Article

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Tamoxifen Alleviates Endometrial Fibrosis Induced by Anhydrous Ethanol in Rats

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Background: As an estrogen receptor modulator, tamoxifen has been utilized in the treatment of fibrotic diseases. However, there is a limited body of research focusing on its potential application in addressing endometrial fibrosis conditions. Our research aims to investigate the effects of tamoxifen at different dosage levels in alleviating endometrial fibrosis and to elucidate its mechanisms of action during the initial stages of endometrial damage.

Methods: A total of thirty sexually mature, unmated female Sprague-Dawley (SD) rats were divided into six distinct groups. To establish the rat uterine adhesion model, the uterine cavity was subjected to perfusion with anhydrous ethanol. The control group received a saline solution, while the treatment group was administered oral estrogen in combination with tamoxifen at doses of 4 mg/kg/d, 20 mg/kg/d, and 40 mg/kg/d. Various techniques, including Hematoxylin and Eosin (HE) staining, Masson's Trichrome staining, Western Blotting analysis, and immunohistochemistry, were employed to assess changes in endometrial thickness, fibrosis, as well as alterations in indicators related to epithelial-mesenchymal transition (EMT), fibrosis, estrogen receptors within the endometrium, and vascular endothelial growth factor (VEGF).

Results: In the model group, the levels of endometrial thickness, E-cadherin, Vimentin, estrogen receptor α (ERα), G protein-coupled receptor 30 (GPR30), and VEGF proteins were significantly lower compared to the control group. Conversely, the levels of collagen accumulation, Smooth Muscle Actin (SMA), Transforming Growth Factor β_1 (TGFβ_1), Drosophila mothers against decapentaplegic protein 2 (Smad2) , and Smad3 were markedly higher than those observed in the control group ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$). In contrast, the low-dose tamoxifen group demonstrated significant increases in endometrial thickness, E-cadherin, Vimentin, ERα, GPR30, and VEGF when compared to the model group ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$). Moreover, the levels of collagen accumulation, TGFβ_1, SMA, Smad2, and Smad3, which are indicative of fibrosis and the TGFβ_1/Drosophila mothers against decapentaplegic (smad) pathway, were notably reduced compared to the model group ($p < 0.05$, $p < 0.0001$).

Conclusions: The results of this study suggest that the administration of a low dose (4 mg/kg/d) of tamoxifen in the early stages of endometrial injury may mitigate epithelial-mesenchymal transition (EMT) indicators and reduce fibrosis within the endometrium induced by anhydrous ethanol.

Keywords: tamoxifen; intrauterine adhesion (IUA); epithelial-mesenchymal transition (EMT); fibrosis; TGFβ_1/smad

Introduction

Intrauterine adhesion (IUA) is typically a consequence of uterine trauma and can result in impaired endometrial repair, leading to a thin endometrium, amenorrhea, recurrent miscarriages, and secondary infertility. Current treatment options for intrauterine adhesions are limited, as existing methods have proven ineffective in promoting endometrial regeneration following injury [1]. Additional research suggests that uterine adhesion is a fibrotic condition characterized by substantial damage to the endometrium and subsequent repair processes. This often results in the accumulation of collagen strands within the uterus, likely due to the extensive penetration of the basal layer, leading to damage to the endometrium. The atypical inflammatory response exacerbates the progression of fibrosis [2,3]. The process of epithelial-mesenchymal transition (EMT) is believed to play a crucial role in the development of organ damage and fibrosis resulting from trauma [4,5]. EMT involves a specific mechanism through which epithelial cells undergo a transformation into a mesenchymal cell phenotype [6]. EMT is thought to be a significant contributor to the development of uterine adhesion, as it leads to the reorganization of the extracellular matrix and the accumulation of numerous fibers on the endothelial surface [7].

Currently, tamoxifen (TAM) stands as the preferred drug for the treatment and prevention of estrogen receptor-positive breast cancer due to its classification as a selective estrogen receptor modulator (SERM). The gynecological community's interest has been piqued by tamoxifen's capacity to stimulate proliferation in the normal endometrium [8,9]. However, the application of such SERMs for the treatment of a compromised endometrium is relatively uncommon. In the realm of fibrotic diseases, tamoxifen has demonstrated an anti-fibrotic effect, with examples including renal fibrosis [10,11], silicone-induced pulmonary fibrosis [12,13], peritoneal fibrosis [14], proliferative scarring [15], hepatic fibrosis [16], and related studies [10,16] indicating that the anti-fibrotic effects of tamoxifen are primarily associated with the inhibition of the Transforming Growth Factor β_1 (TGFβ_1)/Drosophila mothers against decapentaplegic (smad) pathway. Nevertheless, the impact of tamoxifen on the damaged endometrium remains unknown, including its role in the initiation and progression of endometrial trauma.

In this study, we established an animal model of endometrial trauma by introducing anhydrous alcohol into the uterine cavity of rats. We administered varying doses of tamoxifen during the early stages of endometrial injury to observe the effects on the progression and outcomes of endometrial injury. Additionally, we investigated changes in epithelialization indices, mesenchymal indices, and estrogen receptors. We also sought to determine whether tamoxifen's anti-fibrotic activity is linked to the TGFβ_1/smad pathway. Our research aimed to provide an experimental foundation for understanding the pathological processes associated with endometrial trauma and to explore the potential use of tamoxifen in mitigating or preventing endometrial injury.

Approach and Techniques

Animals

Xiamen University's Experimental Animal Centre provided 30 female Sprague-Dawley (SD) rats, approximately ten weeks old, with weights ranging from 220 g to 250 g. The rats were housed in controlled conditions, including a room temperature kept at 21 $\pm$ 2 °C, consistent humidity levels maintained between 40% and 60%, a 12-hour light and dark cycle, and unrestricted access to food and water. All animal experiments strictly followed the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Xiamen University Laboratory Animal Management Ethics Committee permitted the study, with the number XMULAC20190165.

Reagents and Instruments

The following equipment and materials were employed: a Motic microscope (McArdie, Xiamen, China), a paraffin slicer (Jinhua Yidi Medical Equipment Factory, Jinhua, China), isoflurane (Lunanbeit Pharmaceutical Company, Linyi, China), microsurgical tools (Shanghai Jinzhong Instrument Factory, Shanghai, China), medical 7-0 suture (Shanghai Johnson & Johnson, Shanghai, China), and hematoxylin and eosin (Wuhan Xavier Biological Co., Ltd., Wuhan, China).

