Expression and Potential Role of MMP-9 in Intrauterine Adhesion

Congqing Li
first Affiliated Hospital of Anhui Medical University https://orcid.org/0000-0002-0765-9209
Lin Cong (13637095596@139.com)
Youjiang Xu
Second Affiliated Hospital of Anhui Medical University
Wenyan Wang
Second Affiliated Hospital of Anhui Medical University

Research article

Keywords: Matrix Metalloproteinase-9, Intrauterine Adhesion, Fibrosis

Posted Date: April 24th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-24240/v1

Abstract

Background: Intrauterine adhesions will affect the amount of menstrual and fertility, endometrial fibrosis is the last manifestation of the IUA. MMP-9 is closely related to fibrosis. The purpose of the study was to assess the role of MMP-9 in intrauterine adhesion (IUA) rats and patients.

Methods:40 female rats and 24 patients were enrolled in this study. We used immunohistochemistry to detect the MMP-9 expression in rats and human endometrial tissues, as well as detected their protein levels with western blot. In addition, we detected their mRNA expression levels with qRT-PCR.

Results: The expression of MMP-9 in the IUA rats was reduced compared with the Sham group and Ctrl group ($P< 0.05$), and the expression of MMP-9 was also reduced in the IUA patients compared with the Ctrl group ($P< 0.05$). The mRNA levels of MMP-9 in endometrium were presenting similar results ($P< 0.05$).

Conclusions: Our study suggests that MMP-9 may play an important role in fibrosis of the IUA. It may provide a new reference for the treatment of IUA in the future.

1. Background

Intrauterine adhesion (IUA)is an old topic, it has been found more than a century [1], IUA is a very common problem in clinical practice, it is the main cause of menstrual volume reduction, infertility and recurrent abortion. It has been identified that uterine cavity injury, especially induced abortion, can lead to endometrial basal layer injury. Injury is one of the direct causes of uterine cavity adhesion, endometrial fibrosis is the last manifestation of IUA [2]. Fibrosis is a common and difficult problem in clinical. During the healing process of normal wounds, the deposition of extracellular matrix (ECM) led to the occurrence and development of tissue fibrosis. The excessive accumulation or degradation of ECM components in the organs leads to an increase in ECM, fibrosis of the tissue, and ultimately to a decrease or loss of function, such as liver fibrosis, Kidney fibrosis, Pulmonary fibrosis and intestinal fibrosis et al [3]. The mechanism of endometrial fibrosis due to intrauterine adhesions is still unclear. According to conventional speculation, the persistence of ECM components reduced deposition or degradation of the extracellular matrix may be the main cause of fibrosis [3]. The process of fibrosis is interlocking and intricate, these events include the deposition of ECM proteins and related molecules / factors cross-linking of various ECM elements, hydrolysis of ECM proteins, and enzymes that degrade ECM is involved. Among ECM proteolytic enzymes, scholars pay more attention to the research of matrix metalloproteinases (MMPs) [3]. MMPs plays a key role in the balance of fibrosis and anti-fibrosis, when fibrosis and anti-fibrosis are out of balance, the degradation of the extracellular matrix eventually leads to the generation of tissue fibrosis [4, 5]. Based on the understanding of the characteristics of MMP-9 and fibrosis, we aimed to investigate MMP-9 may also be present in IUA fibrosis. In this study, we evaluated the potential role of MMP-9 in the fibrosis of IUA by measuring the expression of MMP-9 in endometrial tissues.

2. Methods

Master Plan Route for this study in Fig. 1.

2.1. IUA patients and controls.

Twenty-four patients were included in our study, of which 12 were IUA patients, the control group was from hysteroscopy infertility patients. The ethics committee of the Second Hospital of Anhui Medical University approved the research plan (PJ-YX2019-016F1). The average age of IUA patients was 29.5 years (24–40 years) and the control group was 24.75 years (21–39 years). We take samples of endometrial fibrosis tissue from May 2018 to July 2018, we score and grade IUA patients, according to criteria devised by the American Fertility Society (AFS) [6]. Endometrial tissue specimens were obtained from hysteroscopy surgery. The criteria for inclusion of surgical specimens excluded infection, any disease in the uterus, chronic inflammation, and malignant diseases. All patients signed written informed consent before surgery and agreed to use endometrial tissue specimens for scientific research.

2.2. Rat Experimental protocol.

The experimental animals for this project were purchased from Animal Experimental Center of Anhui Medical University, animal certificate number: SCXK (Anhui) 2005-001.The experimental protocol follows the requirements of animal ethics and is implemented in accordance with the regulations of the Animal Ethics Committee of Anhui Medical University. These studies were approved by the Institutional Animal Care and Use Committee at Anhui Medical University (LLSC20180085). From January 5th to February 26th, 2017. 40 mature female fertile SD rats were enrolled in this study (weighing 200–250 g, age 8 weeks). The experimental animals were kept in the animal breeding room of the Experimental Center of the Second Affiliated Hospital of Anhui Medical University. Under standard laboratory conditions, the ambient temperature of the animal breeding room is 25 ± 2 ℃, the maximum temperature difference must not exceed 2 ℃; the air humidity is controlled at 60% ±; the indoor noise control is less than 55 decibels (dB), give rats one week to adapt to the breeding environment.

