The Effect of Low Intensity Pulsed Ultrasound on Endometrial Receptivity in Infertile Patients with Adenomyosis

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Abstract

Objective: Adenomyosis (AM) is an important cause of female infertility, and its disease mechanism remains unclear. This study preliminarily investigated the expression of endometrial receptivity markers homeobox A10 (HOXA10) and leukemia inhibitory factor (LIF) in infertile patients with AM and described the effects of low intensity pulsed ultrasound (LIPUS) on it.

Methods: *In vivo*, tissues were obtained from the infertile female AM patient group (AG group, n=10) and healthy control group (CG group, n=11). The expression of HOXA10 and LIF in the two groups was detected by immunohistochemistry (IHC) and western blotting. *In vitro*, primary cells were extracted and cultured from the two groups, and the expression of HOXA10 and LIF protein was detected by western blotting. Then the AG cells were treated with 15, 30, and 60 mW/cm^2 of LIPUS for 7 days (20 min/day), and detected the cell adhesion rate. Finally, treat the AG cells with 30mW/cm^2 LIPUS for 7 days (20 min/day), and detect the expression level of ICAM-1 in the cell supernatant by ELISA. The AG cells was treated with 30 mW/cm^2 LIPUS for 4 days (20 min/day), and the expression levels of HOXA10 and LIF were detected by western blotting, RT-PCR, and agarose gel electrophoresis.

Results: In vivo, IHC staining showed that HOXA10 and LIF proteins were mainly localized in endometrial epithelial cells. Both IHC and western blot showed that the levels of HOXA10 and LIF in the AG group were significantly lower than those in the CG group (P<0.01, P<0.05). In vitro, the expression levels of HOXA10 and LIF protein in the AG cell was significantly lower than those in the CG cell (P<0.001). Then, the cell adhesion ability of the 30 and 60 mW/cm^2 groups was higher than that of the 15 mW/cm^2 group after LIPUS treatment. Finally, The concentration of ICAM-1 in the supernatant of AG cells treated with LIPUS was significantly higher than that of the control group (P<0.01), and the AG cells were treated with 30 mW/cm^2 LIPUS for 4 days (20 min/day), the protein and mRNA expression levels of HOXA10 and LIF were higher than those of the control group (P<0.001).

Conclusion: The reduction of HOXA10 and LIF may be one of the reasons for the decreased endometrial receptivity in AM. The LIPUS promoted the adhesion and the expression of HOXA10 and LIF of EEECs from the AM group, thereby increasing endometrial receptivity.

1. Introduction

Adenomyosis refers to a condition in which the endometrium infiltrates the myometrium and grows diffusely. The main symptoms of this condition are the progressive aggravation of dysmenorrhea, increased menstrual flow, and prolonged menstrual periods(1). Adenomyosis with infertility is not the main symptom of these patients. However, as a women's age at childbirth is delayed, the number of patients with adenomyosis who are complicated by infertility also increases; therefore, the proportion of patients with adenomyosis who undergo an abortion is high (11%-63%)(2). Under the action of estrogen and progesterone, the endometrial structure undergoes specific and periodic changes. The endometrium is usually capable of undergoing embryo implantation 6-9 days after ovulation. This period is called the

“implantation window” for embryo implantation(3). In the “planting window”, the endometrium allows the blastocyst to locate and adhere to the endometrium, and then invades and causes changes in the endometrium stroma. The degree of acceptance is called endometrial receptivity. Endometrial receptivity is used as an evaluation criterion to evaluate the success rate of embryo implantation. However, this receptivity is restricted by both time and space(4). If the receptivity of the endometrium is reduced, embryo implantation will fail(5). Therefore, determining methods to improve endometrial receptivity has become critical in increasing the pregnancy rate. Thus, gaining a better understanding of endometrial receptivity markers has become the basis for research. The improvement of embryo quality and endometrial receptivity are two essential components that could improve the success rate of pregnancy; the improvement of endometrial receptivity is becoming a hot spot for research in reproductive medicine.

Measures to improve the endometrial receptivity and clinical pregnancy rate of patients with adenomyosis include hormonal drug therapy, conservative surgical treatment with the partial removal of lesions, and mechanical stimulation. Hormonal drug therapy is now mainly based on oral gonadotropin-releasing hormone agonist (GnRHa)(6). Studies have shown that GnRHa can improve endometrial thickness and endometrial receptivity in infertile women by increasing endometrial blood perfusion and the expression of genes and proteins related to endometrial receptivity, such as VEGF, ITGB3, and IGF-1. However, because the effect of GnRHa is reversible, the GnRHa treatment is mainly short-term; the long-term effect of this treatment is poor. Surgical resection of the lesion is not the main form of treatment because it is prone to recurrence. Relevant studies have shown that the pregnancy rate can be improved by regular mechanical stimulation of the endometrium(7). This method has been used in a few assisted reproductive centers to improve endometrial receptivity and has achieved certain good results.