Establishment of an Endometrial Injury Model in Rats

Rat vaginal samples were collected twice a day, at 8:00 and 15:00, and subsequently stained with methylene blue to prepare smears. The rats were closely monitored throughout the estrous cycle, and female rats in the diestrus phase were selected for the experiment. Following a 12-hour period of fasting and dehydration, the rats were anesthetized using isoflurane from Lunanbeit Pharmaceutical Company (Linyi, China), administered through a small animal anesthetic recovery system at an inhalation concentration of 2.5%. Subsequently, the rats were immobilized, and their lower abdominal fur was shaved. Disinfection was performed by applying cotton balls soaked in an iodine-volt solution. An incision was made on the outer layer of skin, approximately 2–3 cm above the pubic symphysis, before longitudinally cutting the abdominal wall by 2 cm with scissors. The layers of tissue were incised sequentially to access the abdominal cavity.

The uterine area was exposed, and the initial procedure was performed on the left side. Two No.7 silk threads were used to block the proximal and distal ends of the uterus, secured with tight knots. Sterile gauze strips were then placed underneath the uterus for padding. A 1 mL empty syringe needle was inserted into the uterine cavity, positioned 1.5 cm away from the lower vascular clamp. It was essential to maintain a parallel alignment with the long axis of the uterus while extracting mucus from the uterine cavity. A 1 mL syringe filled with anhydrous ethanol was inserted into the uterine cavity along the original puncture hole. A gradual injection of 1 mL anhydrous ethanol was performed, and the uterine cavity was kept in a filled state for five minutes. Any remaining ethanol in the uterine cavity was aspirated, and the needle was gently withdrawn. Any residual anhydrous ethanol was carefully squeezed from the uterine cavity, followed by a saline flush and releasing the vascular clamp. The contralateral uterus was treated in a similar manner.

The uterus was then repositioned, and the abdominal layers were closed one by one after flushing the abdominopelvic cavity with saline and gently drying it with sterile gauze. The empty control group underwent analogous modifications but received an injection of saline solution.

Grouping and Handling

Six groups were established, each comprising thirty rats: the Control group, the Model group, the Estrogen re-

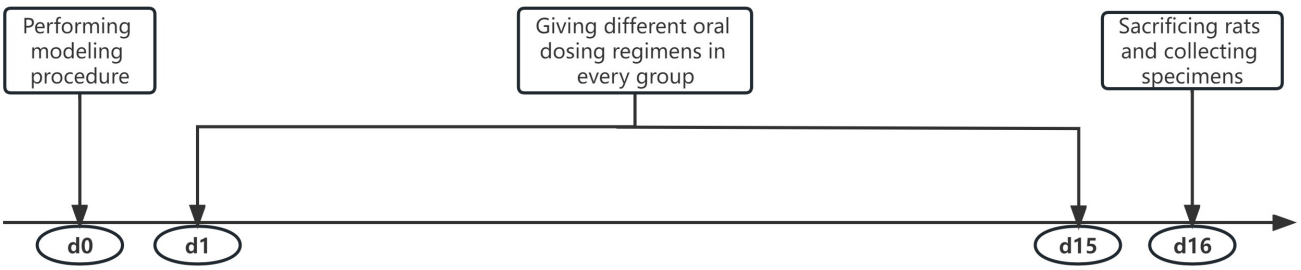

Fig. 1. Timeline for the experimental design.

pair group, the Low dose tamoxifen group, the Medium dose tamoxifen group, and the High dose tamoxifen group. Tamoxifen (Cat# HY-13757A, MCE, Monmouth Junction, NJ, USA) was dissolved in corn oil (Cat# HY-Y1888, MCE, Monmouth Junction, NJ, USA) at a concentration of 40 mg/mL. Rats in the tamoxifen repair group received daily oral doses of tamoxifen at varying levels, specifically 4 mg/kg/d, 20 mg/kg/d, and 40 mg/kg/d. For the estrogen repair group, estradiol (2 mg/kg/d, Cat# HY-B0141, MCE, Monmouth Junction, NJ, USA,) was dissolved in corn oil at a concentration of 12.5 mg/mL and given orally to the rats once a day. Rats in the control and model groups received identical amounts of corn oil orally on a daily basis. Oral dosing commenced the day after the modeling procedure and continued for 15 days, equivalent to three estrous cycles, as detailed in the timeline below (Fig. 1). With the exception of one rat in the model group who succumbed, all rodents remained in good health. On the 16th day, the rats were humanely euthanized by exposure to excessive carbon dioxide gas, and uterine samples were subsequently collected.

Staining with HE and Masson

The rat uterine tissues were sliced into 4 μm sections and prepared for paraffin embedding. Standard techniques from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China) were employed to stain the sections using Hematoxylin and Eosin (HE) as well as Masson's trichrome. The stained sections were examined under a biomicroscope (Olympus BX53, Olympus, Hamburg, Germany). The average width of the endometrium was determined based on HE stained images, involving four measurements taken at 0°, 90°, 180°, and 270° in horizontal sections, utilizing a 20× magnification. Parameters were extracted from images of tissues stained with Masson's trichrome through quantitative morphometry using ImageJ (version1.53, U. S. National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

We followed a standard protocol from ZSGB-BIO (Zhongshan Golden Bridge Co., Ltd., Beijing, China) for conducting Immunohistochemistry (IHC) staining on rat uterine tissues. After antigen retrieval, endogenous peroxidase activity was quenched using a 3% hydrogen peroxide solution. The samples were blocked with 10% normal bovine serum for one hour. To detect the expression of the mentioned proteins, we incubated the sections overnight at 4 °C with antibodies targeting E-cadherin, Vimentin, vascular endothelial growth factor (VEGF), estrogen receptor α (ERα), G protein-coupled receptor 30 (GPR30), and Smooth Muscle Actin (SMA). Subsequently, the slides were incubated with secondary antibodies (goat anti-rabbit or anti-mouse) and stained with DAB (diaminobenzidine, ZLI-9018, ZSGB-BIO) solution for one hour. After a brief staining with hematoxylin solution for 15 seconds, the pathological sections were initially examined under a biomicroscope (Olympus BX53, Olympus, Hamburg, Germany), then digitally scanned and converted into complete sections using the Motic VM (Motic, Xiamen, China) digital section scanning and processing system. The analysis and selection of regions of interest were carried out using the Motic DSAssistant Plus (Motic, Xiamen, China). For quantitative staining intensity analysis, ImageJ was employed, and differential comparisons were made using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, California, USA). A detailed list of the antibodies used can be found in **Supplementary Table 1**.

