

Original Article

ATRA alleviated endometrial fibrosis in a rabbit intrauterine adhesions model through downregulation of the TGF- β 1/smad4 signaling pathway

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Abstract: Background: Intrauterine adhesions (IUA) lead to menstrual and fertility disorders. The therapies to IUA are still difficult and the prognosis is poor up to now. All-trans retinoic acid (ATRA) has been proved to exert anti-fibrosis effect in some fibrosis diseases. In this study, the effects of ATRA on endometrial fibrosis in a rabbit IUA model were evaluated and its possible mechanism was investigated. Materials and methods: The IUA models were established by using mechanical and infectious injury in adult female New Zealand white rabbits. Twenty-four female rabbits were randomly divided into sham, model, and ATRA groups. Eight rabbits in each group were sacrificed on day 14 after operation. Hematoxylin and eosin (H&E) and Masson staining were used for pathological analysis. TGF- β 1 protein expression was observed in endometrium with immunohistochemistry. The expression of TGF- β 1, smad4, COL1A1 and COL1A2 mRNAs were detected by real-time RT-PCR. The expression of TGF- β 1, smad4, COL I proteins was analyzed by Western blot. Results: ATRA treatment decreased endometrial fibrosis and improved endometrial regeneration in a rabbit IUA model. Meanwhile, ATRA decreased the over-expression of TGF- β 1, smad4, COL1A1 and COL1A2 mRNAs and TGF- β 1, smad4, COL I proteins in IUA model. Conclusions: ATRA may ameliorate endometrial fibrosis through downregulation of the TGF- β 1/smad4 signaling pathway.

Keywords: All-trans retinoic acid, intrauterine adhesions, fibrosis, transforming growth factor

Introduction

Intrauterine adhesions (IUA) were first defined by Joseph Asherman in 1948 and characterized as a partial or complete obliteration of the uterine cavity and/or the cervical canal involving partial or complete replacement of the endometrial surfaces with fibrotic tissue [1]. This disease leads to menstrual and fertility disorders, such as hypomenorrhea or amenorrhea, infertility or recurrent pregnancy loss, in reproductive women [2]. The prevalence of IUA has increased with the widespread use of hysteroscopy [3]. IUA were considered to form following intrauterine trauma and infections, resulting in opposing walls of the uterus adhere together with fibrotic tissue. The developments of IUA were associated with the damage to the basal layer of endometrium and endometrial repair disorders [1]. Treatments of IUA involve

hysteroscopic surgery to separate adhesions, as well as preoperative administration with estrogen [4]. The goal is to re-establish a normal uterine cavity and promote endometrial regeneration [5, 6]. Previous studies have focused on treatments for improving endometrial repair and mechanically maintaining the uterine cavity [6-8]. However, these therapies for moderate-to-severe IUA remain difficult and the reproductive prognosis is poor [9]. Consequently it is necessary to search for novel and effective strategies of erasing uterine adhesion formation.

As is known to all, all-trans retinoic acid (ATRA) is a bioactive derivative of vitamin A that exhibits regulating cellular differentiation, proliferation and apoptosis both in vivo and in vitro [10-12]. In addition, recent studies have showed that ATRA exerts anti-fibrogenic effect in some

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Table 1. Primers sequences Used for RT-PCR

Gene		Sequences of primers	bp
TGF- β 1	Forward	5'-ACATCTACACAGTTCCCGGC-3'	192
	Reverse	5'-TCCAGGCTCCAGATGTAGGG-3'	
smad4	Forward	5'-CGCGGATCAACCGAGACATA-3'	118
	Reverse	5'-TACTGGCAGGCTGACTTGTG-3'	
COL1A1	Forward	5'-GCTACTACCGGGCAGATGAC-3'	178
	Reverse	5'-TCTCCGCTCTTCCAGTCAGA-3'	
COL1A2	Forward	5'-CCATCTCGTTTGCCTTCCT-3'	80
	Reverse	5'-GGGCCAACGTCCACATAGAA-3'	
β -actin	Forward	5'-CACCTCTCTCGACGAAACC-3'	152
	Reverse	5'-CTGTACCTTACCCTTCCA-3'	

fibrosis diseases [13-16]. It has been confirmed that transforming growth factor-beta 1 (TGF- β 1) is a hallmark in organ fibrosis which induces epithelial-mesenchymal transition (EMT) and contributes to the excess deposition of extracellular matrix (ECM) as collagens [17, 18]. Song et al found that ATRA could block TGF- β 1 signaling pathway to attenuate bleomycin-induced lung fibrosis in rats [15].

