)%, (17.73 ± 1.89)%. The apoptotic rate of Bcl-2-siRNA group was higher than the negative control siNC group and the blank control group ($P < 0.05$). But there was no significant difference between the latter two groups ($P = 0.42$). Fig. 3d.

4. Discussion

Adenomyosis is a common gynecological disease mostly affecting women of reproductive age. Adenomyosis is closely related to sterility, and the pregnancy rate, implantation rate, and live birth rate are decreased after IVF in patients with adenomyosis [11]. Infertility and dysmenorrhea are difficult points in the treatment. The therapies contain drug therapy (like oral contraceptives and GnRH-a), surgical treatment (e.g. hysterectomy and lesionectomy), interventional therapy (uterine artery embolization), and High Intensity Focused Ultrasound (HIFU). There is no radically and efficiently conservative cure at present.

Bcl-2 is one of the anti-apoptotic factors, first discovered in follicular B-cell lymphoma [12]. The Bcl-2 gene and the expressed protein formed complex biochemical regulators that determined the survival or death of normal cells and cancer cells [13]. Bcl-2 protein inhibited apoptosis by blocking mitochondrial cytochrome C release [14] and interacting with Bax and Bcl-x protein [15]. From the perspective of apoptosis, the cause of tumor was the cells antagonize apoptosis. The apoptosis process was inhibited and abnormal cells accumulated to generate tumors. Reseaches found that the expression of Bcl-2 increased in various malignant tumors. In hepatocellular carcinoma, Bcl-2 was significantly higher than that in benign liver tissue [16]. Increased Bcl-2 in estrogen receptor-positive breast cancer cells inhibited the activity of caspase 3 and cell initiative death, so that the number of surviving cancer cells continued to increase [17,18]. In experiments by Ma et al. [19], targeted silencing of Bcl-2 gene resulted in apoptosis of gastric cancer MFC cells, which significantly inhibited the proliferation of MFC cells. While, overexpression of Bcl-2 proteins accelerated migration of colorectal cancer cells [20].

Here, we researched the expression of Bcl-2 in adenomyosis and its effect on proliferation, migration and apoptosis of endometrial stromal cells. The results of our study were consistent with the previous [21,22]. Our experiments showed that the expression of Bcl-2 in the eutopic endometrium and stromal cells of the adenomyosis was significantly higher than that of the control group. The cell viability and migration ability was stronger and apoptotic rate was lower. Overexpression of Bcl-2 in both proliferative and secretory phases [19] enhanced the anti-apoptosis of endometrial cells, and the sensitivity of cells to apoptosis decreased, allowing cells to escape apoptosis and resulting in implantation in the ectopic sites. Ectopic cells still had strong growth ability to form the ectopic focus. After Bcl-2 was inhibited by siRNA, the expression of Bcl-2 reduced, which can promote the release of cytochrome C indirectly. Once cytochrome C is released, it combines with caspase 9, cell apoptosis protease activator 1(Apaf-1) to form apoptotic enzymes, which activates caspase 3 and initiates apoptosis [23,24]. So, down-regulation of Bcl-2 promoted apoptosis of the endomrtrial stromal cells. The altered proliferation, migration and apoptosis of eutopic endometrium stromal cells possibly elucidate some aspects of the pathophysiology of adenomyosis. In addition, Bcl-2 modulates other cellular functions such as autophagy, mitochondrial fusion, cell differentiation, senescence, cytoskeletal reorganization and cell migration [25]. Bcl-2 interacted with Bcl-xL protein through inositol 1,4,5-triphosphate receptor(IP3R), which regulated the release of Ca^{2+} from endoplasmic reticulum to the mitochondria to promote cell migration [26]. Overexpressed Bcl-2 promoted angiogenesis by interacting with VEGF [27]. And Bcl-2 modulated integrin-dependent cell adhesion [28] to detach cells from the original location. Bcl-2 activated matrix metalloproteinases (MMPs), which degraded extracellular matrix to promote cell migration [29]. The above indicated that Bcl-2 played a key regulatory role in the development of adenomyosis. In clinic, Bcl-2 expression can be inhibited, such as selective Bcl-2 inhibitors. We envisage the possibility of applying them to release the inhibition of Bcl-2 to apoptosis and restore the normal apoptosis pathway of endometrial cells, so as to control the adenomyosis.

