MEDICAL SCIENCE MONITOR

The Effect of Mitomycin C on Reducing Endometrial Fibrosis for Intrauterine Adhesion

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Statistical Analysis C
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Background: Intrauterine adhesion (IUA) is a common reproductive system disease in women, characterized by endometrial stromal cell proliferation, increasing fibroblasts and increasing extracellular matrix secretion. The purpose of this study was to investigate the effect of mitomycin C on reducing endometrial fibrosis for IUA.

Material/Methods: Firstly, a rat IUA model was constructed by intrauterine mechanical injury. The endometrial stromal cells and fibroblasts were isolated and treated with mitomycin C. After that, Cell Counting Kit-8 (CCK-8) assay was used to investigate the endometrial stromal cell viability. Furthermore, cell cycle and apoptosis assays of endometrial stromal cells and fibroblasts were performed, respectively. Finally, the cell viability of human endometrial cells or human uterus adhesion fibroblasts treated with mitomycin C was determined using CCK-8 assay with or without estradiol.

Results: Endometrial stromal cells were isolated from a rat IUA model. Cell cycle assay results showed that mitomycin C inhibited cell viability and promoted G1 cell cycle arrest and apoptosis in rat IUA endometrial stromal cells. Fibroblasts were also isolated from the rat IUA model. We found that mitomycin C inhibited the synthesis and secretion of collagen type I by western blotting analysis. Furthermore, mitomycin C promoted G1 cell cycle arrest and apoptosis in IUA rat uterine fibroblasts. We found that estradiol decreased the inhibitory effects of cell viability of human endometrial cells and human uterus adhesion fibroblasts by mitomycin C.

Conclusions: Our findings revealed that mitomycin C could reduce endometrial fibrosis for intrauterine adhesion.

MeSH Keywords: Cell Cycle • Endometrium • Fibroblasts • Mitomycin

Abbreviations: IUA – intrauterine adhesion; CCK-8 – Cell Counting Kit-8

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Background

Intrauterine adhesion (IUA), also known as Asherman syndrome, is a common complication of endometrial trauma, producing adhesions within the uterine cavity [1]. IUA is mainly caused by damage to the basal layer of endometrium [2]. IUA is most common in trauma or infection, especially when the level of estradiol is low after pregnancy [3]. During the healing process, the opposing walls of the uterus adhere together, causing occlusion of the uterine cavity [4,5]. Patients with IUA may experience amenorrhea, dysmenorrhea, irregular menstruation, infertility or repeated abortion [6]. Currently, IUA patients usually undergoes cervical adhesion resection through hysteroscopy and postoperative hormone replacement therapy, but the recurrence rate is as high as 62.5% [7]. IUA is a main clinical problem in female infertility, however, there is no ideal treatment.

Mitomycin C has been shown to be an antimetabolite that inhibits fibroblast proliferation after surgery [8,9]. It inhibits fibroblast collagen synthesis by inhibiting DNA-dependent RNA synthesis and reducing fiber adhesion [10]. The clinical application of mitomycin C has proven to be effective and safe in preventing postoperative adhesions [11–13]. Mitomycin C has been widely used to reduce scar adhesion formed by ophthalmology and otolaryngology after surgery [14,15]. However, the effects of mitomycin C on IUA is unknown. Thus, in our study, we hypothesized mitomycin C could reduce endometrial fibrosis for IUA.

Material and Methods

Rat IUA model

We used 24 female Sprague-Dawley (SD) rats, aged 9 to 10 weeks old and weighing approximately 220 to 240 g that were purchased from Shanghai Slack Laboratory Animal Co., Ltd. (SCXK 2012-0002). Rats were anesthetized by intraperitoneal injection of 1 mL/kg 3% pentobarbital sodium. Then, the right uterus was exposed through the midline incision of the abdomen, and a longitudinal incision of 3 mm was cut in the lower part of the uterus. The surgical blade No. 21 was used to scrape the intima of the entire uterus until the walls of the uterus were rough, and then the uterus was thoroughly washed with 300 mL of physiological saline. Finally, the uterus incision was sutured, and the abdomen was closed, followed by incision disinfection. The SD rats were sacrificed on the 12th week after surgery and the right uterus was removed. Our research was approved by the Ethics Committee of Wenzhou People's Hospital (No. 2018-17).

Isolation of endometrial stromal cells

Firstly, the removed uterus specimens were washed with phosphate-buffered saline (PBS) and antibiotics. After the epithelial and adipose tissues were trimmed, the remaining connective tissues were cut into small pieces of 1×1×1 mm. Next, the connective tissues were digested with type I collagenase at 37°C for 30 minutes, then filtered through a 100 um mesh and centrifuged at 800 g for 5 minutes. After adding an appropriate amount of complete medium, the isolated cells were cultured in an incubator, and the solution was changed once every 2 to 3 days. After the cells were covered with the bottom of the bottle, they were digested with 0.25% trypsin (Gibco) for 2 minutes. Once the cytoplasmic retraction and enhanced refraction were observed under phase contrast microscopy, 2 mL of medium with serum was immediately added to terminate digestion. Finally, the cell suspension was prepared and counted, followed by inoculation culture.