In this experimental study, we accessed the effect of ATRA on a rabbit IUA model to further understand its role in IUA. Moreover, we explored the potential mechanism underlying the effect of ATRA on IUA-associated endometrial fibrosis.

Materials and methods

Animals

All experimental protocols were approved by the Institutional Animal Ethics Committee. Adult female New Zealand white rabbits weighing 2500 g-3500 g were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, Guangdong, China). The rabbits were housed under standardized laboratory conditions in a temperature-controlled room and light conditions (12-hour light/dark cycle) with free access to food and water.

Animal model and treatment

Adult female New Zealand white rabbits were randomly allocated into three groups (n=8): the sham group; IUA group; ATRA group. The rabbit IUA model was established by using mechanical and infectious injury except the sham group

[19]. Rabbit abdomens were incised to expose the bilateral uteri alone and without any treatment to uteri in the sham group. ATRA (Sigma-Aldrich, USA) were injected intraperitoneally with 2.5 mg/Kg/d dissolved in DMSO (Sigma-Aldrich, USA) 1 day after uterus damage for 14 days; The sham group and IUA group received an equal volume of DMSO. On days 14, all rabbits were sacrificed and the uterine horns were collected.

Histological analysis

The uterine tissues were fixed in 10% buffered formaldehyde before embedded in paraffin. The paraffin sections were routinely stained with hematoxylin and eosin (H&E) and Masson stains respectively to determine the number of endometrial glands and collagen distribution. Histological evaluation was performed by an experienced pathologist under blinded conditions. In the experiment four high-power fields were selected from each stained slice. The number of glands per high-power field was counted. The degree of endometrial fibrosis was analyzed as the ratio between the area of endometrial stromal fibrosis and endometrial area per high-power field using Image-Pro plus system.

Immunohistochemical analysis

After blocking with normal goat serum for 30 min at room temperature, the sections were incubated with monoclonal mouse anti-TGF- β 1 antibody (AbD Serotec, USA) at 1:200 dilution overnight at 4°C. The sections were followed by application with peroxidase-conjugated goat anti-mouse antibody (ComWin Biotech, China) at 1:200 dilution at room temperature for 40 min. The antibody binding sites were visualized by incubation with DAB at room temperature. After immunostaining, sections were counterstained with hematoxylin and mounted in conventional medium. Images were taken and analyzed using Image-Pro plus system.

Quantitative real-time RT-PCR

TGF- β 1, smad4, COL1A1 and COL1A2 mRNAs in endometrial tissues were detected by real-time RT-PCR. Total RNA was extracted from endometrial tissues using Trizol reagent (TaKaRa, Japan). A total of 1 μ g RNA was reversely transcribed into cDNA. The cDNA PCR amplica-