2.2.1. IUA Rat models.

Experimental surgery in rats was performed by the same person and the anesthesia method is intraperitoneal administration of sterile sodium pentobarbital (30 mg / kg). Our goal is to use the rat uterus to build an animal model similar to human uterine adhesions. In clinical work, phenol mucus is used in female sterilization surgery, it is safe and reliable [7]. 40 rats were randomly divided into three groups. Rats were sacrificed 2 weeks after surgery with the carbon dioxide method. Then the target uterus was obtained and endometrial tissue specimens were obtained. After the specimens were removed, the rats' bodies were harmlessly treated.

2.2.2. IUA Model Group.

20 rats were included in the IUA group. We used one rat's uterine cavity as the experimental research object, and injected 0.04 ml of phenol mucilage into the selected uterine cavity, composition of phenol mucilage: phenol solution 25% v / v, gum Arabic 5% v / v, glycerol 20% v / v). This method resulted in adhesion within 2 weeks. (the surgical procedure is shown in Fig.2).

2.2.3. Sham Group and the Control Group.

10 rats were operated, the operative procedure was same to the IUA group, the difference was that normal saline was injected into the right uterine cavity instead of phenol mucilage. The control group was also 10 rats. The difference was that nothing was injected into the right uterine cavity.

2.3. Collection Tissue Samples from patients and rats.

Endometrial tissue samples we will get divides into two, one portion should be kept at room temperature in formalin, the other was stored at -80℃ until use.

2.4. HE Staining and Masson Staining of Rats Tissue Samples.

Tissue specimens were fixed in 4% neutral buffered formalin, and paraffin sections were routinely made, and then stained according to the HE procedure. Masson staining methods and procedures are performed according to the requirements of the reagent manufacturer [8].

2.5. Immunohistochemistry of Human and Rats Tissue Samples.

IHC reagents were purchased from the Beijing zhongShanJinQiao Company and conducted according to the manufacturer's instructions. Sections undergo conventional immunohistochemical (IHC) staining [9,10]. To measure the relative expression of MMP-9 in different groups of endometrial tissues, we calculated the expression score by evaluating the percentage of positive cells and the intensity of the staining signal. Calculate expression score by multiplying percentage with intensity score, and then converted into a relative expression.

2.6. Western Blot Analysis.

Western blot reagents were purchased from the Beyotime Company (China) and conducted according to the manufacturer's instructions. Molecular imaging systems (Bio-Rad, Philadelphia, PA, USA) were used to capture the bands, finally, the relative expression value is calculated.

2.7. Analysis of mRNA of MMP-9 by RT-qPCR.

The obtained endometrial tissue samples were thawed at room temperature. Total RNA was extracted using Trizol reagent (Invitrogen), and RT kit (Takara) was used for reverse transcription reaction according to the instructions. MMP-9 was amplified by PCR using cDNA as a template. Real-time quantitative PCR to detect their relative mRNA levels. Internal control is using GAPDH. The primer sequences are shown in Table 1. The PCR conditions consisted of 5 min at 95°C for one cycle followed by 45 cycles of 95°C for 10 s, 60°C for 40 s, and 72°C for 90 s.

2.8. Statistical Analyses.

All Statistical Analyses were performed using SPSS software (version 19.0, SPSS, Chicago, IL), the results were expressed as mean ± S.E.M. Comparisons between groups were analyzed by unpaired t-tests or analysis of variance (ANOVA). A low P -value lesser than 0.05(P□0.05) is considered statistically significant.

Target mRNA	Primer sequence
MMP-9-forwards:	5□-TTGACAGCGACAAGAAGTGG-3□
MMP-9-reverse:	5□-CCCTCAGTGAAGCGGTACAT-3□
GADPH-forwards:	5□-GGTTGAGCAGGTACTTT-3□
GADPH-reverse:	5□-AGCAAGAGCACAAGAGGAAG-3□

Table 1
Primers used for PCR analysis

3. Results

3.1. Basic information about the Study Groups.

There was no statistically significant difference in age between IUA patients and the control group. Totally 40 female rats were randomly divided among three groups.

3.2. HE staining and Masson staining of Rats Tissue Samples.

HE staining showed a narrowing of the uterine cavity in the IUA group. Masson staining results showed that severe fibrosis occurred in the endometrial tissue of the IUA group, compared with the control group and the sham operation group. (Fig.3).

3.3. MMP-9 Expression in IUA rats and Patients.

It is a known fact that MMP-9 plays an important role in fibrosis, we measured the tissue samples expression of IHC, protein and mRNA MMP-9 in IUA rats and patients to assess whether the differential expression in IUA or not. We performed IHC staining of the tissue samples from IUA rats and patients revealed that the IUA groups showed negative MMP-9 staining. The differences of them were significant in the analysis ($P < 0.05$) (Fig.4). We demonstrated protein expression of MMP-9 by Western blot, significant decreases of protein expression in IUA rats and patients compared to controls, the protein expression of MMP-9 difference was significantly ($P < 0.05$) (Fig.5). In addition, mRNA expression of MMP-9 was significantly decreased ($P < 0.05$) in IUA rats and patients compared with controls ($P < 0.05$) (Fig.6).