Western Blotting Analysis

Approximately 30 mg of uterine tissue was weighed and homogenized, and then 300 microliters of a lysis solution were added. The uterine tissue was crushed using a ball mill (GT300, LAB-BIOGEN, Kunming, China) operating at 25–30 Hz to extract proteins, and the protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (#C05-02001, Bioss, Beijing, China). Subsequently, electrophoresis was performed, and the separated proteins were transferred to PVDF membranes (EMD Millipore, Wilmington, Delaware, USA) using a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, California, USA). Prior to incubation with the primary antibody (estrogen receptor α, GPR30, E-cadherin, VEGF, Vimentin, TGFβ_1, SMA, Drosophila mothers against decapentaplegic protein 2 (Smad2), Drosophila mothers against decapentaplegic protein 3 (Smad3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 4 °C overnight, the membranes

were initially treated with 5% skimmed milk for 1 hour at room temperature. Afterward, chemiluminescence was used to detect the intensity of each protein band following a 1-hour incubation with the secondary antibody (goat anti-rabbit or anti-mouse) at room temperature. Image-Pro Plus (version 6.0, Media Cybernetics, Baltimore, Maryland, USA) was employed to analyze and determine the intensity of each protein band. GAPDH served as the internal control. Bar graphs were generated using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, California, USA).

Statistical Analysis

The data from our study is presented as the mean ± standard deviation (SD). Each experiment was performed independently in triplicate. To compare the two groups, we used the Student's *t*-test. Multiple group comparisons were conducted using one-way analysis of variance (ANOVA). Statistical significance thresholds, denoted as $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, were calculated using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, California, USA).

Results

Effects of Different Doses of Tamoxifen on Endometrial Thickness and Collagen Accumulation in Rats

In Fig. 2A, the uterine plasma membrane, myometrium, endometrium, and cavity epithelium remained intact and connected in the control group. The glandular and cavity epithelia primarily consisted of a single layer of cuboidal cells. The endometrium displayed a regular arrangement of functional and basal layers, maintaining its undulating shape and abundance of tubular glands and blood vessels (Fig. 2A,a).

Upon the establishment of the model group, a noticeable decrease in the thickness of the endometrial layers was observed, accompanied by a more significant disruption in the endometrium. The functional and basal layers underwent changes in their original form, losing their undulating shape. Glands and blood vessels became scarce, and the uterine cavity enlarged. The epithelial cells appeared compressed, adopting a low columnar or flattened shape (Fig. 2A,b). In the estrogen repair group, the uterine cavity remained visible, with a more pronounced reduction in endometrial thickness and an absence of observable glandular or vascular components (Fig. 2A,c). The endometrial composition and uterine cavity in the low dose tamoxifen group closely resembled those of the control group, showing a significant reduction in glands and no substantial thinning of the endometrium (Fig. 2A,d). However, both the tamoxifen groups with medium and high doses displayed a narrowed uterine cavity and diminished reparative capacity (Fig. 2A,e,f).

In comparison to the control group (Fig. 2B,a,b), the model group exhibited a significantly reduced uterine cavity size and a thinner endometrium ($p < 0.0001$). Collagen accumulation was significantly higher in the model group (Fig. 2C,a,b, $p < 0.001$). The thickness of the regenerated endometrium in the low dose tamoxifen group (455.98 ± 71.71 µm) was significantly greater than that in the model group (182.98 ± 30.77 µm) (Fig. 2B,b,d, **Supplementary Table 2**, Fig. 2D, $p < 0.01$). Masson staining revealed that the level of collagen accumulation in the low dose tamoxifen group was significantly lower compared to the model group (Fig. 2C,b,d, Fig. 2E, $p < 0.05$). There were no significant differences observed in the thickness of the endometrium and collagen deposition among the groups in the estrogen repair group, medium dose tamoxifen group, and high dose tamoxifen group compared to the model group (Fig. 2B,c,e,f,2C,c,e,f,2D,2E).

Effects of Different Doses of Tamoxifen on E-cadherin and Vimentin

In the control group, E-cadherin (Fig. 3A,a) and Vimentin (Fig. 3B,a) were observed in the cytoplasm of endometrial epithelial and stromal cells. However, in the model group, the levels of E-cadherin (Fig. 3A,b) and Vimentin (Fig. 3B,b) were significantly lower than in the control group (Fig. 3A–D, $p < 0.05$ and $p < 0.01$). In the low dose tamoxifen group, there was a notable increase in the number of cells positive for E-cadherin (Fig. 3A,d, Fig. 3C, $p < 0.0001$) and Vimentin (Fig. 3B,d, Fig. 3D, $p < 0.01$) compared to the model group. In the high dose tamoxifen group, there was a notable increase in the number of cells positive for E-cadherin (Fig. 3A,f, Fig. 3C, $p < 0.001$) compared to the model group. However, no significant differences were observed in the levels of E-cadherin between the estrogen repair group, the medium dose tamoxifen group and the model group (Fig. 3A,c,e,3C). No significant differences were observed in the levels of Vimentin between the estrogen repair group, the medium dose tamoxifen group, the high dose tamoxifen group and the model group (Fig. 3B,c,e,f,3D).

Effects of Different Doses of Tamoxifen on ERα, GPR30, and VEGF

In the control group, ERα (Fig. 4A,a) and GPR30 (Fig. 4B,a) were localized in the nucleus and cell membrane of endometrial epithelial cells. Most cells expressing VEGF (Fig. 4C,a) displayed staining. A substantial number of VEGF-positive cells were distributed throughout the endometrial layer, myometrium, and perivascular area. However, in the model group, ERα, VEGF levels significantly decreased compared to the control group (Fig. 4A,a,b,4C,a,b,4D,4F, $p < 0.05$ and $p < 0.0001$), no significant difference was observed in GPR30 (Fig. 4B,a,b,4E). After treatment, the low dose tamoxifen group and the estrogen repair group