Ultrasound therapy has the advantage of being precise and noninvasive compared to drugs, conservative surgical excision, and mechanical stimulation while improving symptoms and other treatments. At present, there are three known biological effects induced by ultrasound: mechanical effect, thermal effect, and cavitation effect(8). LIPUS mainly exerts mechanical effects and can produce mechanical stimulation and induce biological effects through ultrasonic conduction within the body. *In vitro* cell experiments(9) have found that cellular damage can be improved by the administration of LIPUS as this technique increases cell adhesion and proliferation. In the present study, we collected samples of eutopic endometrium from patients with adenomyosis complicated by infertility and women of normal reproductive age. We then analyzed the differences in expression of endometrial receptivity markers (HOXA10 and LIF) in samples of eutopic endometrium from the two groups by western blotting and immunohistochemistry. We also isolated EEEC cells from the two groups. After culture and identification, western blotting was also used to analyze the differences in expression of endometrial receptivity markers in the two different types of EEECs. The eutopic endometrial cells from patients with adenomyosis were stimulated with LIPUS at different intensities to investigate changes in adhesion ability. Then, we selected the optimal intensity. EEECs from patients with adenomyosis and infertility were then treated with the selected optimal LIPUS intensity, and the supernatant was collected from cultured cells. Changes in the levels of markers of cell adhesion ability were then detected by ELISA, and the optimal LIPUS effect intensity that caused biological effects on the EEECs from patients with

adenomyosis was determined. EEECs from patients with adenomyosis were cultured at this intensity and changes in the levels of endometrial receptivity markers were compared before and after treatment. We aimed to investigate the biological effects and possible mechanisms of action for LIPUS on the eutopic endometrium of patients with adenomyosis and infertility at the cellular and molecular levels to provide a theoretical and experimental basis for the further rational application of LIPUS in clinical practice.

2. Materials And Methods

2.1 Subjects

Ten eutopic endometrial tissues were acquired from infertile patients with adenomyosis attending the First Affiliated Hospital of Chongqing Medical University during the mid-secretion phase to form the AG group. Patient age ranged from 27 to 45 years with a mean age of 36.80±8.21 years.

The normal eutopic endometrial group (the CG group) featured 11 healthy women from the First Affiliated Hospital of Chongqing Medical University. Age ranged from 26–45 years with a mean age of 35.91±9.61 years. Endometrial samples were taken during the normal secretion phase.

There was no significant difference in age between the two groups ($P > 0.05$). Each patient underwent curettage or surgery to obtain endometrial samples, and no endometrial lesions were confirmed by pathology. All specimens were selected from January 2014 to March 2015 in the First Affiliated Hospital of Chongqing Medical University and confirmed by surgery and pathology. The study was approved by the Ethics Committee of Chongqing Medical University and all patients provided informed and signed consent.

The inclusion criteria for the AG were as follows (1) women of reproductive age, 25–45-years-old, with a normal sexual life, no contraception and no pregnancy for 1 year, or having a history of pregnancy and not becoming pregnant again for 1 year; (2) normal menstrual cycle of 21–35 days with a bipolar basic temperature; (3) no abnormalities with regards to basic endocrinology; (4) no hormone therapy over the previous three months; (5) no organic lesions detected in the ovary by B ultrasound; (6) bilateral oviduct patency was confirmed by salpingography, and (7) there was no abnormality with regards to semen analysis in the spouse. The inclusion criteria for the CG group were as follows: (1) women of reproductive age, 25–45-years-old, with a normal sexual life, a history of pregnancy, and no history of infertility; (2) normal menstrual cycle of 21–35 days with a bipolar basic temperature; (3) no abnormalities with regards to basic endocrinology; (4) no hormonal therapy over the previous three months; and (5) no organic lesions in the uterus and ovary.

Patients were excluded if they (1) had infertility caused by abnormal follicles or other reasons; (2) had serious primary diseases of the cardiovascular system, liver, and kidneys, combined with medical and surgical diseases; (3) were unable to cooperate, such as those with neurological or mental disorders, or were unwilling to cooperate; or (4) had an allergic constitution.

2.2 Specimen collection

Twenty-one samples of eutopic endometrium were obtained by surgery and curettage. All cases were confirmed by laparoscopy or laparotomy and verified by pathological sections. All pathological specimens were collected and divided into three parts: one part was immediately placed into D-Hanks solution pre-packed in an icebox and sent to the laboratory within 1–2 h to prepare for subsequent cell culture tests. One part was quickly placed in liquid nitrogen to prepare for western blotting. The last part was placed in 10% formalin, fixed for 24 h, and then embedded in paraffin. This part was used for HE staining, endometrial staging, and immunohistochemistry.