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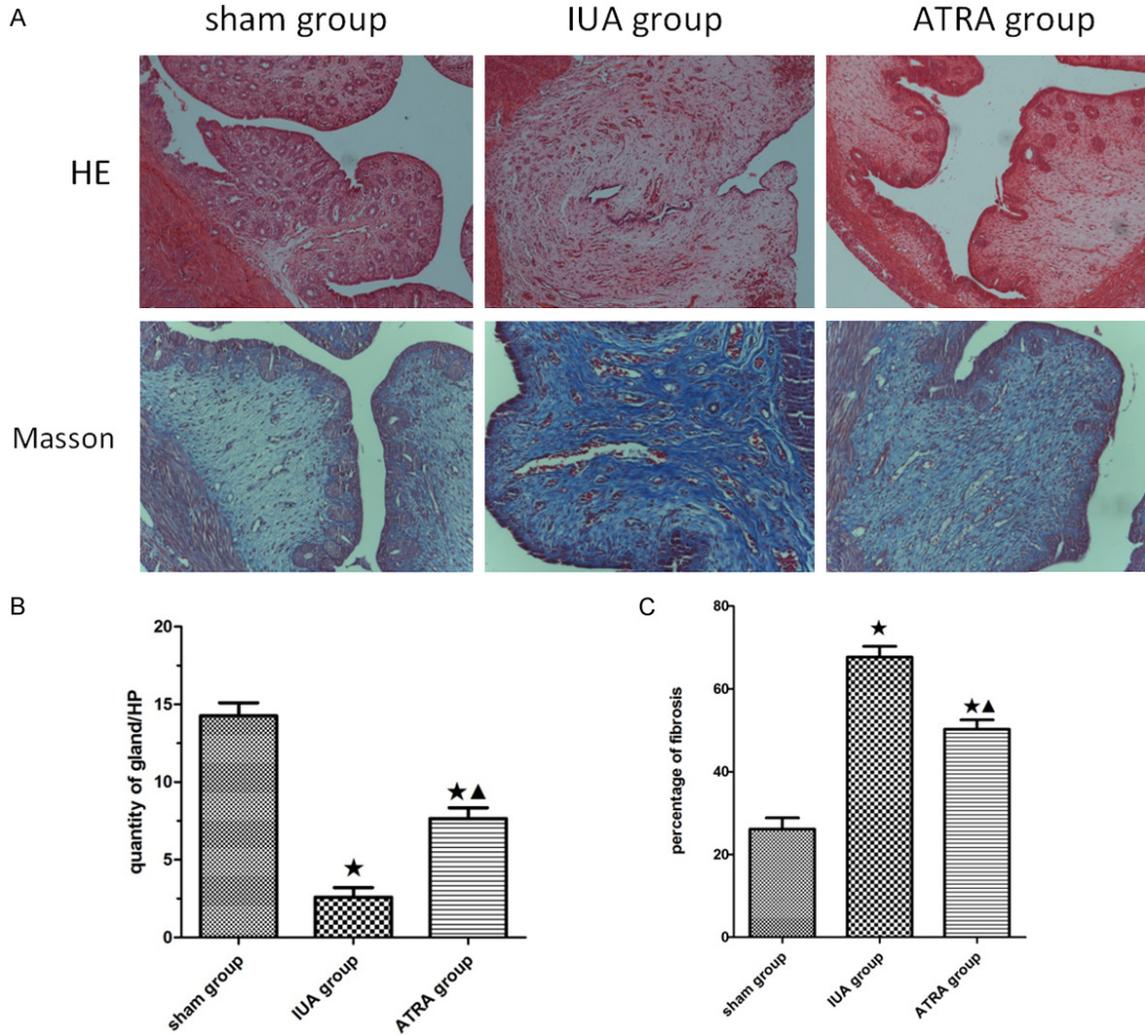


Figure 1. Anti-fibrotic effects of ATRA in IUA model. A. The histological changes in the three groups are shown by HE staining (magnification, $\times 100$) and Masson staining (magnification, $\times 200$). B. The quantity of glands in the three groups. * $P < 0.05$ compared with the sham group; ▲ $P < 0.05$ compared with IUA group. C. The percentage of fibrosis area in the three groups. * $P < 0.05$ compared with the sham group; ▲ $P < 0.05$ compared with IUA group.

tion conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s. Melt curves were maintained at 95°C for 15 s, 55°C for 45 s and 95°C for 15 s. In this study β -actin was used as an internal control. The relative gene expression was quantitatively analyzed by the comparative C_t method ($2^{-\Delta\Delta C_t}$). The sequences of primers used in the present study are given in **Table 1**.