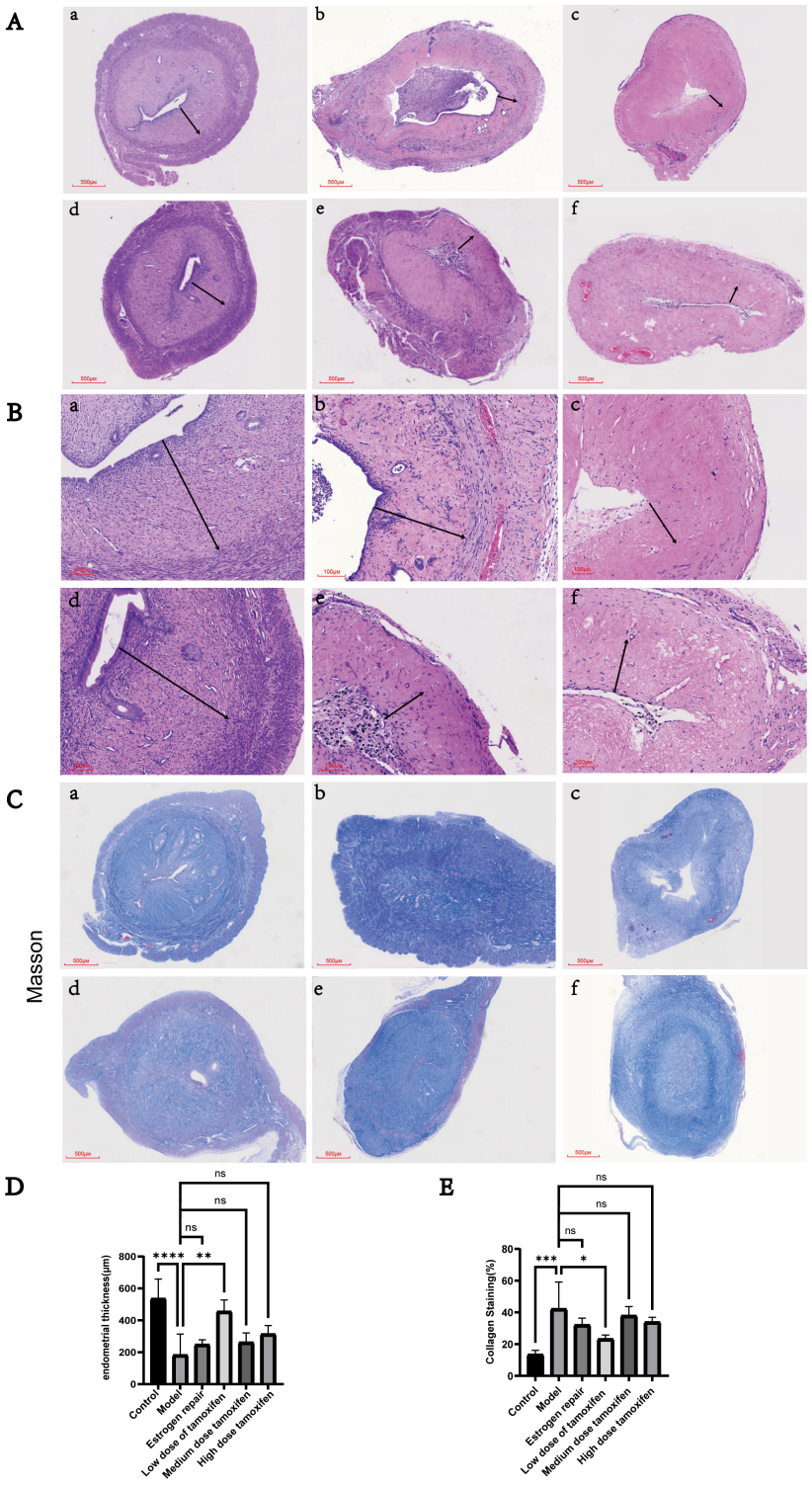

Fig. 2. The figure displays the Hematoxylin and Eosin (HE) and Masson staining of the endometrium in all rat groups. The groups consisted of the control group (a), the model group (b), the estrogen repair group (c), the low dose tamoxifen group (d), the medium dose tamoxifen group (e), and the high dose tamoxifen group (f). (A) HE staining of rat uterine tissue, with a scale bar of 500 μm. (B) Measurement of rat endometrial thickness indicated by solid black arrows, with a scale bar of 100 μm. (C) Masson staining was performed on rat endometrial tissue, with a scale bar of 500 μm. (D,E) Comparative bar charts presenting the endometrial thickness in rats and Masson staining results, with average values and standard deviations. Significance levels are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns, not significant; n = 4.

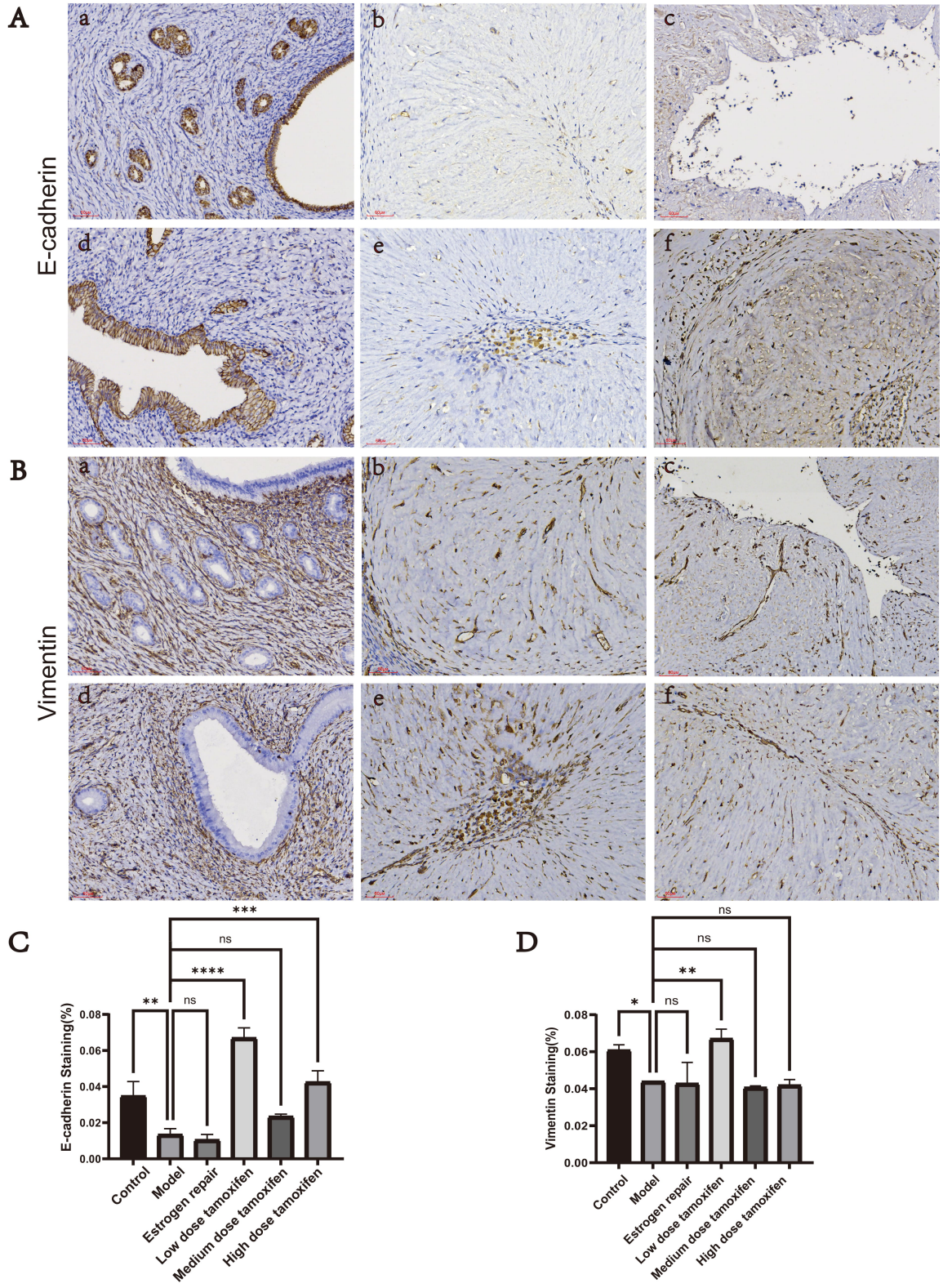

Fig. 3. The figure displays the immunohistochemical analysis of E-cadherin and Vimentin levels in the endometrium of rats from all groups. The groups in this analysis included the control group (a), the model group (b), the estrogen repair group (c), the low dose tamoxifen group (d), the medium dose tamoxifen group (e), and the high dose tamoxifen group (f). Immunohistochemistry (IHC) was employed to assess the expression of E-cadherin (A) and Vimentin (B) proteins in each group. Brown-yellow particles were observed, and the scale bar was set at 60 µm. The histograms (C,D) depict the percentage of E-cadherin and Vimentin staining in the rat endometrium for each group, as well as a comparative analysis between the groups. Statistical significance is denoted by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns, not significant; n = 4.