2.3 Experimental groups

In vitro experiments are divided into two groups: (1) CG: eutopic endometrium from healthy women during the mid-secretion phase (n=11) and (2) AG: eutopic endometrium from infertile women with adenomyosis during the mid-secretion phase (n=10).

In vivo, the endometrial cells were divided into two groups: (1) CG: eutopic endometrial cells in mid-secretion of healthy women. (2) AG: eutopic endometrial cells in mid-secretion of infertility in adenomyosis.

In vivo, AG groups cells treated with LIPUS were divided into two groups: (1) Control: No LIPUS treated AG groups cells. (2) LIPUS: 30 mW/cm^2 LIPUS treated AG groups cells for 4 days (20 min/day)

2.4 LIPUS applied to cells

Apply special ultrasonic adhesive to make the bottom of the 6-well plate close to the ultrasonic probe on the support table. The low intensity pulsed ultrasonic instrument is provided by the key laboratory of biomedical engineering college, Chongqing medical university. The ultrasonic frequency is 1.5MHz, the ultrasonic gap time is 800μs, the pulse frequency is 1kHz, and the action time is 200μs. After 24 hours of cell culture, LIPUS was applied to the cultured EEEC from infertile patients with adenomyosis *in vitro* with ultrasonic intensities of 15, 30, and 60mW/cm^2, respectively. Ultrasound stimulation of different intensity (20min/ day) was started for 1, 2, 3, 4, 5, 6, 7 days. Continue to incubate after sonicating the cells every day.

2.4 IHC

IHC staining methods were carried out following the manufacturer's instructions (Biotin Labeling Immunohistochemical Kit SP-9000 Sheep Resistance Rabbit /Mouse/Guinea pig/Rat IgG; Beijing Zhongshan Cor, China). Sections (4 μm thick) were incubated with H_2O_2 to eliminate endogenous

peroxidase activity for 10 min and then blocked with 10% goat serum at 37ºC for 30 min. The slices were incubated with rabbit monoclonal HOXA10 antibody (1/1,000, GeneTex, USA), rabbit monoclonal LIF antibody (1/800, Abcam, USA), rabbit monoclonal CK19 antibody (1/800, Abcam, UK) overnight at 4ºC. The next morning, appropriate conjugated Sheep Resistance Rabbit IgG antibodies (Beijing Zhongshan Cor, China) were incubated with the slices at 37ºC for 40 min. The sections were then washed and developed with DAB as a chromogen. Sections were then coverslipped and sealed with neutral resin. Representative images were then acquired by microscopy. The histological integral H-score method proposed by Budwig-Novotny was used for semi-quantitative analysis; the formula was h-score =ΣPi(i+l) (10). Pi represents the percentage of the number of positive cells with the same staining intensity in the immunohistochemical staining range of each tissue relative to the total number of cells to be tested; that is, Pi = the number of positive cells/total number of cells to be tested. Staining intensity was graded as 1 (+), 2 (++), and 3 (+++). The maximum H-score was 4, and 1 is the correction factor.

2.5 Western blotting

The tissues were mixed with RIPA protein lysis buffer (Bi Yuntian Biotechnology Institute, China) and fully homogenized, lysed, and centrifuged. The supernatants were then collected. Protein concentration was determined by the BCA method (BCA Protein Concentration Quantitative Kit; Biyuntian Biotechnology Institute China). Adjust the protein concentration to 10ug/ul, and the protein loading volume is 10ul/well. Protein samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (upper glue: 90v, 20min, lower glue: 120v, 90min), and then transferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). After being blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 for 1 h. The membranes were incubated with primary antibodies at 4℃ overnight as follows: rabbit monoclonal HOXA10 antibody (1/1,000; GeneTex, USA); Rabbit monoclonal LIF antibody (1/800, Abcam, UK). The next morning, membranes were washed and incubated with secondary antibody (sheep anti-rabbit IgG, Abcam, UK; 1:2,000). Positive binding was detected with an ECL Kit (Biyuntian Biotechnology Institute, China) and a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA). The optical density of each band was analyzed by ImageJ.

2.6 Cell adhesion experiment

Matrigel (BD Bioscience, USA) was used to cover 96-well plates and sealed with 2% BSA. For experimentation, we selected EEECs from the CG and AG that had entered the logarithmic growth phase. Samples from the cell cultures were digested and centrifuged. This allowed us to count the number of live cells, adjust the cell density to 5×10^4 cells/mL; we then added 200 μL of the cell suspension to each well for culture. After incubation for 4 h, 200 μL of serum-free DMEM-F12 culture medium and 20 μL of MTT (Sigma-Aldrich, USA) solution were added for 4 h. The culture medium was then drained from each well and 200 μL of DMSO solution was added. Then, the 96-well plates were placed on an oscillator for 10 min until fully dissolved. The absorbance (A570) value of each well at 570 nm wavelength was then

measured by an automatic enzyme reader and the adhesion rate was calculated. The experiment was repeated three times. Cell adhesion rate = experimental well A570/ control well A570 × 100%.