Western blotting

Total proteins were extracted from endometrial tissues and separated on 10% SDS-PAGE. Separated protein was transferred to a PVDF membrane that was blocked in 5% BSA at room temperature for 1 hour and probed with prima-

ry antibodies overnight at 4°C . The diluted concentrations of the primary antibodies were as follows: TGF- $\beta 1$ (AbD Serotec, USA), 1:1000; Smad4 (Abcam, USA), 1:1000; collagen I (Abcam, USA), 1:1000; β -actin, 1:1000 (Abcam, USA). Secondary antibodies (EarthOx, USA) included HRP-labeled and were diluted 1:2000 with 0.2% TBS-T incubated for 1 hour at room temperature. Protein bands on Western blots were visualized using ECL reagent (Millipore, German). Relative band densities of proteins in Western blots were normalized against β -actin.

Statistical analysis

All data were expressed as the mean \pm standard deviation. One-way ANOVA with S-N-K test

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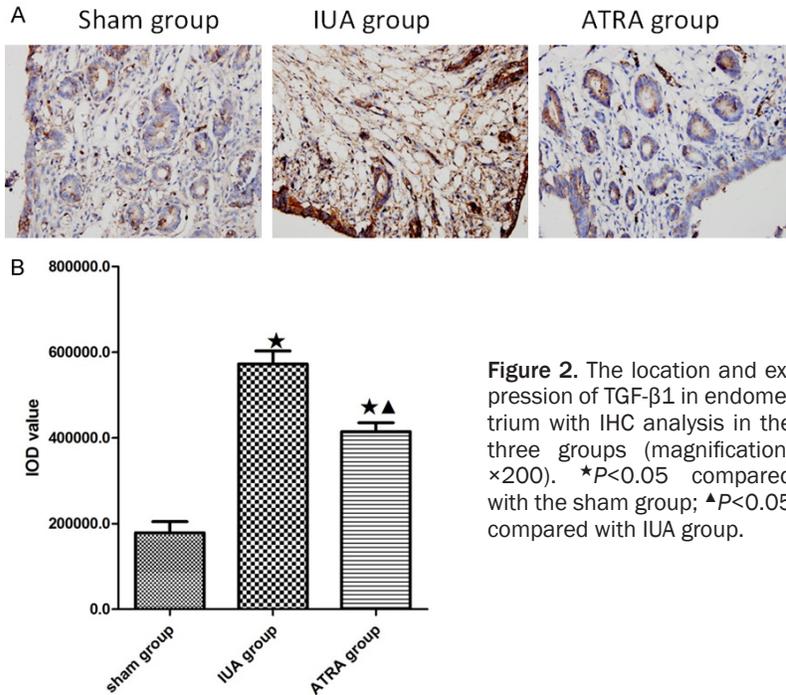


Figure 2. The location and expression of TGF- β 1 in endometrium with IHC analysis in the three groups (magnification, $\times 200$). $\star P < 0.05$ compared with the sham group; $\blacktriangle P < 0.05$ compared with IUA group.

was used for comparison between three groups. Data analysis was performed by SPSS software 13.0 (SPSS, Chicago, IL). A value of $P < 0.05$ was considered significantly different.

Results

Anti-fibrotic effects of ATRA in rabbit IUA model based on histopathological findings

The rabbits in the sham group exhibited a typical histopathology of uterine tissue. The endometrium was composed of polypoid proliferation without fibrosis formation and the epithelial cells are columnar. Round glands were detected in the endometrial submucosa or basal layer.

In IUA model the fibrosis changes of endometrium were detected by histological evaluation and Masson staining. The epithelial cells were flattened or low columnar and showed strong Masson staining for collagens deposition after dual injury by using mechanical and infectious injury. These histological characteristics of endometrial fibrosis were significantly attenuated in ATRA-treated groups. Masson analysis indicated that ATRA-treated group had lower collagen content compared with IUA group. In addition, ATRA treatment significantly increased the number of endometrial glands compared

with IUA group. The results indicated ATRA treatment significantly alleviated endometrial fibrosis and improved endometrial regeneration in IUA model (**Figure 1A-C**).