Cell culture

The rat or human endometrial stromal cells and fibroblasts were seeded in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS) and cultured in a saturated humidity incubator (37°C, 5% CO_2).

Immunofluorescence

Firstly, the prepared cell slides were washed with PBS. The slides were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After washing with PBS 3 times for 5 minutes, the slide was incubated with 0.1% Triton X-100 solution (sc281181; Santa Cruz, CA, USA) for 10 minutes at room temperature. After that, 1% BSA solution was added to each well and blocked at 37°C for 90 minutes. The slides were incubated with primary antibodies including anti-vimentin (1: 100; 10366-1-AP; Proteintech, USA) and anti-cytokeratin 19 (1: 100; 10712-1-AP; Proteintech, USA) overnight at 4°C in a refrigerator. Next, the cells were incubated with goat anti-rabbit IgG (H+L) 488 fluorescent secondary antibody (1: 800; A11034; Life Technologies, USA) at 37°C for 1 hour in the dark. The nucleus was stained with DAPI (D9452; SIGMA, Canada) for 5 minutes at room temperature. Finally, the slides were observed under a fluorescence microscope.

Cell Counting Kit-8 (CCK-8) assay

The rat endometrial stromal cells were seeded in 96-well plates (1×10³/well) and placed in an incubator for 24 hours. After that, the cells were cultured with different concentrations (0, 1.25, 2.5, 5, 10, 20, and 40 ug/mL) of mitomycin C. Then 100 µL of different concentrations of mitomycin C were added to each well. After 24 hours and 48 hours, cell viability was detected
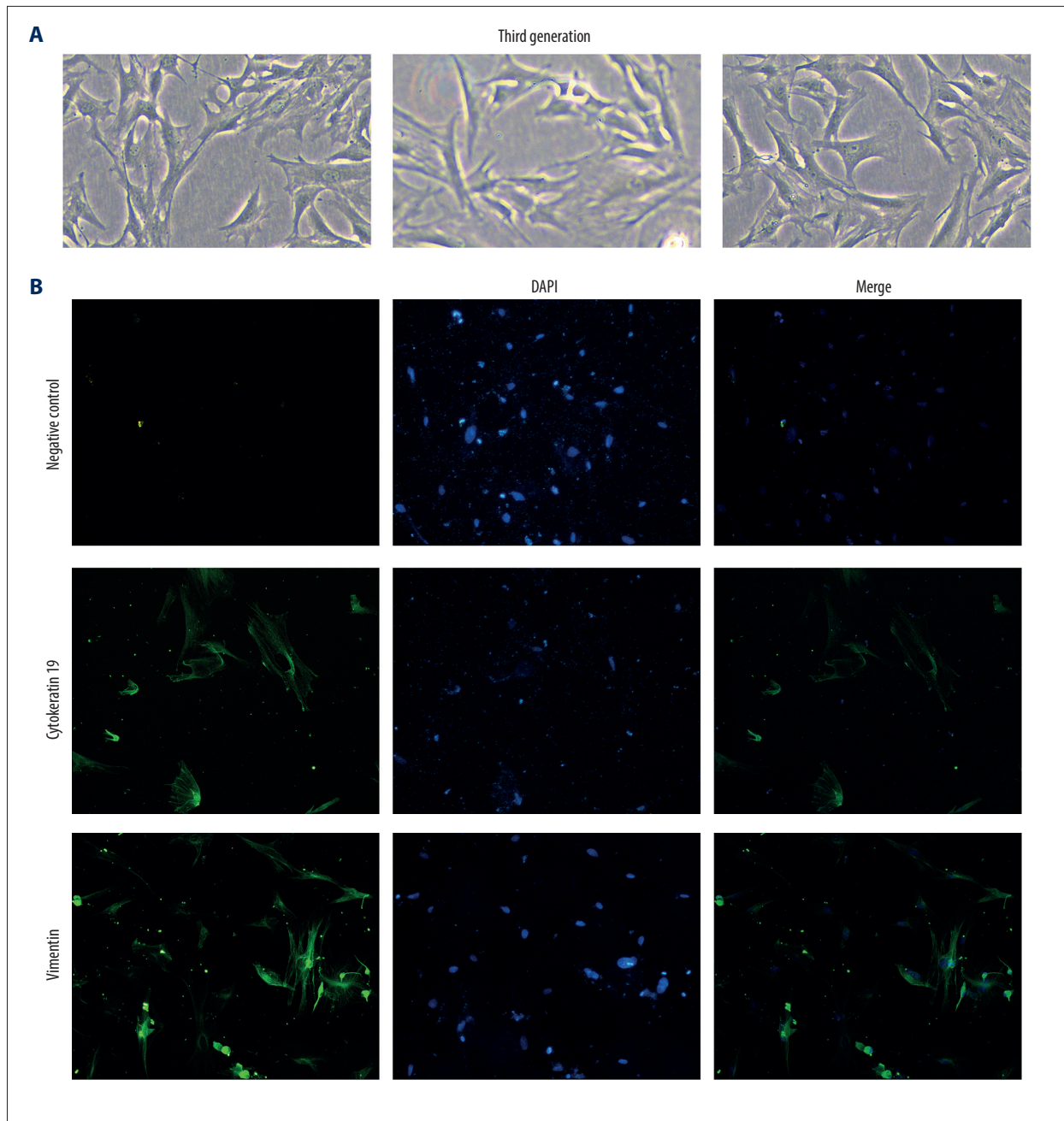
Figure 1. Identification of endometrial stromal cells in a rat IUA model. (**A**) Morphology of endometrial stromal cells in a rat IUA model. White bar is 10 μm. (**B**) Immunofluorescence results showing the expression of vimentin and cytokeratin 19 in endometrial stromal cells of rat IUA model. Representative images are shown.