2.7 ELISA

The concentration of ICAM-1 in the supernatant of EEEC *in vitro* cultured of infertile patients with adenomyosis was determined by ELISA. The specific steps were carried out step by step according to the instructions [human intercellular adhesion molecule-1, ICAM-1 ELISA Kit; Abcam, UK]. Final determination (within 15 min after adding stop solution): set the blank well to zero, and then measure the absorbance (OD) of each well in sequence at 450 nm using an ELX800 microplate reader (Bio-Tech, USA). The actual concentration of each target factor was calculated according to the standard curve. The experiment was repeated three times.

2.8 RT-PCR and agarose gel electrophoresis

Cells were lysed by Plus (Invitrogen, USA). RNA was then isolated, precipitated, washed, and dissolved. The ratio of RNA A260/A280 was then determined. Samples with a ratio of 1.8-2.0 were used for RT-PCR experiments. A GeneQuant was used to determine the concentration of each sample. RNA concentration (μg/μL) = concentration × dilution ratio. Reverse transcription was performed according to the instructions of the reverse transcription PCR kit (TAKARA, Shiga Japan). The primers were designed and synthesized by TAKARA. Next, 5 μL of the reaction products were mixed with sample loading buffer for electrophoresis. All RT-PCR results were captured by a gel imager and analyzed by QuantityOne software. Results were expressed as the ratio of the optical density value of a given band for the target mRNA to GAPDH.

The mRNA expression levels of HOXA10 and LIF in EEECs cultured *in vitro* from infertile patients with adenomyosis during the mid-secretion phase after LIPUS treatment was detected by RT-PCR. The sequences of the forward (F) and reverse (R) primers were as follows: HOXA10, F-5'-ACTGCTCGAAATGGCCCCTGTC-3', R-5'-CGGCTCCTTTGCACCA TTGACC-3'; LIF, F-5'-GCCCACCCTGTCCTTGTTTCTG-3', R-5'-CGGGCCACAGGAGGAGCTT-3', GAPDH, F-5'-TGGGGTGATGCTGGTGCTGAGT-3', R-5'-AGGTTTCTCCAGGCGGCA TGTC-3'. The product length was as follows: HOXA10 (132 bp), LIF (117 bp), GAPDH (500 bp).

2.9 Statistical analysis

SPSS version 22.0 statistical software (IBM Cor, USA) was used for analysis, and values are expressed as mean ± standard deviation. The Mann-Whitney test was used to compare age between the CG and AG groups. The chi-square test, rank-sum test, analysis of variance, and *t*-test were used to compare data between groups. $P<0.05$ was considered statistically significant.

3. Results

3.1 IHC detection of HOXA10 and LIF in the CG and AG groups

Immunohistochemical analysis showed that HOXA10 and LIF proteins were mainly expressed in endometrial epithelial cells, although lower levels of expression were detected in the stromal cells. HOXA10 was mainly expressed in the cytoplasm or nucleus, while LIF was mainly expressed in the cytoplasm. All 21 endometrial samples were positive for these two proteins. The expression of HOXA10 and LIF in the CG group was significantly higher than that in the AM group (P<0.01). The expression levels of HOXA10 and LIF in normal endometrium were significantly higher than those in infertile patients with AM (Figure 1A). H-score results showed that the levels of HOXA10 and LIF secreted during the mid-stage phase and detected in endometrial biopsies from the AG group were lower than those in the CG group (Figure 1B).

3.2 Western blot results for HOXA10 and LIF proteins in the CG and AG groups

The expression of HOXA10 and LIF was detected in all 21 endometrial tissues. The expression of HOXA10 and LIF in the AG was significantly lower than that in the CG (P<0.05). The expression levels of HOXA10 and LIF in infertile patients with AM were significantly lower than those in normal endometrium and were closely related to the occurrence of adenomyosis and infertility (Figure 2).

3.3 Isolation and identification of EEECs in the CG and AG groups and western blotting of HOXA10 and LIF proteins in CG and AG cells cultured *in vitro*

Previous research showed that keratin exists in epithelial cells(11, 12); therefore, we performed immunocytochemical staining with epithelial cell-specific cytokeratin (CK19) antibodies. Positive immunostaining appeared as a brownish yellow color in the cytoplasm and blue-purple nuclei in epithelial cells (Figure 3A).