4. Discussion

In general, massive granulation tissue hyperplasia and fibrosis in the uterine cavity after the abortion or curettage, IUA occur 5 to 7 days after an injury usually, when a sufficient proportion of fibrosis occurs, its own regulatory mechanism can hinder the regeneration of the endometrium and the formation of intrauterine adhesions [11].Generally, in the initial stage of tissue damage, the damaged and dead cells will release anti-fibrinolytic coagulation factors, which will trigger platelet activation, generate high levels of MMPs, destroy ECM and allow inflammatory mediators to recruit inflammatory cells to the injury site. On the other hand, the microenvironment of the injury site will also change accordingly. The pro-inflammatory response will lead to the activation of matrix-expressing cells and will also enhance the formation of fibers [4].

MMP expression is not isolated in the body, it is affected by other members of the family, and it is controlled by a series of endogenous inhibitors (collectively referred to as metalloproteinase tissue inhibitors (TIMP)). During the inflammatory response, many cell types, including macrophages [4], were identified as the producers of MMPs and TIMPs. From this perspective, it is suggested that many inflammatory diseases include the participation of MMPs. MMPs can degrade many components of ECM and have a strong acting capacity, which has been widely studied by scholars, especially in the fibrosis of lung, liver, and heart diseases [12]. Basic research finds that MMPs are not only a physiological medium for extracellular matrix turnover, but also a key factor in the remodeling process under pathological conditions [13]. Fibrosis is a basic connective tissue lesion that is pathologically characterized by an

increase in fibrous extracellular matrix (ECM) components in tissues or organs. MMPs is widely studied because they are important enzymes that degrade ECM. Under normal physiological conditions, MMPs / TIMPs are in a state of dynamic equilibrium, once MMPs / TIMPs are out of balance during tissue remodeling, the normal synthesis and degradation of ECM components are out of balance, leading to the occurrence of fibrosis. [14]. Previous findings suggest that increased expression of MMPs appears to be associated with various fibrous diseases. However, some studies have shown that matrix metalloproteinases have the ability to promote and combat tissue fibrosis at the same time. [15–22]

The specific details during the fibrosis process are unknown, but we should realize that the rules for specific MMPs may not be the same in different organ systems [23]. IUA is the manifestation of tissue fibrosis, here we found that MMP-9 expression is decreased in both IUA rats and patients, IHC, Western blot and RT-qPCR were used to detect the MMP-9 from multiple perspectives. Some studies have shown that over expression of MMP-9 and reduced levels of TIMP-1 will improve fibrosis of the disease. [24–28] However, no relevant studies have been discovered in the IUA. We speculate that MMP-9 should also have an anti-fibrotic effect in IUA. In this study, whether in IUA rat models or IUA patients, MMP-9 in their endometrial tissues showed a low expression state, and the decline in MMP-9 may lead to reducing degradation of ECM and promoted the formation of fibrosis effect. After abortion or curettage, raising the level of MMP-9 in the endometrium may be a method to prevent IUA in the future, or it may improve the prevention of re-IUA after the hysteroscopic adhesiolysis.

Previous studies of fibrosis of other organ diseases have shown that MMP-9 is an up-regulated state, why are the MMP-9 we detect decreased? The reasons may be as follows. First, MMP-9 changes dynamically at different stages of fibrosis, and maybe a high expression state in the early stage. MMP-9 appears to be down-regulated as the fibrosis progresses. Second, in this study, maybe the degree of fibrosis of IUA had an impact on the results. In addition, our research team explored the pathogenesis of IUA from the perspective of inflammation, and found that CXC chemokine ligand − 5 (CXCL5) is under-expressed, the results of this research are being submitted, a further study is needed to confirm the intracellular signaling pathway of CXCL5/Pi3k/AKT/MMP-9 in IUA, especially find and confirm other key factors on this pathway, and the relationship between these key factors.

For the treatment of fibrosis, there is a method using a non-active mutant of MMP-9 as TIMP- 1 antagonist or inducing agent [29]. After injecting these drugs in laboratory animals, it has been shown to reduce fibrosis and reduce the accumulation of ECM components that form scars and disrupt normal tissue structure. The MMP-based drug delivery system can directly inhibit its proteolytic activity or use its proteolytic activity to release drug-active forms in specific tissues [30]. These studies need to be further developed, and it is hoped that they can also be used for IUA endometrial fibrosis treatment.

5. Conclusion

In summary, this study establishes an animal model platform for studying the mechanism of the IUA. Furthermore, we identified MMP-9 as a novel factor of fibrosis in IUA. MMP-9 may be the treatment entry

point for the treatment of IUA and the prevention of recurrence of adhesions after IUA separation surgery.