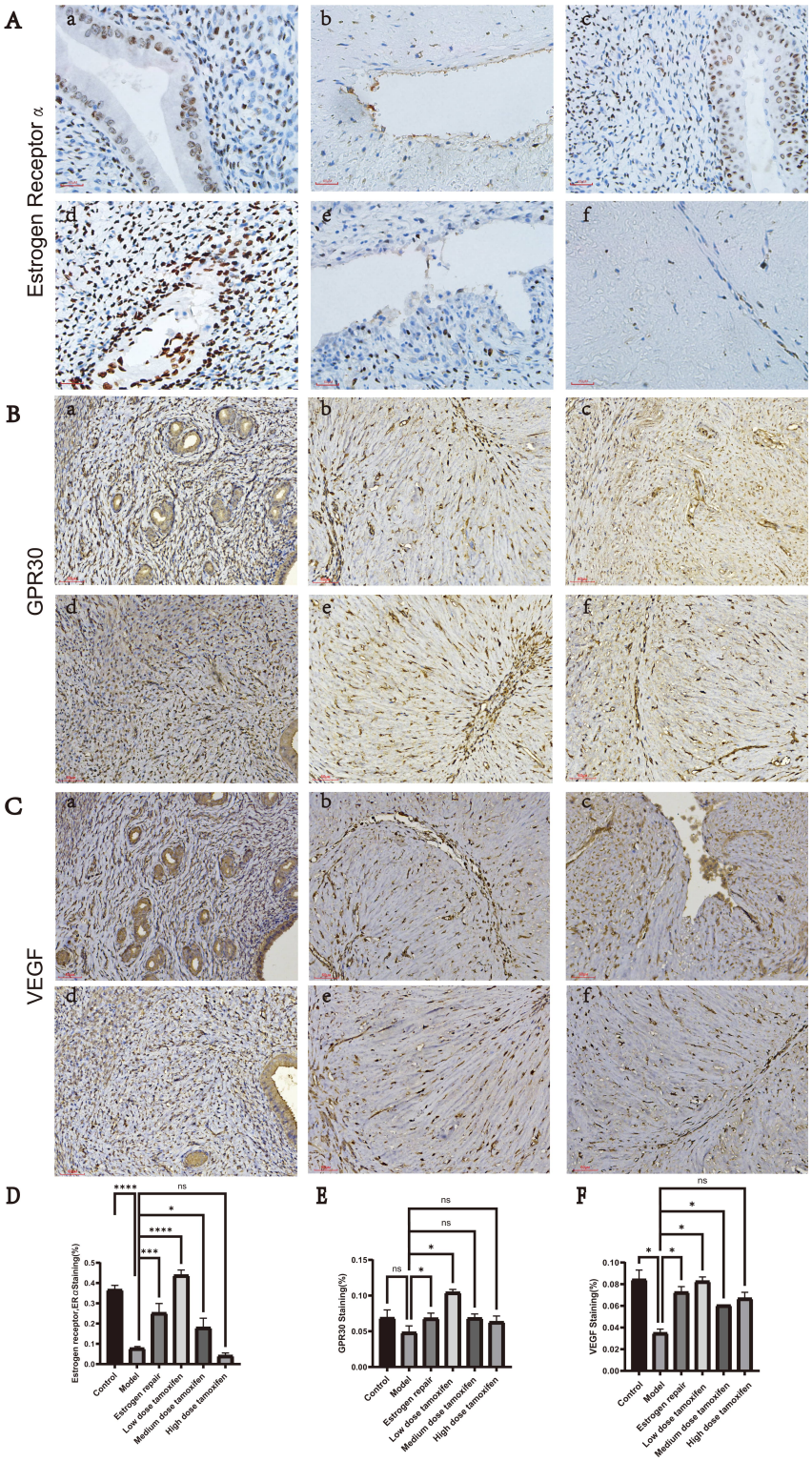

Fig. 4. The figure displays the ERα, GPR30 and VEGF expression by immunohistochemistry staining in the rat endometrium of different groups. The groups were categorized as follows: (a) control group, (b) model group, (c) estrogen repair group, (d) low dose tamoxifen group, (e) medium dose tamoxifen group, and (f) high dose tamoxifen group. The expression of ERα (A), GPR30 (B), and VEGF (C) in each group was detected using IHC, with the presence of brown-yellow particles. The scale bar represents 60 µm. The histograms (D–F) compare the percentage of ERα, GPR30, and VEGF staining in the endometrium of each group of rats. Statistical significance is indicated as $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$, ns, not significant; n = 4. ERα, estrogen receptor α; GPR30, G protein-coupled receptor 30; VEGF, vascular endothelial growth factor.