The expression of HOXA10 and LIF protein in EEECs from the CG and AG groups was detected by western blotting. The expression of HOXA10 and LIF in the AG cell was significantly lower than that in the CG cell

3.4 The cell adhesion ability of cells from the AG group with different intensities and periods of LIPUS treatment

Endometrial epithelial cells were treated with LIPUS at different intensities (15, 30, and 60 mW/cm^2) for 7 days (20 min/day). Adhesion ability test results showed that the 30 and 60 mW/cm^2 groups was significantly 1.5 times higher than that in the 15 mW/cm^2 group (P<0.05). However, there was no significant difference between the 30 and 60 mW/cm^2 groups. Importantly, the adhesion ability of endometrial epithelial cells gradually increased on days 3-4 and reached the highest on day 4 (P<0.01) (Figure 4).

3.5 The elisa detection of ICAM-1 in AG cell with 30mW/cm2 LIPUS for 7 days, and the western blot, RT-PCR of HOXA10 and LIF proteins in AG cell with 30mW/cm^2 of LIPUS for 4 days

We selected 30 mW/cm^2 over 7 days (20 min/day) as the LIPUS treatment. ELISA showed that the concentration of ICAM-1 in the supernatant of AG cells treated with LIPUS was significantly higher than that of the controls, and the concentration of ICAM-1 increased significantly on days 3-4 (P<0.05); the concentration of ICAM-1 stabilized after the 4th day (Figure 5A).

Then, we tested an intensity of 30 mW/cm^2 for 4 days for LIPUS treatment. The protein expression levels of HOXA10 and LIF in the LIPUS group were significantly higher than those in the control group (P<0.05) (Figure 5B, C). RT-PCR (Figure 5D) and agarose gel electrophoresis (Figure 5E) further indicated that the mRNA expression of HOXA10 and LIF in the AG group, when treated with LIPUS, was significantly higher than the controls(P<0.001).

Discussion

Implantation is accomplished by the blastocyst contacting and invading the endometrium. Adhesion molecules play a major mediating role in the interaction between the blastocyst and endometrium. The homeobox (*HOX*) gene is a transcription regulatory gene that plays a biological role mainly by binding with DNA to activate or inhibit a target gene and is a member of a multi-gene family. The *HOXA10* gene is mainly expressed in the human endometrium; its function is to maintain a normal morphology in the endometrium, promote proliferation and differentiation, establish endometrial receptivity, and mediate implantation and development of embryos(13). Ashary(14) previously found that HOXA10 was detected in every stage of the menstrual cycle. Compared with early proliferation, late proliferation, and early secretion, the expression levels of HOXA10 are known to be significantly increased in the middle and late secretion stages, thus suggesting that the expression levels of HOXA10 are closely related to embryo implantation. In a previous study, Bagot also found that normal mouse embryos could not implant into the uterus of mice in which the *HOXA10* gene had been deleted(15, 16). The expression of the *HOXA10* gene was found in both ectopic and eutopic endometrium of patients with endometriosis; the expression levels were low in the epithelial glands of both types of tissue. In addition, leukemia inhibitory factor (LIF),

a secretory glycoprotein, is a member of the interleukin-6 family and exerts many biological functions. It is known that delayed implantation involves strict hormonal regulation and complex molecular signal exchange between the mother and embryo. For example, it was first found that embryos could not implant successfully in wild-type homozygous female mice in which the *LIF* gene had been deleted. However, when these mice were injected with LIF, the ability of embryos to implant was restored. The attachment and invasion of the embryo are realized through interaction between trophoblast cells and the endometrium. LIF is directly anchored on the surface of trophoblast cells and regulates the differentiation of trophoblast cells, thus playing an important role in the process of embryo implantation(17). Studies have shown that LIF is highly expressed in the secretory phase of the menstrual cycle in women of normal childbearing age; however, only low expression levels have been detected in the endometrium of infertile women with implantation failure(18). These results suggest that HOXA10 and LIF may play an important role in embryo implantation.

In this study, the eutopic endometrial tissues of infertile patients with adenomyosis and healthy women were compared. Immunohistochemical experiments showed that the levels of HOXA10 and LIF were significantly lower in the pathological tissues than in normal tissues. The expression of HOXA10 and LIF was mainly located in the endometrial glandular epithelial cells, with a small amount of expression in the stromal cells. Endometrial tissue culture is an advanced method used to study human living cells and tissues and has obvious advantages compared with animal experiments(19). Subsequently, western blotting was used to detect the protein expression of HOXA10 and LIF in primary cultured eutopic endometrial glandular epithelial cells. The expression of HOXA10 and LIF in infertile patients with AM was significantly lower than that in the healthy group. The decreased expression levels of LIF in infertile patients with AM may be related to the reduced endometrium proliferation, differentiation, and decidualization, thus resulting in reduced levels of endometrial receptivity. The reduced expression of HOXA10 may lead to the abnormal regulation of embryo implantation. A reduction in endometrial receptivity may affect embryo implantation, thus increasing the risk of infertility in patients with adenomyosis.