Abbreviations

IUA: Intrauterine adhesion; MMP-9: Matrix metalloproteinases 9; IHC: Immunohistochemistry.

Declarations

Ethics approval and consent to participate

The experimental protocol follows the requirements of animal ethics and is implemented in accordance with the regulations of the Animal Ethics Committee of Anhui Medical University. These studies were approved by the Institutional Animal Care and Use Committee at Anhui Medical University (LLSC20180085). The study was approved by the Medical Ethics Committee of the Second Hospital of Anhui Medical University (PJ-YX2019-016 F1). Each participant provided written informed consent to be included in the study.

Consent for publication

Not applicable

Availability of data and materials

All materials and data used in the study are available and can provide as necessary by contacting the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Funding

This research did not receive specific funding.

Authors' contributions

CQ conceived and designed the study, performed analysis, interpretation of data and drafted the manuscript. LC assisted with the design, conception and interpretation of data, and critically reviewed the manuscript. YJ assisted with the design, conception and interpretation of data. WY assisted with the design, conception and interpretation of data. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Shiying Sun and Lei Zhan for technical assistance and valuable discussions.

References

[1] Fritsch.H.Ein Fall von volligenSchwund der GebaumutterhohlenachAuskratzung, Zentralblattfur Gyn ¨ akologie¨.1894;18: 1337-1342.

[2] Diamond MP, Freeman ML.Clinical implications ofpostsurgical adhesions. Human Reproduction Update.2001;6: 567-576.

[3] Liu Y:Cellular and molecular mechanisms of renal fibrosis.Nature Reviews Nephrology.2011; 12: 684-96.

[4] Afratis NA, Klepfish M,Karamanos NK, Sagi I.The apparent competitive action of ECM proteases and cross-linking enzymes during fibrosis: Applications to drug discovery. Advanced Drug Delivery Reviews.2018; 129: 4-15.

[5] Eckes B, Zigrino P, Kessler D, et al.Fibroblast-matrix interactions in wound healing and fibrosis.Matrix Biology.2000;19: 325-332.

[6]American Fertility Society.The American Fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, Müllerian anomalies and intrauterine adhesions.Fertility and Sterility.1988; 49: 944-955.

[7]Richart RM.Female sterilization using chemical agents.Research Frontiers in Fertility Regulation.1981;1: 1-12.

[8] Wang X, Ma N, Sun Q, Huang C, Liu Y, Luo X. Elevated NF-κB signaling in Asherman syndrome patients and animal models.Oncotarget.2017; 8: 15399-15406.

[9] Zhang S, Li P, Yuan Z, Tan J.Platelet-rich plasma improves the appetite effects of menstrual blood-derived stomatal cells in the rat model of intrauterine adhesion.Stem Cell Research &Therapy. 2019;10:1-12.

[10] Kobayashi S, Yamada-Okabe H, Suzuki M, et al. LGR5-Positive ColonCancer Stem Cells Interconvert at Drug Resistant LGR5-Negative Cells and are Capable of TumorReconstitution.Stem Cells.2012; 30: 2631-2644.

[11]Schenker JG.Etiology of and therapeutic approach to synechia uteri.European Journal of Obstetrics Gynecology and Reproductive Biology.1996; 65: 109-113.

[12]Vu T,Werb Z, Gelatinase B structure, regulation, and function.Biology of Extracellular Matrix.1998;115-148.

[13] Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Current Opinion in Cell Biology.2004;16:558-564.

[14] Robert S, Gicquel T, Victoni T,et al. Involvement of matrix metalloproteinases (MMPs) and inflammatory pathway in molecular mechanisms of fibrosis.Bioscience Reports.2016;36: 1-11.

[15] Jelena K,Santibanez JF.Transforming Growth Factor-Beta and Matrix Metalloproteinases: Functional Interactions in Tumor Stroma-Infiltrating Myeloid Cells.The Scientific World Journal.2014;2014: 1-14.

[16] Geurts N, Opdenakker G,Van den Steen PE.Matrix metalloproteinases as therapeutic targets in protozoan parasitic infections.Journal of Pharmacology and Experimental Therapeutics.2012;133: 257-279.

[17]Parks WC,Wilson CL,López-Boado YS.Matrix metalloproteinases as modulators of inflammation and innate immunity.Nature Reviews Immunology.2004;4: 617-629.

[18] Xue M, March L,Sambrook PN,Jackson CJ.Differential regulation of matrix metalloproteinase 2 and matrix metalloproteinase 9 by activated protein C: relevance to inflammation in rheumatoid arthritis,"Arthritis Rheumatology.2007;56: 2864-2874.

[19]Rutschow S,Li J,Schultheiss HP, Pauschinger M. Myocardial proteases and matrix remodeling in inflammatory heart disease.Cardiovascular Research.2006;69: 646-656.

[20]Polyakova V, Loeffler I, Hein S,et al.Fibrosis in envisage human heart failure: severe changes in collagen metabolism and MMP/TIMP profiles.International Journal of Cardiology. 2011;151: 18-33.