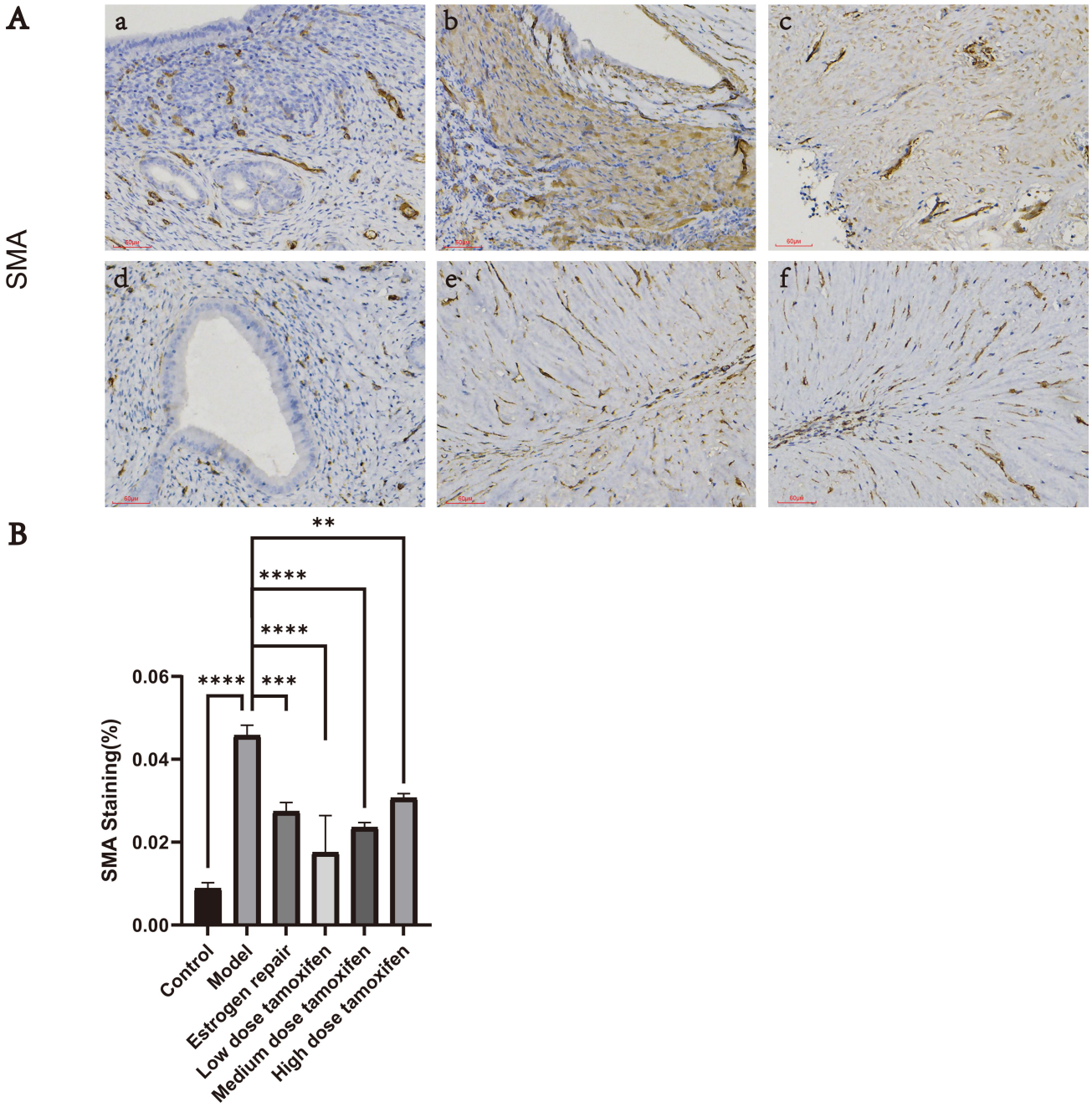

Fig. 5. The figure displays the Immunohistochemistry staining result of SMA proteins in different rats' groups. The groups were categorized as follows: (a) control group, (b) model group, (c) estrogen repair group, (d) low dose tamoxifen group, (e) medium dose tamoxifen group, and (f) high dose tamoxifen group. Immunohistochemistry was employed to detect the protein expression of SMA in the rat endometrium (A). The scale bar is 60 µm. The histogram (B) illustrates the distribution of SMA in different groups of stained rat endometrium and provides comparisons between each group. Statistical significance is denoted as $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$; n = 4. SMA, Smooth Muscle Actin.

showed significant increase in the total count of ERα, GPR30, and VEGF when compared to the model group (Fig. 4A,c,d,4B,c,d,4C,c,d,4D–4F, $p < 0.05$, $p < 0.001$ and $p < 0.0001$). In the medium dose tamoxifen group, ERα, VEGF levels significantly decreased compared to the model group (Fig. 4A,b,e,4C,b,e,4D,4F, $p < 0.05$), but no significant difference was observed in the GPR30 (Fig. 4B,b,e,4E). No significant differences were observed in the reported indices when comparing the model group with the high dose tamoxifen repair group (Fig. 4A,b,f,4B,b,f,4C,b,f,4D–4F).

Effects of Different Doses of Tamoxifen on SMA

The levels of Smooth Muscle Actin (SMA) in the model group showed a significant increase compared to the control group (Fig. 5A,a,b, Fig. 5B, $p < 0.0001$). However, the low dose tamoxifen group exhibited a significant decrease in SMA protein expression levels compared to the model group (Fig. 5A,b,d, Fig. 5B, $p < 0.0001$). Furthermore, the levels of SMA expression in the estrogen repair group, medium dose tamoxifen group, and high dose tamoxifen group were significantly lower than those in the model group (Fig. 5A,b,c,e,f, Fig. 5B, $p < 0.01$, $p < 0.001$, $p < 0.0001$).

Effects of Different Tamoxifen Dosages on the Protein Expression Levels of the Indicators of EMT and Fibrosis in Rat Uterine Tissues

Western Blotting (WB) was employed to assess the expression of estrogen receptors, markers of EMT, and endometrial fibrosis-related proteins following treatment. In comparison to the control group, the model group exhibited a significant decrease in the levels of E-cadherin, Vimentin, ERα, GPR30, and VEGF proteins (Fig. 6A–F, $p < 0.05$ and $p < 0.01$, respectively). Moreover, TGFβ_1 and SMA protein expression increased considerably in the model group (Fig. 6G,H, $p < 0.01$). Compared to the model group, the low dose tamoxifen group and estrogen repair group showed a marked increase in the levels of E-cadherin, Vimentin, ERα, GPR30, and VEGF proteins, while the expression of TGFβ_1 and SMA proteins significantly decreased (Fig. 6A–H, $p < 0.05$, $p < 0.01$, and $p < 0.001$). The indices of SMA in the medium dose tamoxifen and high dose tamoxifen repair groups did not exhibit any notable variation compared to the model group (Fig. 6A,H). In the model group, there was a significant increase in the levels of Smad2 and Smad3 proteins, while a marked decrease was observed in the low dose tamoxifen group (Fig. 6I,J, $p < 0.05$ and $p < 0.01$). These findings suggest that tamoxifen has an impact on the TGFβ_1/smad pathway.

Discussion

Intrauterine adhesion (IUA) is a common condition resulting from endometrial trauma that often leads to a thin endometrium. This condition significantly affects natural conception rates and embryo implantation rates in assisted reproductive techniques (ART). Estrogen is frequently used in clinical therapy to prevent and treat uterine adhesions, but the outcomes are inconsistent [17]. According to limited clinical studies [18], the administration of tamoxifen to patients with a history of previous endometrial damage during intrauterine insemination or frozen embryo transfer significantly improves endometrial thickness and pregnancy outcomes. However, due to the ethical considerations surrounding the endometrium, the mechanism by which tamoxifen enhances the thickness of the injured endometrium has not been extensively explored. To investigate the mechanism of tamoxifen's repair of the injured endometrium, we created an endometrial damage model by perfusing the uterine cavity of rats with anhydrous alcohol [19]. We administered varying doses of tamoxifen and estrogen for repair. Different doses of tamoxifen (4 mg/kg/d, 20 mg/kg/d, 40 mg/kg/d) were used to observe the drug's effects on various endometrial injury indicators. These indicators included endometrial morphology, thickness, estrogen receptors, VEGF, epithelial-mesenchymal transition markers, and changes in fibrosis markers. The study revealed that the use of 4 mg/kg/d tamoxifen during the early stages of endometrial injury increased endometrial thickness, preserved endometrial morphology, elevated ERα, GPR30, and vascular endothelial growth factor in the damaged endometrium, and reduced the occurrence of epithelial-mesenchymal transition and fibrosis.