It is generally believed that LIPUS causes biological effects *via* mechanical action. In recent years, many clinical studies have been carried out on the special biological effects of low intensity ultrasound. Studies have confirmed that the LIPUS used in fractures could stimulate the proliferation and migration of endothelial cells to complete tissue remodeling(20). Zhang et al. applied LIPUS to fracture patients and found that the healing speed of fractures after low intensity ultrasound action was significantly higher than that of patients without LIPUS action(21). The specific mechanism underlying the action of LIPUS was that the adhesion of bone cells at the fracture healing site was significantly enhanced, and the migration ability of preosteoblasts to the defect site is also significantly enhanced. According to these studies, it can be concluded that LIPUS affected cell proliferation and differentiation *via* the mechanical action on biological cells, thus changing cellular function such as cell migration and adhesion ability. Whether LIPUS can change the specific biological activity of endometrial cells in infertile patients with adenomyosis has not been reported.

ICAM-1 (CD54) is one of the main members of the cell adhesion molecule family(22). ICAM-1 specifically binds to extracellular signal molecules and activates cell functions through information transmission; it also mediates the adhesion between cells and the extracellular matrix and affects the mutual stability between cells and the mobility of cells. Through changes in the expression levels of ICAM-1, the adhesion ability of cells can be increased or decreased. Endometrial receptivity is closely related to the success rate of embryo implantation. The expression levels of ICAM-1 in endometrial cells is directly related to the formation of endometrial receptivity and the stability of blastocyst adhesion to the endometrium. Kuesse et al. found that the expression of ICAM-1 in the eutopic endometrium was significantly lower than that of the ectopic endometrium in endometriosis, and was significantly lower than that of the normal endometrium(23). The low expression levels of ICAM-1 in the eutopic endometrium of endometriosis may indicate that the endometrium undergoes key changes, thus reducing the adhesion between endometrial epithelial cells and between the epithelium and the basement membrane; this makes it difficult for the blastocyst to adhere to the endometrium; this is not conducive to the occurrence of pregnancy.

It is currently believed that the abnormal expression of cell adhesion molecules in the endometrial epithelial cells of patients with adenomyosis affects blastocyst implantation(24). Our tissue assay confirmed that the expression of HOXA10 and LIF markers associated with endometrial receptivity were abnormally reduced in infertile patients with adenomyosis. These markers were closely related to endometrial adhesion ability, and the location of embryo implantation. The reduced expression of these markers may reduce endometrial receptivity. In this study, LIPUS was used to treat the endometrial epithelial cells of infertile patients with adenomyosis; we observed that the adhesion ability of EEECs cultured *in vitro* during the mid-secretory phase of infertile patients with adenomyosis increased after LIPUS stimulation with different intensities. From day two to five, cell adhesion of EEECs gradually increases, and the adhesion ability reached the highest on the 5th day. These results indicated that LIPUS could promote the adhesion of endometrial epithelial cells, thus increasing the receptivity of the endometrium and improving the microenvironment of the endometrium; these changes were more conducive to blastocyst implantation. LIPUS at 30 mW/cm^2 was used to treat endometrial epithelial cells taken from the mid-secretory phase of infertile patients with adenomyosis and cultured *in vitro*; the concentration of ICAM-1 in endometrial epithelial cells of infertile patients with adenomyosis was significantly increased from day 3 to day 4. We confirmed that the adhesion ability of endometrial epithelial cells and ICAM-1, an important regulatory molecule controlling the adhesion of embryos to the endometrium, were significantly increased compared with previous stages. These results suggested that LIPUS may have a positive effect on improving the endometrial receptivity of infertile patients with adenomyosis and has a positive guiding significance for reducing the risk of infertility associated with adenomyosis. Pregnancy difficulties and infertility often occur in patients with endometriosis, adenomyosis, polycystic ovary syndrome, and other diseases. The underlying factors are mainly related to defects in endometrium receptivity and the loss of stability of blastocyst adhesion to the endometrium. In our preliminary experiment, the expression levels of HOXA10 and LIF markers of endometrial receptivity in infertile patients with adenomyosis and healthy fertile women were compared by western blotting; these markers were significantly reduced in adenomyosis. Following LIPUS treatment, the adhesion of

endometrial epithelial cells cultured *in vitro* from infertile patients with adenomyosis was significantly enhanced. Our results showed that the expression levels of HOXA10 and LIF in the endometrium of patients with adenomyosis were significantly increased by LIPUS.