[21]Itoh T,Matsuda H,Tanioka M,Kuwabara K,Itohara S,Suzuki R.The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis.The Journal of Immunology.2002;169: 2643-2647.

[22]Edsparr K,Basse PH,Goldfarb RH,Albertsson P.Matrix metalloproteinases in cytotoxic lymphocytes impact on tumor infiltration and immunomodulation.Cancer Microenvironment.2010;4: 351-360.

[23] Giannandrea M,Parks WC.Diverse functions of matrix metalloproteinases during fibrosis. Disease Models and Mechanisms.2014;7:193-203.

[24]Wang K,Lin B,Brems JJ,Gamelli RL.Hepatic apoptosis can modulate liver fibrosis through TIMP1 pathway.Apoptosis. 2013;18:566-577.

[45]Yoshiji H,Kuriyama S,Yoshii J,et al.Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse.Hepatology.2002;36:850-860.

[26] Iredale J.Defining therapeutic targets for liver fibrosis: exploiting the biology of inflammation and repair.Pharmacological Research. 2008;58:129-136.

[27]Yoshiji H,Kuriyama S,Miyamoto Y, et al.Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model.Hepatology. 2000;32:1248-1254.

[28]Medeiros NI, Gomes J,Correa-Oliveira R.Synergic and antagonistic relationship between MMP-2 and MMP-9 with fibrosis and inflammation in Chagas' cardiomyopathy.Parasite immunology.2017;39:e12446.

[29] Roeb E, Behrmann I, Grotzinger J, et al. An MMP-9 mutant without gelatinolytic activity as a novel TIMP-1-antagonist. FASEB J. 2000;14:1671-1673.

[30]J. Vandooren, G. Opdenakker, P.M. Loadman, et al. Proteases in cancer drug delivery[J], Adv. Drug Deliv. Rev. 2016;97:144-155.

Figures

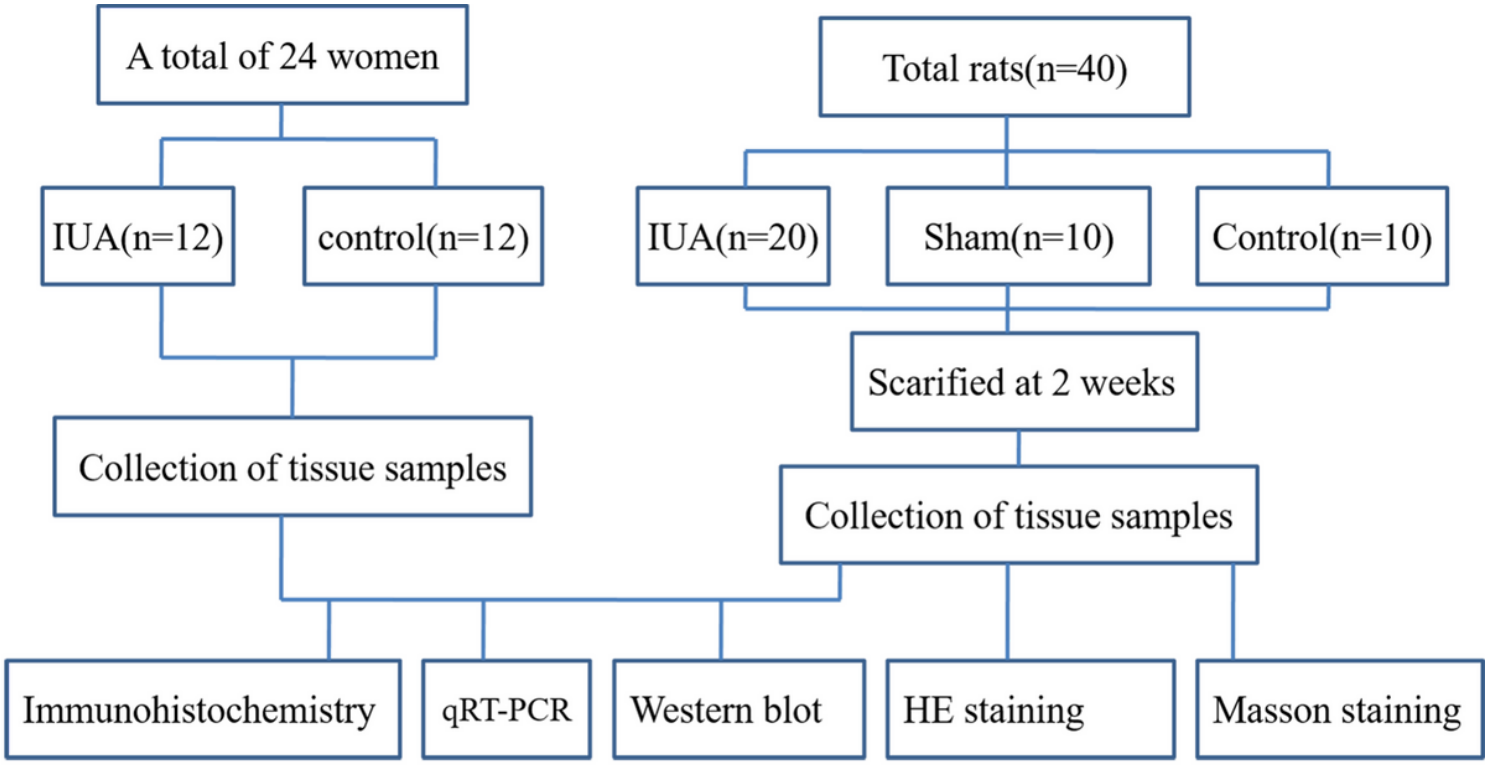


Figure 1

Master Plan Route for this Study.