by CCK-8 assay. 10 μL of CCK-8 detection solution was added to each well at 37°C for 1 hour in the dark. The optical density (OD) value of each well was read at 450 nm. Cell viability (%)=OD value of mitomycin C group/OD value of control group×100%.

Flow cytometry

The third generation of endometrial stromal cells or the eighth of uterine fibroblast were selected for apoptosis and cell cycle assays. The cells were seeded in 96-well plates (1×10^3/well) for 24 hours, and then cultured with 4 mL different concentrations (0, 5, 10 ug/mL) of mitomycin C for 48 hours. After adding 300 uL of 1×Binding Buffer to each tube of cells, the apoptotic
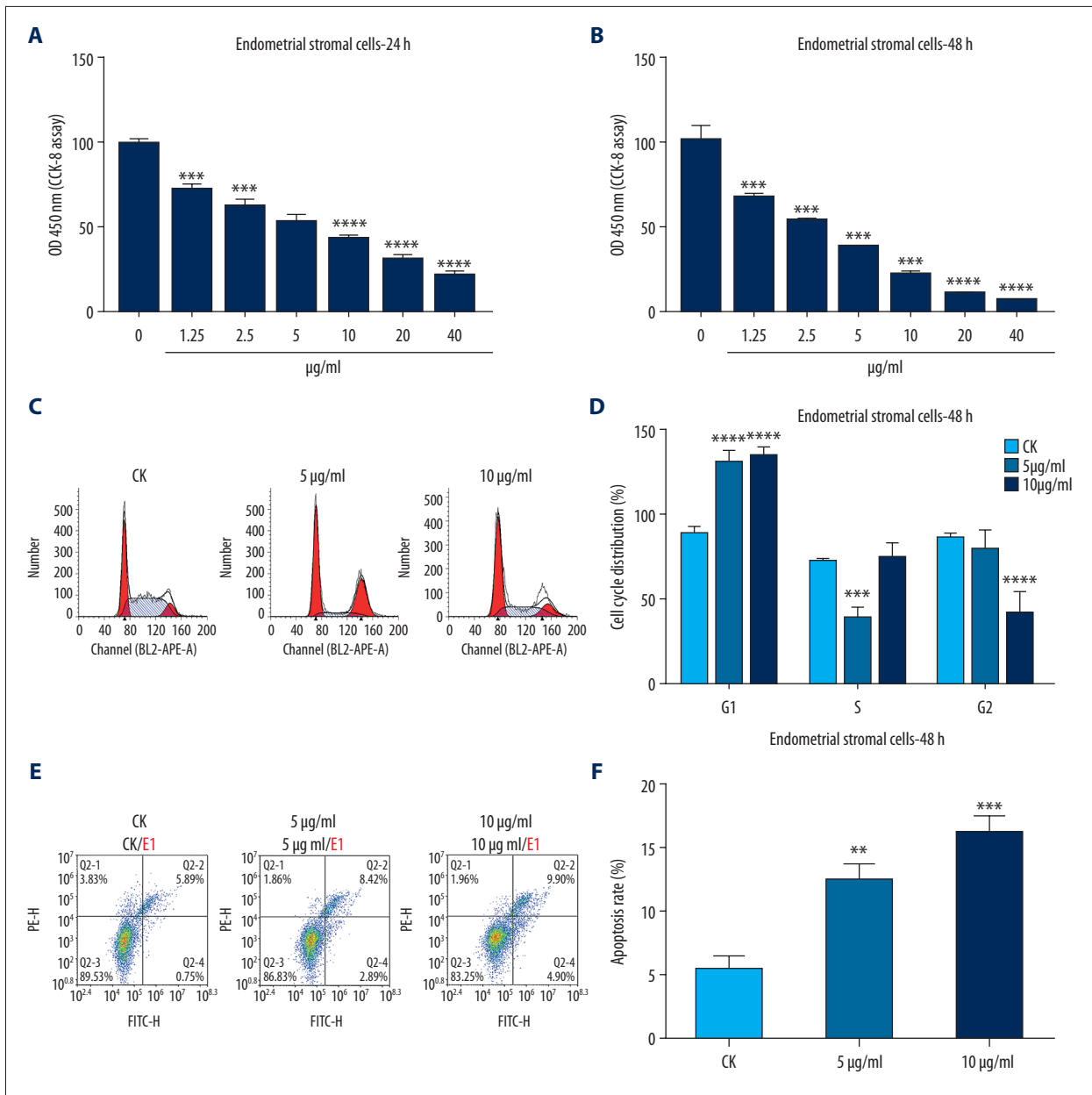
Figure 2. Mitomycin C inhibits cell viability and promotes G1 cell cycle arrest and apoptosis in rat intrauterine adhesion (IUA) endometrial stromal cells. (**A, B**) Cell Counting Kit-8 (CCK-8) assay showing the effects of different concentrations of mitomycin C on cell viability of rat IUA endometrial stromal cell after treatment for 24 hours and 48 hours. (**C, D**) Cell cycle assay showing the cell cycle distribution of rat IUA endometrial stromal cell after mitomycin C treatment (CK; 5 µg/mL; 10 µg/mL) for 24 hours and 48 hours by flow cytometry. (**E, F**) Apoptosis assay showing the cell apoptosis of rat IUA endometrial stromal cell treated by (CK; 5 µg/mL; 10 µg/mL) mitomycin C for 48 hours. ** $P<0.01$; *** $P<0.001$ and *** $P<0.0001$.