In summary, we found Bcl-2 was overexpressed in adenomyosis. After reduced the expression of Bcl-2 in endometrial stromal cells, cell viability and migration weakened, and the apoptotic rate increased. These results indicate that Bcl-2 play an important role in the occurrence and development of adenomyosis and may be a potential novel target for adenomyosis treatment.

Conflict of interest

None.

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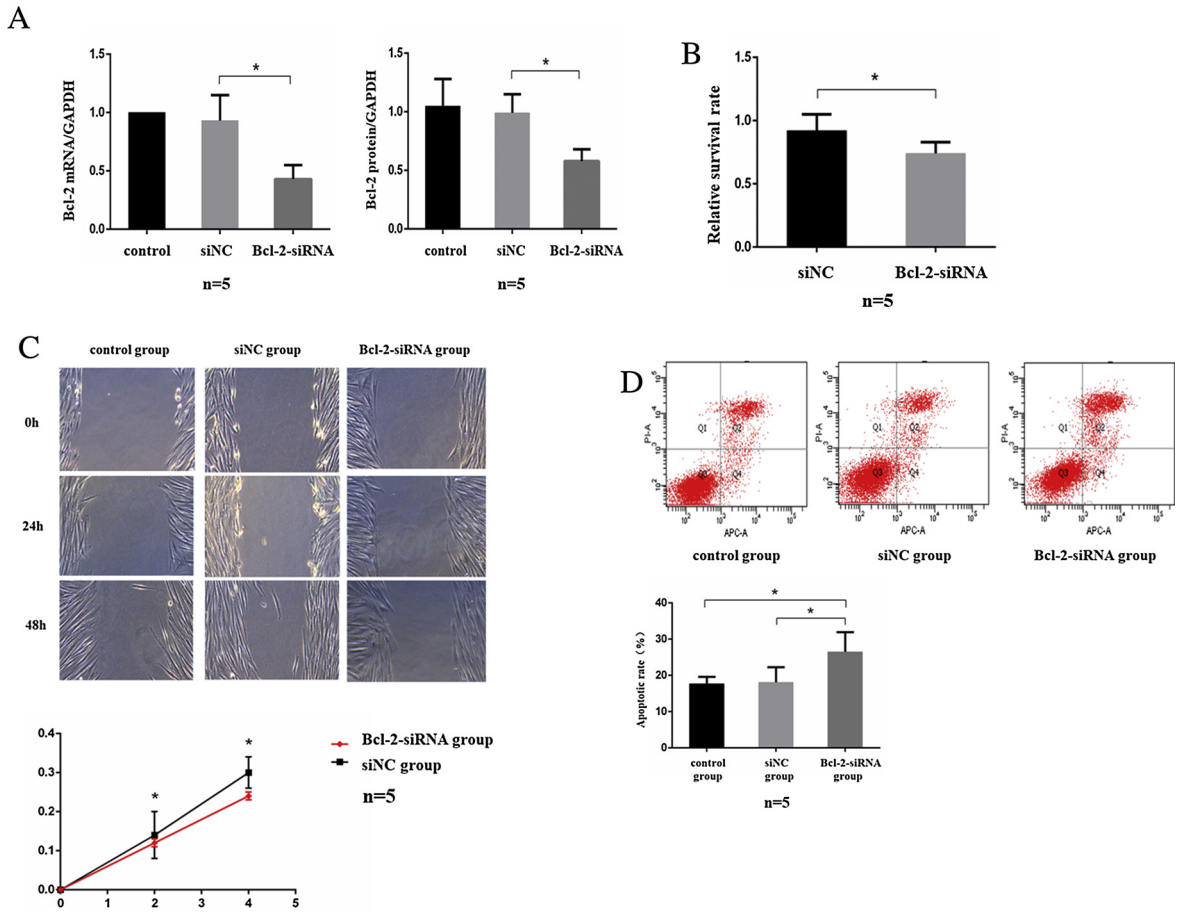

Fig. 3. Effects of Bcl-2 on cell proliferation, migration and apoptotic rate after siRNA was transiently transfected into endometrial stromal cells of adenomyosis for 48 hours. The expression of Bcl-2 was significantly inhibited after siRNA transfected into the cells for 48h(Fig. 3a). Relative survival rate of cells after Bcl-2 gene interference for 48h(Fig. 3b). The relative migration distance of cells transfected by Bcl-2-siRNA for 48h(Fig. 3c). Apoptosis of cells transfected by Bcl-2-siRNA for 48h(Fig. 3d). n = 5, * $P < 0.05$.

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