Intrauterine adhesion is closely associated with epithelial-mesenchymal transition (EMT). The decrease in E-cadherin within epithelial cells indicates a reduction in intercellular gap junctions and a gradual acquisition of mesenchymal cell characteristics. Vimentin, an intermediate filament protein, plays a role in maintaining cell integrity and stabilizing the cytoskeleton. Additionally, E-cadherin and Vimentin serve as indicators of EMT, reflecting endometrial damage and recovery [20]. Moderate EMT contributes to tissue repair, while excessive EMT can lead to the replacement of normal tissue with fibrotic tissue or scarring, resulting in tissue and organ fibrosis. Intrauterine adhesion is fundamentally a fibrotic condition, and its development is associated with continuous and excessive endometrial EMT. Elevated Smooth Muscle Actin (SMA) levels in fibroblasts are a critical indicator of fibrosis development, as they signify the transformation of fibroblasts into myofibroblasts. Inhibiting EMT can help reduce the progression of endometrial fibrosis [21]. According to our findings, the endometrium of rats in the model group was significantly thinner, with fewer glands, and the morphology of the endometrial epithelial cells had changed to form elongated spindle-shaped cells, indicating a gradual transformation of epithelial cells into fibroblasts. These signs suggest that the endometrium in the model group had been injured. The IHC staining results for E-cadherin and Vimentin in the model group were considerably lower than those observed in the control group, indicating an ongoing epithelial-mesenchymal transition. In contrast, the endometrial thickness and epithelial morphology of the rats in the low dose tamoxifen group were significantly better than those in the model group. The levels of E-cadherin and Vimentin in this group were significantly higher than those in the model group, indicating better reepithelialization and repair capacity, with a certain degree of reversal of EMT. Additionally, we found that the content of SMA in the endometrium of rats in this group was significantly lower than that in the model group. Masson staining revealed that the content of collagen fibers in the endometrial

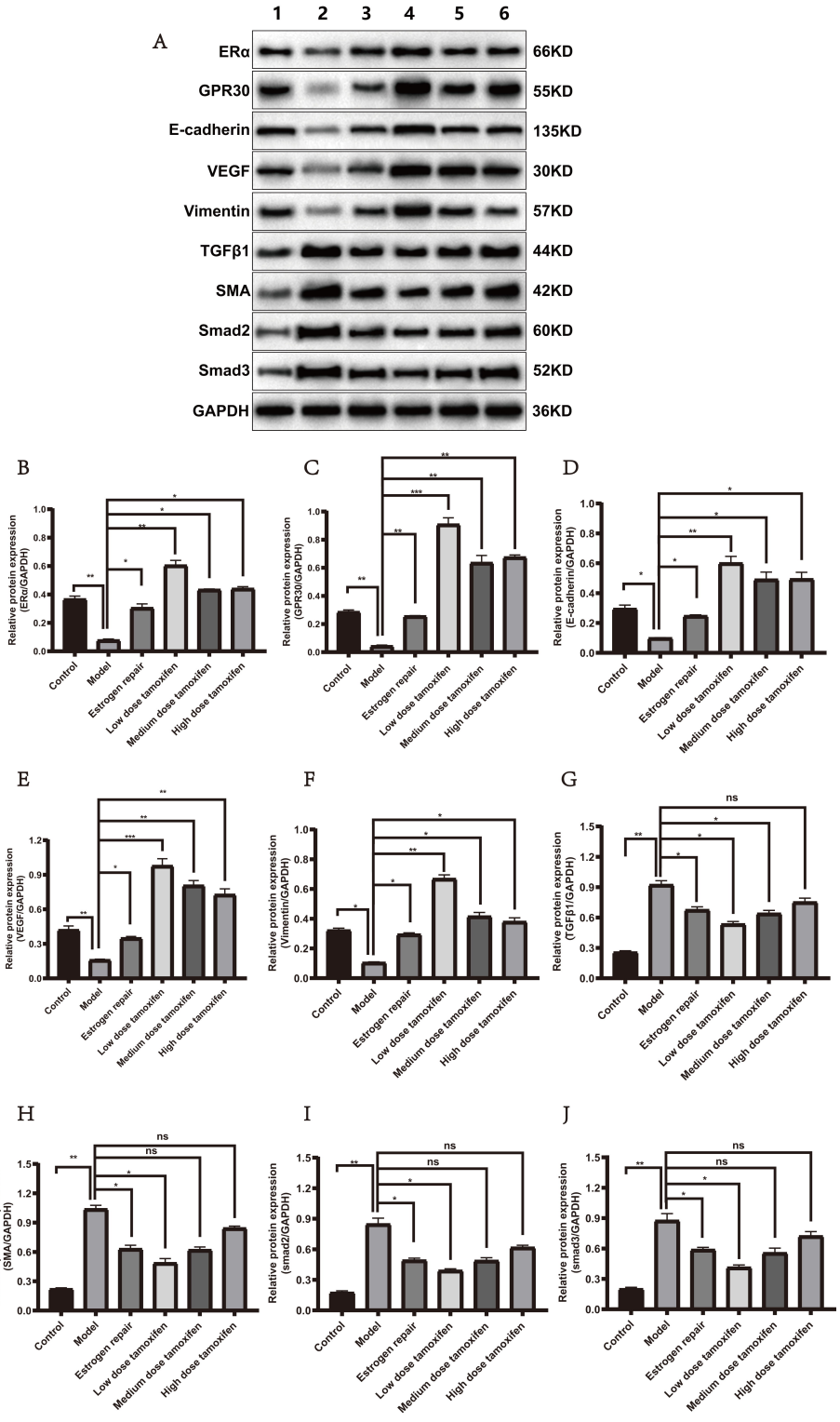

Fig. 6. The figure displays the protein expression of markers for estrogen receptors, epithelial-mesenchymal transition (EMT) and endometrial fibrosis with Western Blotting. The Western Blotting technique was utilized to analyze the markers of endometrial EMT, fibrosis, and the Transforming Growth Factor $\beta 1$ (TGFβ_1)/smad pathway. The groups were categorized as follows: (1) control group, (2) model group, (3) estrogen repair group, (4) low dose tamoxifen group, (5) medium dose tamoxifen group, and (6) high dose tamoxifen group. (A) Western Blotting analysis was performed on various endometrial markers in separate rat groups. (B–J) The grey values of each marker were compared using histograms. Statistical significance is denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, not significant; n = 4. Smad2, Drosophila mothers against decapentaplegic protein 2; Smad3, Drosophila mothers against decapentaplegic protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

epithelium of rats in this group was significantly lower than that in the model group. These findings suggest that the use of an appropriate amount of tamoxifen (4 mg/kg/d) can provide better repair of endometrial injury in rats. Using tamoxifen early in the endometrial damage phase can reverse the EMT process and minimize endometrial fibrosis.