cells were marked with 5 uL of Annexin V-FITC and incubated for 15 minutes at room temperature in the dark. Then 5 uL of propidium iodide (PI) was then added and incubated for 5 min at room temperature in the dark. Finally, after adding 200 uL of 1×Binding Buffer, apoptotic cells were analyzed by NovoCyte flow cytometry (ACEA, China). For cell cycle, the cells were only stained with PI and analyzed on the flow cytometry.

Western blot analysis

The cells were cultured with 4 mL different concentrations (0, 5, and 10 ug/mL) of mitomycin C for 48 hours. After adding 300 uL of 1×Binding Buffer to each tube of cells, the cells were lysed radioimmunoprecipitation assay (RIPA) buffer (P0013B; Beyotime, China) supplemented with phenylmethylsulfonyl
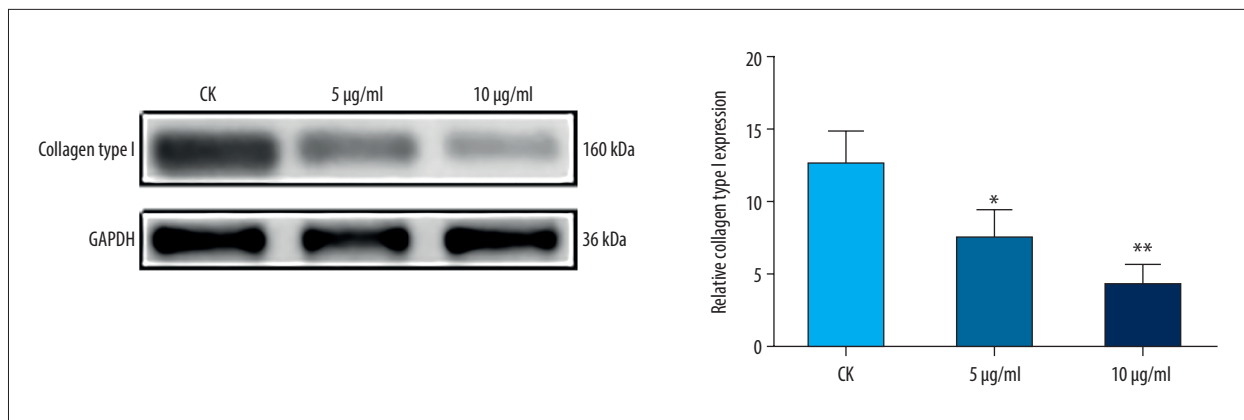
Figure 3. Western blotting analysis showing the expression of collagen type I in intrauterine adhesion (IUA) rat uterine fibroblasts treated with (CK; 5 µg/mL; 10 µg/mL) mitomycin C for 48 hours. GADPH was used as an internal control. * *P*<0.05 and ** *P*<0.01.

fluoride (PMSF) (ST506; Beyotime, China). After separating on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (P0015L; Beyotime, China), the samples were transferred onto polyvinylidene difluoride (PVDG) membrane. Then, the membrane was blocked by 5% skim milk powder at room temperature for 2 hours. The membrane was incubated with collagen type I primary antibody (10712-1-AP; Proteintech, USA) at 4°C overnight, and then incubated with secondary antibody at room temperature for 2 hours. GAPDH was used as an internal control. Finally, the protein blots were visualized using ECL (Beyotime, China).

Statistical analysis

All statistical analysis was performed using Graphpad Prism 7.0 (San Diego, CA, USA). Data were expressed as the mean±standard deviation (SD). The differences between 2 groups were analyzed using Student's *t*-test. *P*-value <0.05 was considered statistically significant.