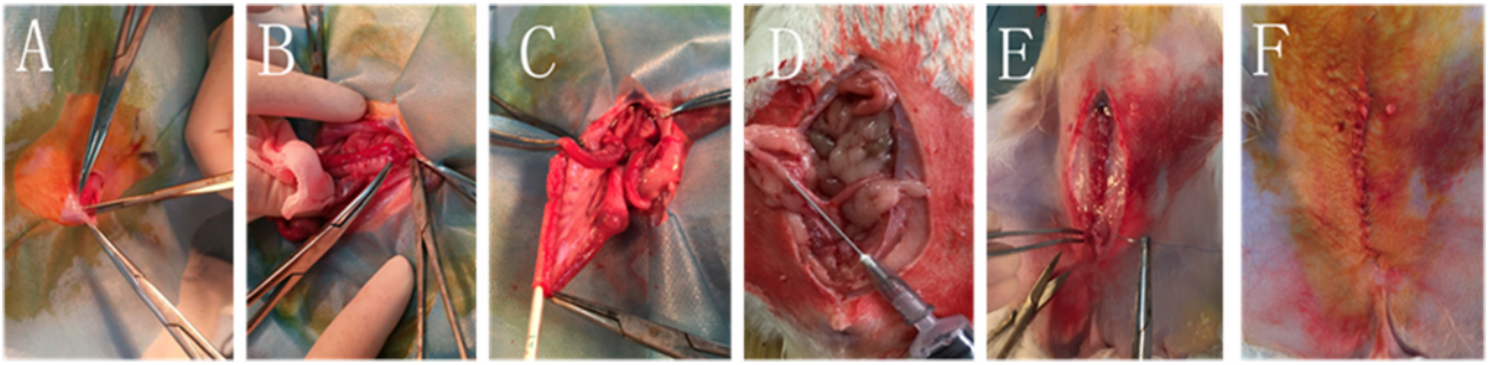
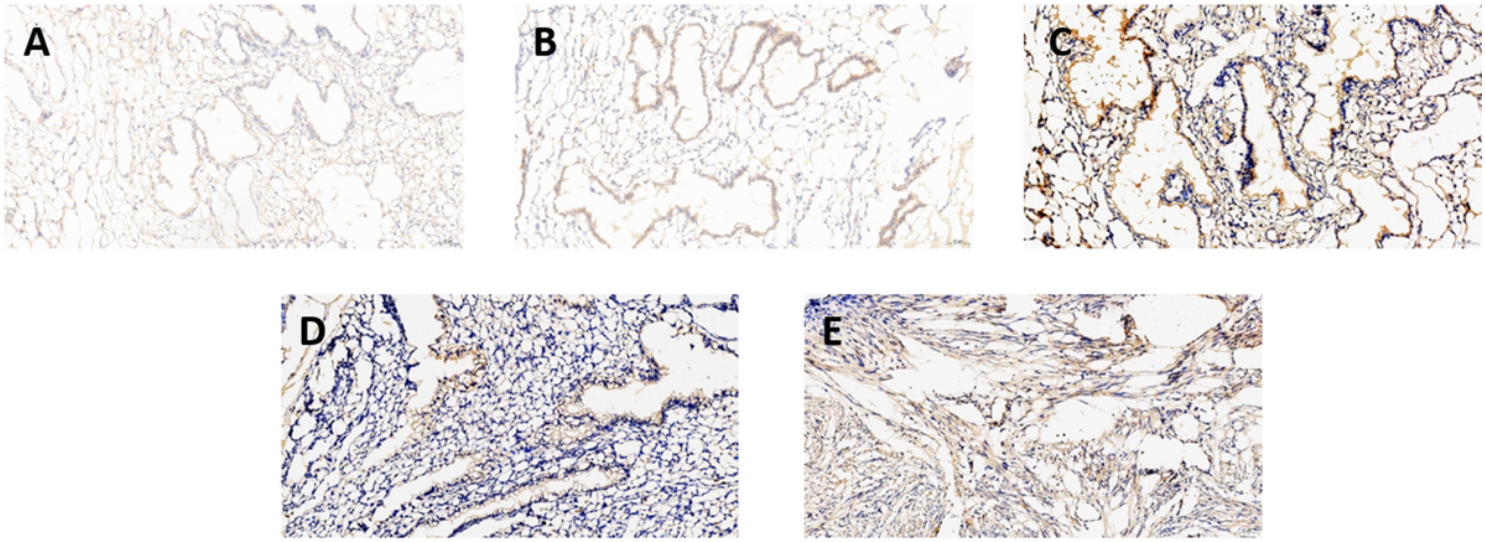