Effect of ATRA on the protein expression of TGF- β 1 in uterine tissue with IHC

TGF- β 1 was mainly observed in the endometrial epithelial cells by IHC (**Figure 2**). The positive expression was markedly increased in IUA group compared with the sham group which also proved TGF- β 1 as a profibrogenic cytokine in IUA model. In ATRA group, the IOD value was lower than in IUA group. That means

ATRA may decrease the overexpression of TGF- β 1 protein in IUA model (**Figure 2A, 2B**).

Effect of ATRA on the expression of TGF- β 1, smad4, COL1A1 and COL1A2 mRNAs in uterine tissue

In this study, mRNA levels of TGF- β 1, smad4, COL1A1 and COL1A2 in uterine tissue were analyzed respectively. TGF- β 1 was confirmed to be a central cytokine in the process of organ fibrogenesis [20]. Smads are important intracellular molecules of the TGF- β 1 signaling pathway and smad4 plays a central role in transduction of TGF- β 1 signals. Activation of TGF- β 1 signaling pathway is associated with an excessive accumulation of collagens in the process of fibrosis. The levels of COL1A1 and COL1A2 mRNA reflect collagen synthesis. TGF- β 1, smad4, COL1A1 and COL1A2 mRNAs were all elevated in IUA group compared with the sham group, but the expression of which were lower obviously in ATRA group (**Figure 3A-D**).

Effect of ATRA on the protein expressions of TGF- β 1, smad4, collagen in uterine tissue with Western blot analysis

The protein expression levels of TGF- β 1, smad4, collagen I on Western blot were in concordance with the results of the mRNA measurements.