Results

### Endometrial stromal cells in a rat IUA model

Firstly, a rat IUA model was constructed by endometrial injury and endometrial stromal cells were isolated. We observed the morphology of isolated endometrial stromal cells under a microscope and the growth characteristics of endometrial stromal cells are shown in Figure 1A. To further identify endometrial stromal cells, immunofluorescence staining of vimentin and cytokeratin 19 was performed. Immunofluorescence results showed that the expression of cytokeratin 19 was decreased and the expression of vimentin was elevated (Figure 1B). The aforementioned results showed that the isolated endometrial cells are mainly endometrial stromal cells.

Mitomycin C inhibited cell viability and promoted G1 cell cycle arrest and apoptosis in rat IUA endometrial stromal cells

To investigate the effects of mitomycin C on cell viability of rat IUA endometrial stromal cell, CCK-8 assay was performed. The results showed that mitomycin C significantly inhibited cell viability of rat IUA endometrial stromal cell with a concentration-dependent manner after treatment with mitomycin C for 24 hours and 48 hours (Figure 2A, 2B). We also observed cell cycle of rat IUA endometrial stromal cells via flow cytometry. The rat IUA endometrial stromal cells were treated with different concentrations of mitomycin C (CK; 5 µg/mL; 10 µg/mL). We found that rat IUA endometrial stromal cells were arrested at G1 after treatment with 5 µg/mL or 10 µg/mL mitomycin C (Figure 2C, 2D). To examine the effects of mitomycin C on cell apoptosis of rat IUA endometrial stromal cells, apoptotic cells were marked with Annexin V-FITC and analyzed by flow cytometry. We found that mitomycin C promoted cell apoptosis with a concentration-dependent manner after mitomycin C treatment for 48 hours (Figure 2E, 2F). Above results showed that mitomycin C inhibits cell viability and promotes G1 cell cycle arrest and apoptosis in rat IUA endometrial stromal cells.

Mitomycin C inhibited IUA rat uterine fibroblasts to secrete collagen type I

To investigate the ability of IUA rat uterine fibroblasts to secrete type I collagen, fibroblasts were isolated and cultured for 6 to 9 generations. Western blotting analysis was performed, and the results showed that, compared to controls, the expression of collagen type I was significantly inhibited in IUA rat uterine fibroblasts treated with mitomycin C for 48 hours, with a concentration-dependent manner (Figure 3). These results indicated that mitomycin C inhibits IUA rat uterine fibroblast to secrete collagen type I.
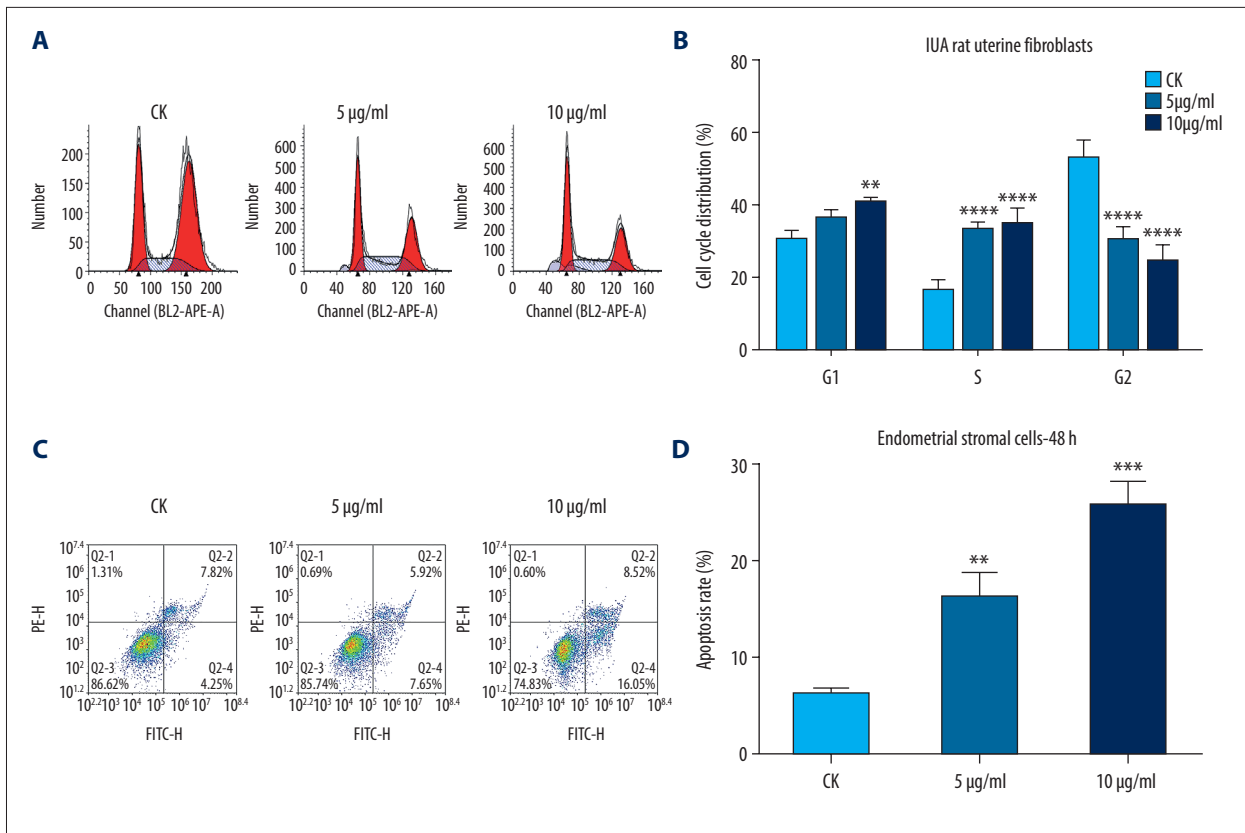
Figure 4. Mitomycin C promotes G1 cell cycle arrest and apoptosis in intrauterine adhesion (IUA) rat uterine fibroblasts. (**A, B**) Cell cycle assay showing the cell cycle distribution of IUA rat uterine fibroblasts after mitomycin C treatment (CK; 5 µg/mL; 10 µg/mL) for 48 hours by flow cytometry. (**C, D**) Apoptosis assay showing the cell apoptosis of IUA rat uterine fibroblasts treated by (CK; 5 µg/mL; 10 µg/mL) mitomycin C for 48 hours. ** $P<0.01$; *** $P<0.001$ and *** $P<0.0001$.