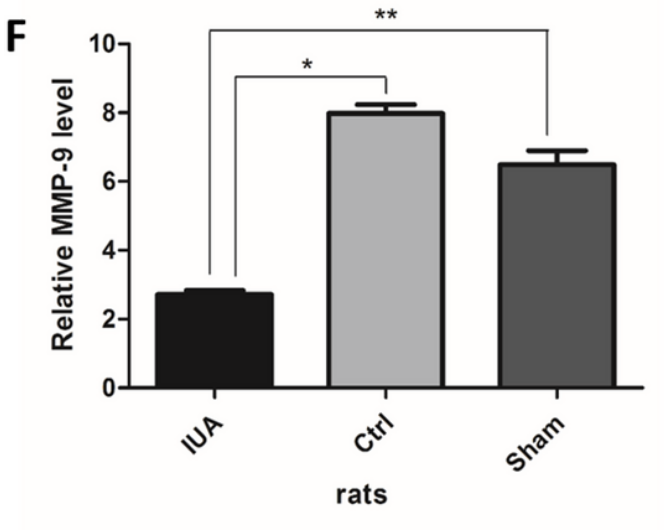
Figure 2

The operation procedure of IUA rat model. (A)Select the incision sign. (B)Incise the skin and subcutaneous tissue. (C) Rat uterus was observed, and one side uterine cavity was probed. (D) Phenol mucilage was slowly injected into rat uterine cavity with the syringe. (E)After the operation, the abdominal incision was closed. (F)After suturing the skin, sterilize the incision again.

Figure 3

HE and Masson staining revealed in rats. (A,D) IUA group (phenol mucilage treatment), (B,E) sham group (saline treatment) and (C,F)control group (no treatment) ,scale bar = 200μm.





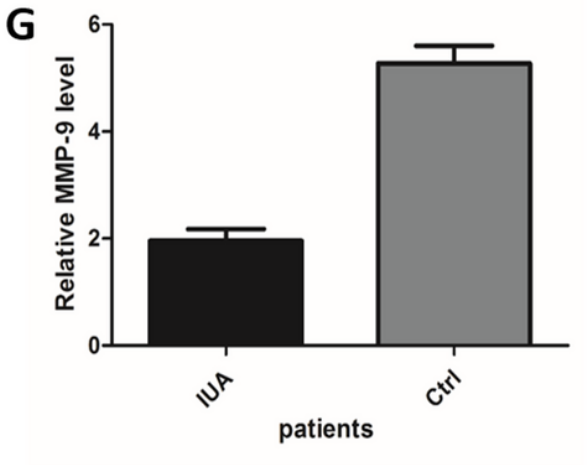


Figure 4

IHC of MMP-9 in rats and patients. Rat groups: A(IUA),B(Sham),C(control). Patient group: D(IUA) and E(Control). Scale bar=50μM, (F) Relative MMP-9 level in rats, comparison of IUA to Sham and Ctrl,

*P<0.05, **P<0.05. (G) Relative MMP-9 level in patients, comparison of IUA to Ctrl, P<0.05.