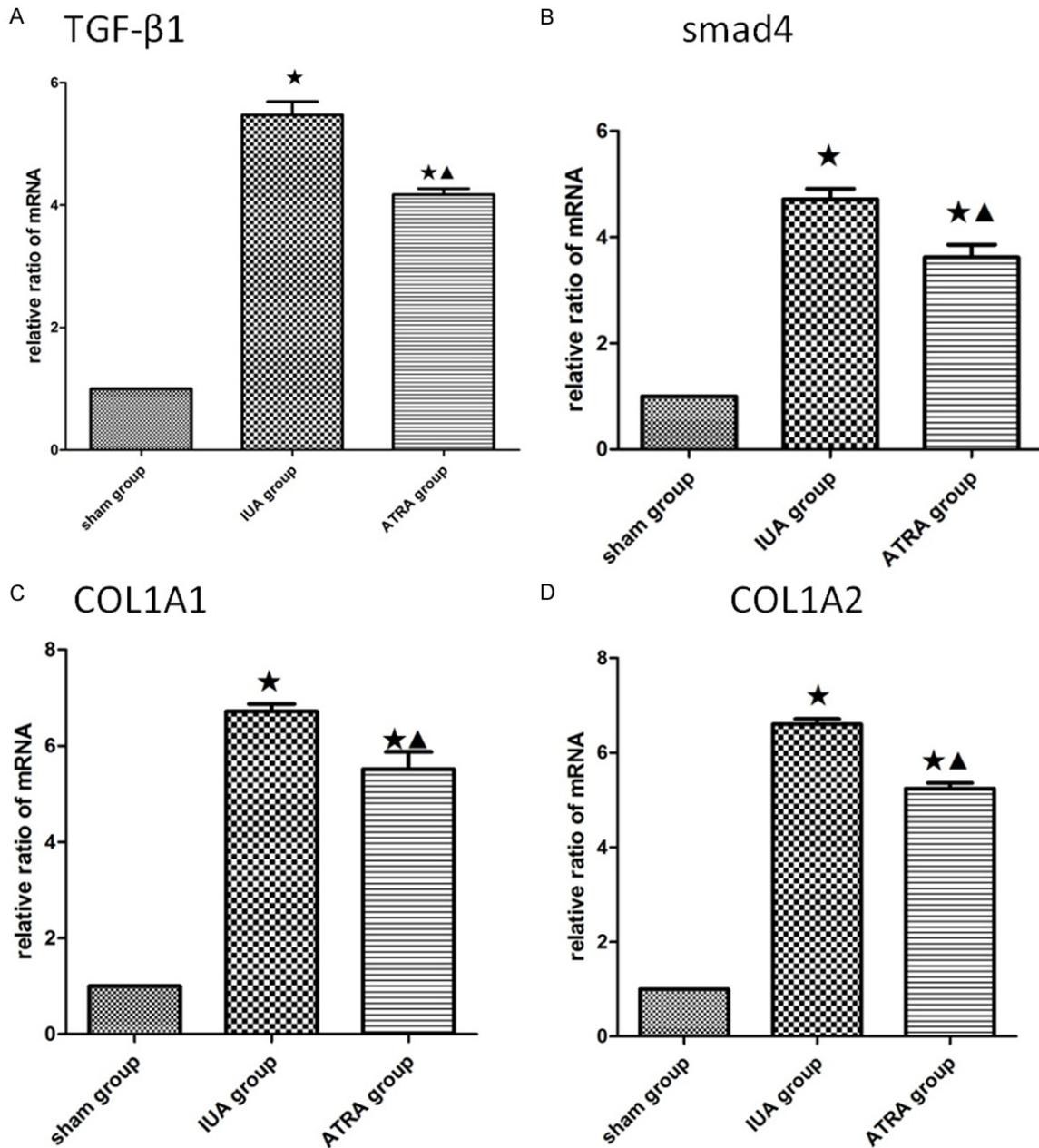


Figure 3. Analysis of TGF-β1, smad4, COL1A1 and COL1A2. mRNA levels by qRT-PCR. * $P < 0.05$ compared with the sham group; ▲ $P < 0.05$ compared with IUA group.

Compared with the sham group, overexpression of TGF-β1, smad4, collagen I proteins were observed in IUA group, and that was also a confirmed evidence of fibrosis in the rabbit uterus after mechanical and infectious injury. The decreased protein expression of TGF-β1, smad4, collagen I was observed in ATRA treatment group which indicated that ATRA did interfere with the endometrial fibrosis (Figure 4A-D).

Discussion

It has been known that the etiology of IUA was related to the damage to the uterine which could lead to menstrual abnormalities, miscarriage and infertility [3]. Hysteroscopic surgery has revolutionized the treatments of IUA and it is the established gold standard technique [21]. Many preoperative and postoperative treatments have been adopted to improve the

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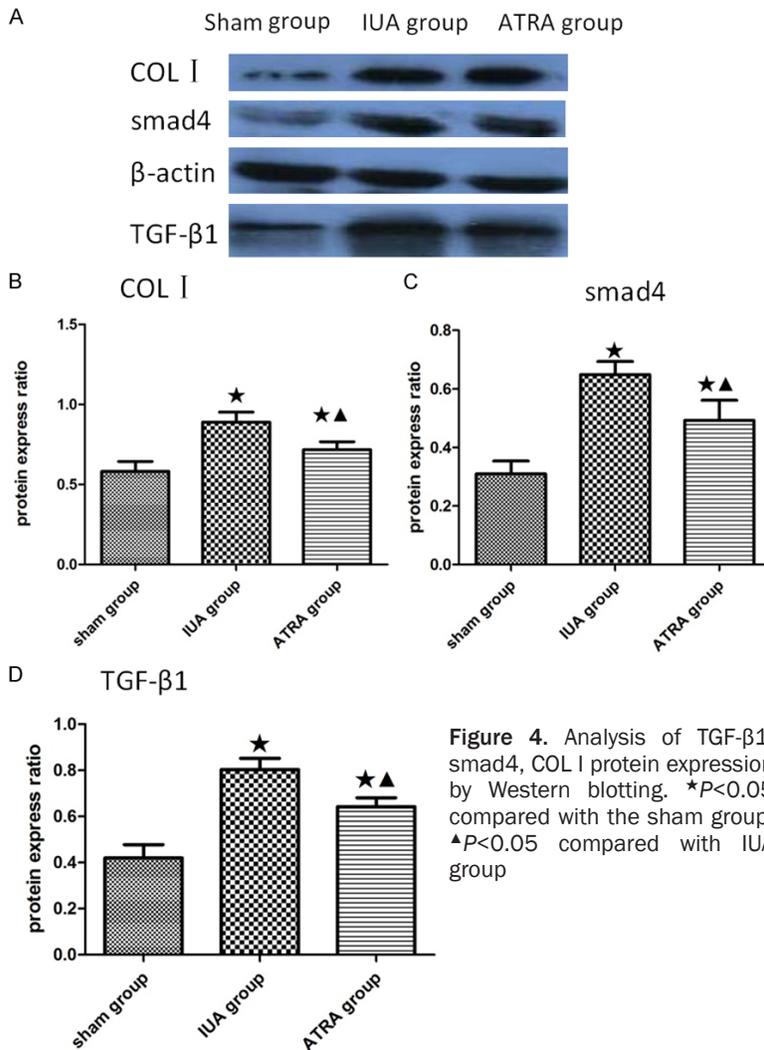