Mitomycin C promoted G1 cell cycle arrest and apoptosis in IUA rat uterine fibroblasts

Cell cycle and apoptosis assays were performed using flow cytometry. The cell cycle assay results showed that IUA rat uterine fibroblasts were arrested at G1 phage after treatment with mitomycin C for 48 hours with a concentration-dependent manner (Figure 4A, 4B). Furthermore, the apoptosis assay results suggested that mitomycin C promoted IUA rat uterine fibroblast apoptosis, with a concentration-dependent manner (Figure 4C, 4D). These results revealed that mitomycin C promotes G1 cell cycle arrest and apoptosis in IUA rat uterine fibroblasts.
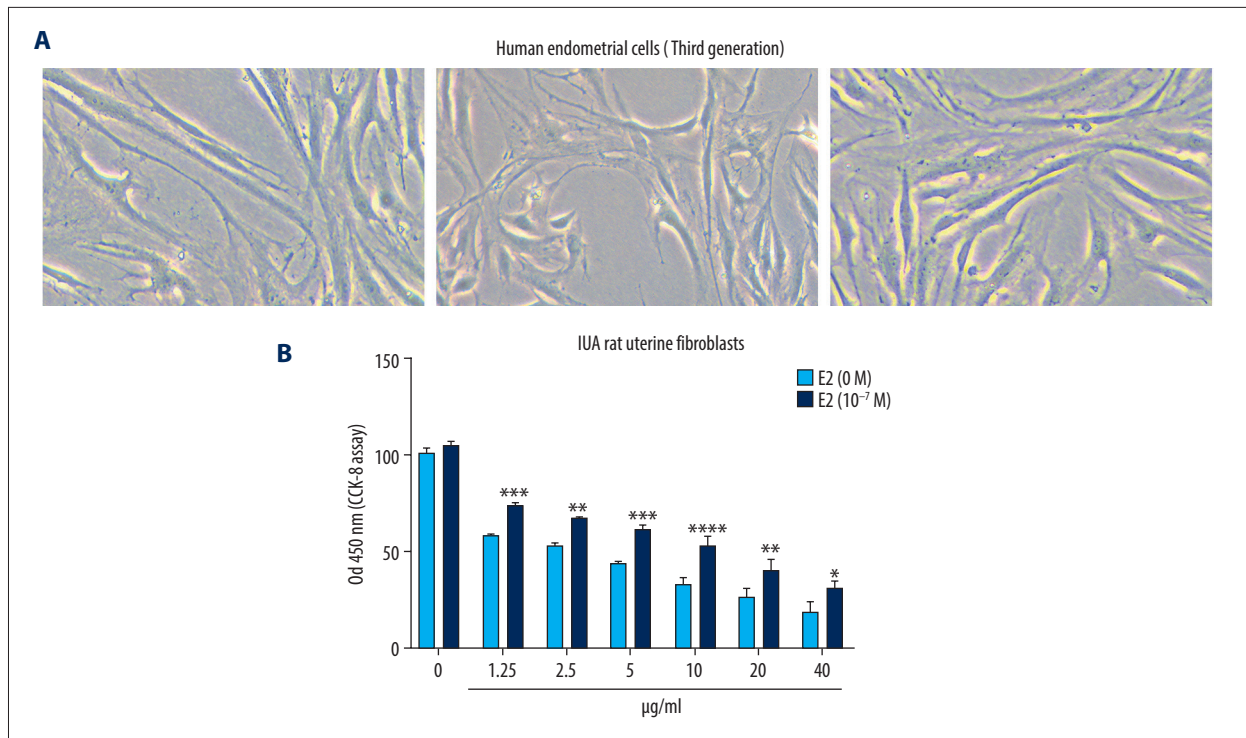
Estradiol decreased the inhibitory effects of cell viability of human endometrial cells by mitomycin C

We further investigated the effects of mitomycin C on cell viability of human endometrial cells by CCK-8 assay. Firstly, we observed the morphology of human endometrial cells under a microscope (Figure 5A). The CCK-8 results showed that mitomycin C inhibited cell viability of human endometrial cells,

with a concentration-dependent manner (Figure 5B). After adding estradiol, we found that the inhibitory effects of human endometrial cell viability were decreased. The aforementioned results indicated that estradiol decreases the inhibitory effects of cell viability of human endometrial cells by mitomycin C.