Most studies suggest that embryo implantation is a process involving trophoblast cell adhesion and invasion of the endometrium. In the present study, LIPUS was used to treat endometrial glandular epithelial cells that were obtained from the mid-secretory phase of patients with adenomyosis and cultured *in vitro*. The expression of LIF in the endometrium changes during the menstrual cycle and increases during the implantation window(25). Previous studies have shown that the expression levels of LIF were reduced in the endometrium of women with recurrent implantation failure and unexplained infertility(26, 27). In our experiments, we demonstrated that the expression of LIF was abnormally reduced in the eutopic endometrium during the mid-secretory phase in adenomyosis. LIPUS was applied to endometrial epithelial cells cultured *in vitro* from infertile patients with adenomyosis during the mid-secretory phase. We found that the levels of LIF protein and mRNA were significantly improved after the ultrasound, as determined by western blotting and RT-PCR. This suggested that the endometrium produced more LIF after low intensity pulsed ultrasound to improve the receptivity of the endometrium.

During the process of embryo development, the abnormal expression levels of HOXA10 will also lead to the abnormal development of implanted embryos(14). In the normal menstrual cycle, the peak expression of HOXA10 occurs during the implantation window; this is highly consistent with the increased levels of progesterone(28). However, our previous study observed that in EEECs cultured *in vitro*, that were taken from infertile patients with adenomyosis during the mid-secretory phase, the levels of HOXA10 were significantly lower than those of the healthy group. Western blotting and PCR were used to detect HOXA10 in EEECs cultured *in vitro* from infertile patients with adenomyosis and treated with LIPUS. We found that the levels of HOXA10 were significantly increased after ultrasound compared. The biological effect of increased HOXA10 levels in the endometrium improved the receptivity of the endometrium; this was more conducive to blastocyst implantation. The levels of HOXA10 and LIF, which are related to embryo implantation, were found to be increased by LIPUS in EEECs cultured *in vitro* and harvested from infertile patients with adenomyosis. However, the specific target, the detailed mechanism of the effect, and the signal transduction pathway involved have yet to be elucidated.

Conclusions

In this study, we demonstrated that the levels of HOXA10 and LIF in the endometrium of the mid-secretory phase of infertile patients with adenomyosis were significantly lower than those in healthy fertile women. This suggested that the reduced receptivity of the endometrium in these patients was an important factor underlying infertility, and that the endometrial epithelial cells played an important role in the establishment of endometrial receptivity. Our data suggested that the adhesion of endometrial epithelial cells in infertile patients with adenomyosis should be promoted and a favorable environment can be created for blastocyst implantation by the application of LIPUS. Furthermore, the expression levels of

HOXA10 and LIF in endometrial epithelial cells of infertile patients with adenomyosis were enhanced by LIPUS, thus improving endometrial receptivity and facilitating blastocyst implantation.

Abbreviations

HOXA10: homeobox A10; LIF: leukemia inhibitory factor; EEEC: eutopic endometrial epithelial cells; AM: adenomyosis; LIPUS: low intensity pulsed ultrasound; CG: control group; AG: adenomyosis infertility group; gonadotropin-releasing hormone agonist (GnRHa); IHC: immunohistochemistry; AM-EEEC: adenomyosis eutopic endometrial epithelial cells; ELISA: enzyme-linked immunosorbent assay. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Declarations

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Authors' contributions

L.L performed a literature search and drafted the manuscript. X.H collected clinical specimens and revise manuscripts. L.Z completed basic experiments and data sorting. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets of the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Chongqing Medical University.

Consent for publication

The patients signed informed consent regarding publishing their data.

Competing interests

The authors declare that they have no competing interests.