Figure 4. Analysis of TGF-β1, smad4, COL I protein expression by Western blotting. * $P < 0.05$ compared with the sham group; ▲ $P < 0.05$ compared with IUA group

(ECM) and might play a protective role against IUA formation. Currently, no reports are available to assess the roles of ATRA on IUA disease. Our study may provide a proof for further study of ATRA on IUA in clinical.

TGF-β1 has been considered a strong fibrosis cytokine which responsible for simulating the synthesis of collagens and inhibiting their degradation in the fibrosis. Furthermore, TGF-β1 mRNA and/or protein expression is increased in fibrotic diseases including pulmonary fibrosis, liver cirrhosis, renal fibrosis, systemic sclerosis, and cardiac fibrosis [13-17]. Chen et al have found that elevated expression of TGF-β1 may accelerate endometrial fibrosis and IUA in human study [22]. In our study, the up-regulation of TGF-β1 detected in IUA model confirmed the induction of fibrosis by TGF-β1 in endometrium. It has been demonstrated that TGF-β1/smads signaling pathway play a pivotal role in the fibrogenesis which also has

surgical outcomes, but the operative effects and reproductive prognosis especially for moderate-to-severe IUA are unsatisfied due to reformation of IUA and inconsistent endometrial growth [5]. In order to effectively treat IUA, purposeful efforts should be made toward endometrial fibrosis during the formation of IUA.

Previously, we reported that the mechanical and infectious injury-induced IUA in rabbit is characterized by endometrial stromal fibrosis and delayed regeneration of endometrial epithelial cells [19]. In present study, ATRA was shown to effectively ameliorate IUA formation in rabbit. After treated with ATRA, number of endometrial glands increased significantly. Masson trichrome staining also revealed reduced endometrial fibrosis achieved with ATRA. Our findings suggest that ATRA attenuates the deposition of extracellular matrix

been activated in IUA model from our evidence. Smads are important intracellular signal transductive molecules in the TGF-β1 signaling pathway. Smad4, a common smad, combines with phosphorylated smad2/smads3 and then form a heteromeric complex, which translocates to the nucleus and binds to the DNA elements to regulate the expression of target genes [23]. In the present study, IUA model was characterized by activation of TGF-β1 signaling via elevated smad4 by using this signaling in result with increased COL1A1/COL1A2 mRNA and collagen I protein. Furthermore, ATRA administration inhibited TGF-β1 signaling via decreased smad4 mRNA/protein in IUA model. Thus, it can be suggested that ATRA regresses endometrial fibrosis that originate from activated smad4-dependent-TGF-β1 signaling in IUA model.

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In conclusion, our study suggested that ATRA has a significant inhibitory effect on the formation of IUA. This inhibitory effect is mediated mainly through its suppression of TGF- β 1 signaling in the IUA model. Therefore, ATRA can be considered an option for improving endometrium regeneration in IUA with reduced fibrosis.

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Disclosure of conflict of interest

None.

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