Estradiol decreased the inhibitory effects of cell viability of human uterus adhesion fibroblasts by mitomycin C

We further observed the effects of mitomycin C on cell viability of human uterus adhesion fibroblasts using CCK-8 assay. The morphology of human uterus adhesion fibroblasts is shown in Figure 6A. The CCK-8 results showed that mitomycin C suppressed human uterus adhesion fibroblast viability, with a concentration-dependent manner (Figure 6B). Furthermore, we found that estradiol decreased the inhibitory effects of human uterus adhesion fibroblast viability. These results indicated that estradiol decreases the inhibitory effects of cell viability of human endometrial cells by mitomycin C.

A Human endometrial cells (Third generation)

B IUA rat uterine fibroblasts

Figure 5. Estradiol decreases the inhibitory effects of cell viability of human endometrial cells by mitomycin C. (**A**) Morphology of human endometrial cells. White bar is 10 μm. (**B**) Cell Counting Kit-8 (CCK-8) showing the effects of mitomycin C on cell viability of human endometrial cells with or without estradiol (E2). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ and *** $P<0.0001$.

A Human fibroplast in uterine adhesion (Ninth generation)

B
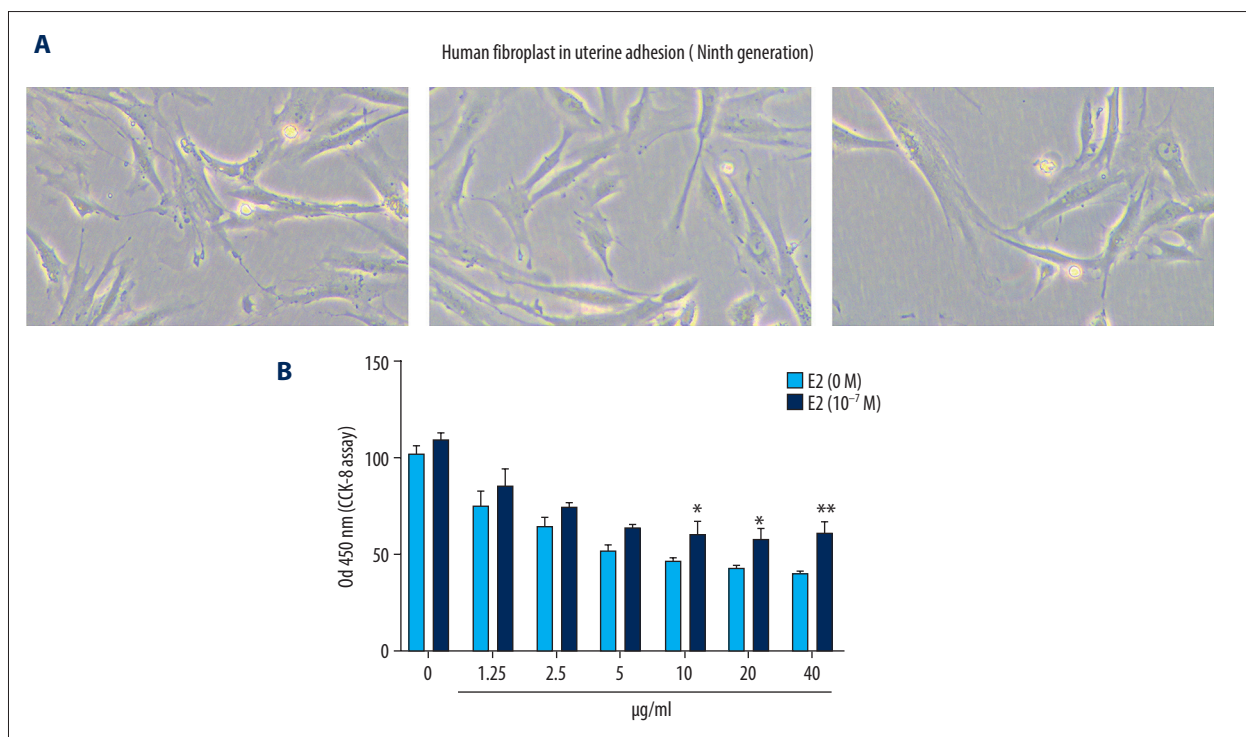
Figure 6. Estradiol decreases the inhibitory effects of cell viability of human uterus adhesion fibroblasts by mitomycin C. (**A**) Morphology of human uterus adhesion fibroblasts. White bar is 10 μm. (**B**) Cell Counting Kit-8 (CCK-8) showing the effects of mitomycin C on cell viability of human uterus adhesion fibroblasts with or without estradiol (E2). * $P<0.05$ and ** $P<0.01$.