Tamoxifen is known to modulate the estrogen receptor [22] and has varying effects on the endometrium depending on the surrounding levels of estrogen. It acts as an estrogen receptor antagonist in breast tissue, inhibiting cancer cell proliferation [23]. In premenopausal women with elevated estrogen levels, tamoxifen demonstrates an estrogen-blocking effect. Conversely, in postmenopausal women with low estrogen levels, tamoxifen exerts an estrogen-agonistic effect [24,25]. Tamoxifen's ability to stimulate endometrial hyperplasia has garnered attention in breast cancer patients. The endometrial tissue contains estrogen receptors, including ERα, ERβ, and GPR30. ERα and ERβ have distinct signaling pathways and mediate different biological responses. ERα primarily promotes the differentiation and proliferation of endometrial epithelial cells, while ERβ mainly induces apoptosis [26]. GPR30, a distinct estrogen receptor found on the cell membrane, operates with a unique and rapid mechanism compared to ERα and ERβ [27]. GPR30 has been suggested to be the primary mediator of immediate cellular responses triggered by estrogen. GPR30 expression is often elevated in pathological conditions where high levels of GPR30 are present, such as in focal tissues in endometriosis and endometrial tissue in endometrial hyperplasia [28–32].

However, there has been limited research on how tamoxifen affects the various endometrial estrogen receptors in the context of endometrial injury. In our study, we discovered that ERα and GPR30 levels were significantly lower in the model group compared to the control group, indicating that endometrial injury leads to reduced ERα and GPR30 levels in the endometrium. This may explain why the damaged endometrium becomes less responsive to estrogen stimulation. Both the low dose tamoxifen and estrogen repair groups showed some elevation of ERα and GPR30 levels in the endometrium, with the low dose tamoxifen having a greater enhancing effect than estrogen. Therefore, tamoxifen might play a role in endometrial repair by increasing ERα and GPR30 levels in the injured endometrium. However, we found that increasing the tamoxifen dose in the medium dose and high dose tamoxifen groups did not further raise ERα and GPR30 expression levels compared to the low dose tamoxifen group. This suggests that increasing the tamoxifen dose did not lead to a more significant agonistic effect on the estrogen receptor. This may be due to a limited number of estrogen receptors and receptor saturation, making it ineffective to increase the tamoxifen dose regularly for damaged endometrium.

Tamoxifen does not solely stimulate endometrial proliferation by activating estrogen receptors. A clinical trial employed microarray technology to conduct genetic testing on the endometrium of postmenopausal women undergoing treatment with estradiol and tamoxifen [33]. The results revealed significant differences in gene expression within their endometrial tissues, with an estimated 75% disparity in the genetic composition between the two groups. This indicates that the growth-promoting effects of tamoxifen and estrogen on the endometrium may be attributed to different genetic factors and pathways. Tamoxifen's role in stimulating endometrial proliferation extends beyond its function as an estrogen receptor agonist. Angiogenesis is a critical process in endometrial regeneration, ensuring tissue perfusion, which, in turn, promotes endometrial cell proliferation and faster tissue expansion. Additionally, uterine blood vessels play a pivotal role in endometrial metaplasia, embryo implantation, and the development of tiny spiral arteries [34]. Vascular endothelial growth factor (VEGF) plays a key role in promoting endothelial cell proliferation, altering permeability, facilitating migration, and aiding in the formation of capillaries. This factor is crucial for the accelerated growth of the vascular system in the human endometrium [35], particularly in the context of endometrial regeneration.

In our study, we observed that VEGF levels in the endometrium of rats in the model group were significantly lower than those in the control group, indicating an impaired ability for blood vessel regeneration following endometrial injury. However, when we used estrogen and tamoxifen to repair the rat endometrium, VEGF levels were substantially higher than in the control group. Remarkably, tamoxifen exhibited a greater potential to increase VEGF levels in the injured endometrium compared to estrogen. Furthermore, we found that the proper dose of tamoxifen (4 mg/kg/d) significantly elevated the level of vascular endothelial growth factor in the damaged endometrium. Consequently, we propose that tamoxifen may promote endometrial regeneration by not only enhancing estrogen receptors but also by increasing the number of uterine blood vessels through the activation of VEGF expression in the injured endometrium.

The mechanism through which tamoxifen inhibits endometrial fibrosis involves its impact on the TGFβ_1/smad pathway. Studies have consistently shown that smad2/3-mediated TGFβ_1 signaling is a key driver of the fibrotic process [5]. Building on previous research on tamoxifen's effects in various fibrotic conditions [10,16,36], we investigated the molecules within the TGFβ_1/smad pathway. Our findings revealed that in the model group, the levels of TGFβ_1, smad2, and smad3 were significantly increased compared to the control group. However, in the low dose tamoxifen group, these molecules were significantly decreased compared to the model group. This indicates that the early administration of tamoxifen in the context of endometrial injury has a notable effect on the TGFβ_1/smad pathway, potentially contributing to the inhibition of endometrial fibrosis.

Conclusions

In summary, our research has shown that the early use of a low dose of tamoxifen (4 mg/kg/d) when endometrial damage begins has a positive impact on preventing such damage. Tamoxifen is more effective than estrogen in repairing the endometrium and restoring its morphology and structure to normal following injury. Moreover, the early administration of tamoxifen in the course of endometrial trauma significantly reduces the progression of endometrial fibrosis. This effect may be attributed to tamoxifen's ability to reverse EMT and influence the TGFβ_1/smad pathway. The findings of this study offer new perspectives on the prevention and treatment of intrauterine adhesions.

Availability of Data and Materials

Data of this manuscript is available from the corresponding author based on reasonable request.

Author Contributions

HJ and JS performed the most of the experiments and wrote the manuscript. XH contributed to the rat model construction and sample collection. MY contributed to the data analysis, HW prepared the figures and interpreted the data. HJ and XD designed the experiments and revised the manuscript. XH, MY, HW were involved in the drafting and revising of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The research received the endorsement of the Ethics Committee for Laboratory Animal Management at Xiamen University, under the reference XMULAC20190165.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436180.15.

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