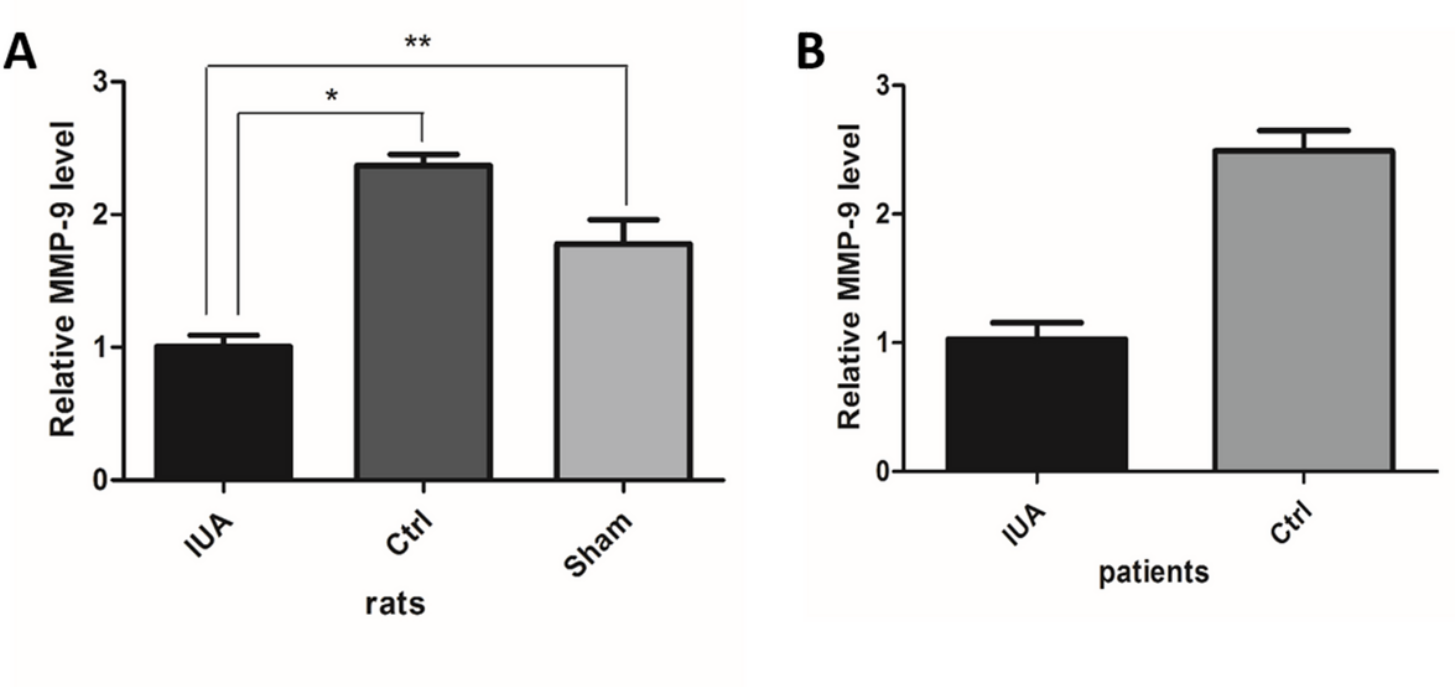


Figure 5

Determination of MMP-9 protein expression in IUA rats and patients. (A) Relative MMP-9 protein expression in three groups. Comparison of IUA to Ctrl, Sham group, *P<0.05 **P<0.05. (B) Relative MMP-9 protein expression in patients. Comparison of IUA to Ctrl, P<0.05.

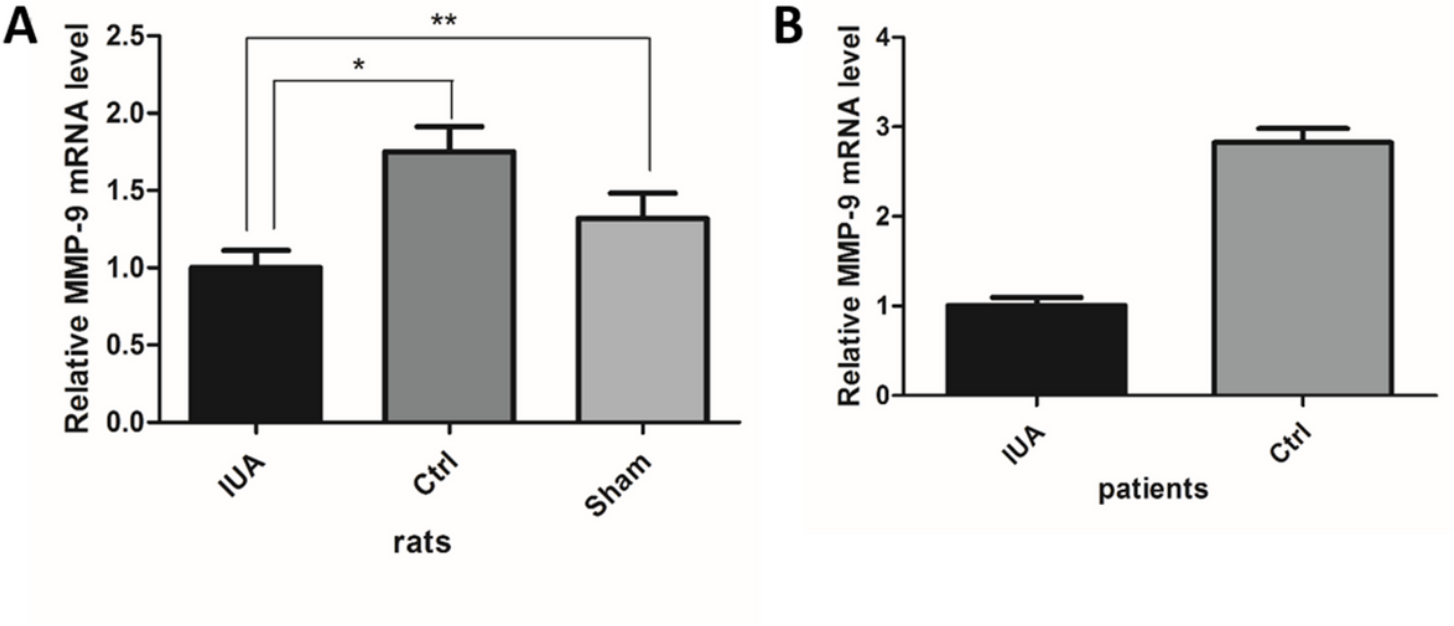


Figure 6

qRT-PCR analysis of MMP-9 mRNA expression. (A) rats. *P<0.05, **P<0.05. (B) Patients, P<0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Checklist.pdf
- SupplementaryMaterials.pdf