Discussion

IUA is a major health problem that causes female infertility, irregular menstruation and repeated abortions [16]. Unfortunately, there is currently no effective strategy for treating IUA [17,18]. Mitomycin C has been shown to inhibit fibroblast proliferation, promote fibroblast apoptosis, and ultimately prevent adhesion formation [19]. However, the effect of mitomycin C on IUA remains unclear. Therefore, in our study, we successfully constructed a rat IUA model and our findings revealed the inhibitory effect of mitomycin C on endometrial fibrosis for IUA.

The human endometrium is a highly regenerative tissue. The endometrium is composed of epithelial cells, stromal cells, vascular smooth cells, and vascular endothelial cells [20]. From a functional point of view, the endometrium consists of an outer functional layer and an inner base layer. The matrix is the main part of the inner base layer. The regeneration of the endometrial surface epithelium is due to stromal cell differentiation. However, IUA can cause damage to the endometrial basal layer [4]. When the uterine cavity is anatomically restored, endometrial fibrosis will determine reproductive outcomes [21]. Increased evidence suggests that endometrial fibrosis is associated with the development of IUA, but there has been no satisfactory progress in effective treatment [22]. Therefore, more studies are needed to elucidate the mechanisms of endometrial fibrosis and to develop new prevention and treatment strategies. In this study, we successfully constructed a rat IUA model through endometrial injury. Although it has been recognized that IUA development in human is more complex than in rat models, and the pathophysiology of rat models may be a little different from human, the rat model based on endometrial injury has been considered as a successful NEC model. Furthermore, endometrial stromal cells were isolated from rat IUA model. Our immunofluorescence results showed that the expression of cytokeratin 19 was decreased and the expression of vimentin was elevated, suggesting that the isolated endometrial cells were mainly endometrial stromal cells. We found that mitomycin C inhibited endometrial stromal cell viability with concentration dependent manners. Furthermore, endometrial stromal cells were arrested at G1 cell cycle. We also observed that mitomycin C promoted the apoptosis of endometrial stromal cells. IUA is characterized by the destruction of the basal layer of the endometrium [23]. Histologically, endometrial fibrosis contributes to IUA, which results in impaired endometrial function, uterine cavity deformation and stenosis. In the IUA, the endometrial stroma is primarily replaced by large fibrous tissues [24]. In addition, the IUA destroys the boundary between the basal layer and the functional layer of the endometrium [25]. Our study showed that mitomycin C inhibited cell viability and promoted apoptosis of endometrial stromal cells from a rat IUA model.

Endometrial fibrosis is considered to be a key pathological event in the development of IUA [26,27]. Fibroblasts play a key role in the pathogenesis of fibrous scar formation. Fibrosis may limit the activity of the myometrium, reduce the perfusion of estrogen and progesterone, and eventually lead to atrophy [28]. In our study, we found that IUA rat fibroblasts were arrested at G1 after treatment with mitomycin C. Moreover, mitomycin C promoted IUA rat fibroblast apoptosis. Mitomycin C inhibited IUA rat uterine fibroblasts to secrete collagen type I, a fibrotic marker that provides mechanical stability for tissues and serves as a functional environment for cells [29,30]. Endometrial fibrosis is associated with the development of IUA. Therefore, prevention of endometrial fibrosis is the basis of IUA treatment. Our study suggested that mitomycin C could prevent endometrial fibrosis by inhibiting the proliferation of fibroblasts.

Treatment of IUA includes adhesions to dissolve and prevention of adhesion recurrence [31,32]. The main purpose of treatment is to restore the normal shape and volume of the uterine cavity [33]. High-dose estrogen therapy is often used to prevent recurrence of uterine adhesions and promote endometrial repair [34]. However, the use of estrogen alone is not effective and is often associated with adverse effects, which greatly limits its widespread use. We found that estradiol could decrease the inhibitory effects of cell viability of human endometrial cells or human uterus adhesion fibroblasts by mitomycin C.

Taken together, our study found that mitomycin C could reduce endometrial fibrosis for intrauterine adhesion. However, a limitation of this study should be pointed out. This was a study in an animal model, and the findings require further validation by both *in vitro* and *in vivo* studies and human toxicity testing.

Conclusions

In our study, we found that mitomycin C inhibited cell viability and promoted G1 cell cycle arrest and apoptosis in rat IUA endometrial stromal cells. Mitomycin C inhibited the synthesis and secretion of collagen type I. Furthermore, we found that mitomycin C promoted G1 cell cycle arrest and apoptosis in IUA rat uterine fibroblasts. Estradiol decreased the inhibitory effects of cell viability of human endometrial cells and human uterus adhesion fibroblasts by mitomycin C. Our findings revealed that mitomycin C could have an inhibitory effect on endometrial fibrosis for IUA.

Conflicts of interest

None.

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