Authors' information

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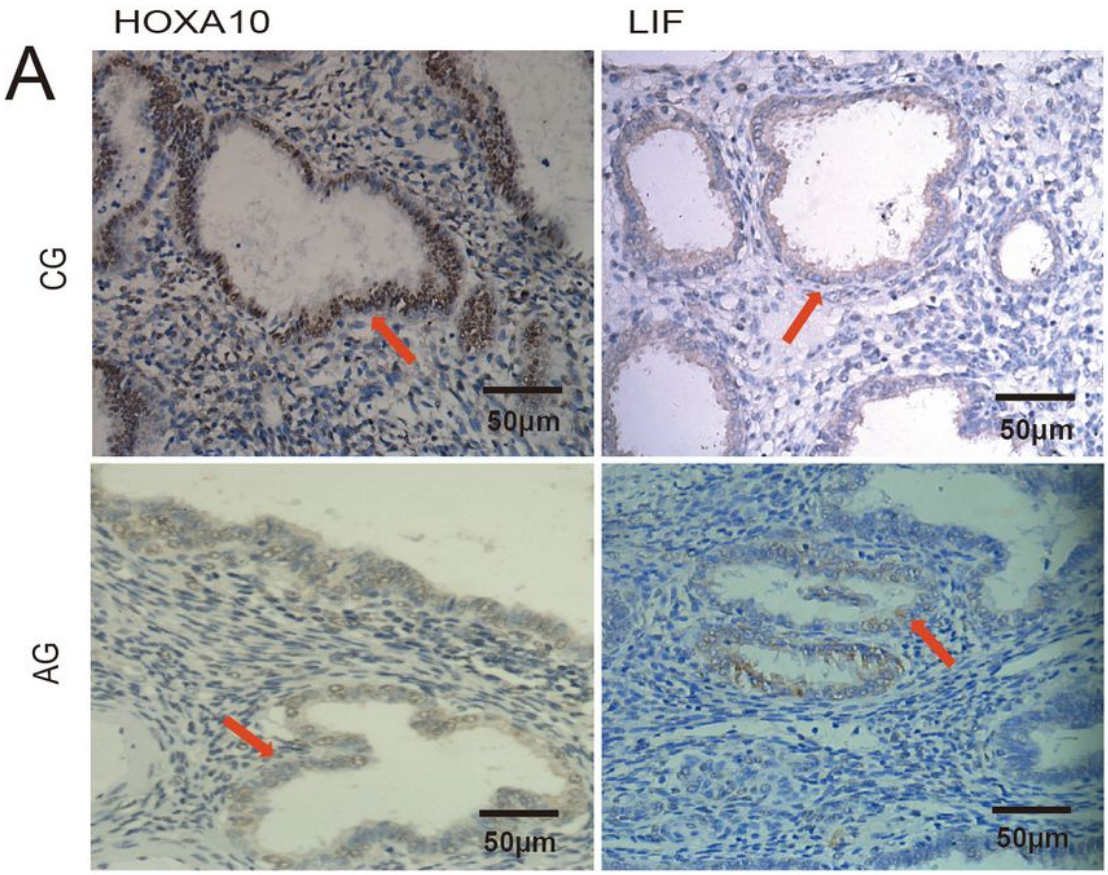
Figures



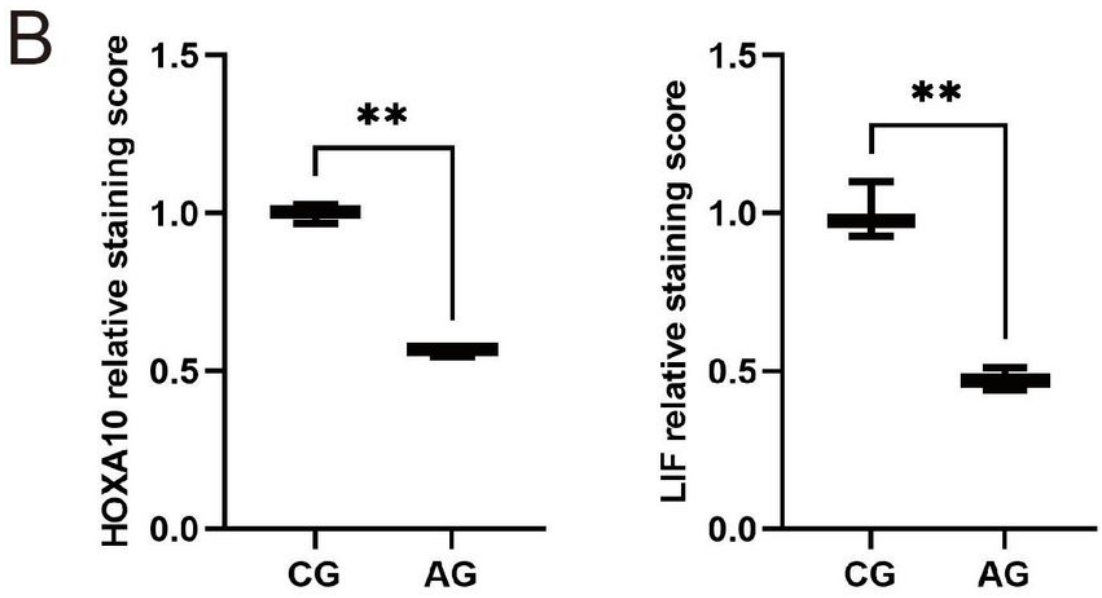


Figure 1

The expression, localization and H score of HOXA10 and LIF proteins. A: The tissue expression and localization of HOXA10 and LIF in eutopic endometrium tissue samples from the CG and AG groups. B: The H score of HOXA10 and LIF of eutopic endometrial biopsies from the mid-secretory phase of patients in the CG and AG groups. CG, control group; AG, adenomyosis infertility group (compared with the CG group, $^{**}P<0.01$).

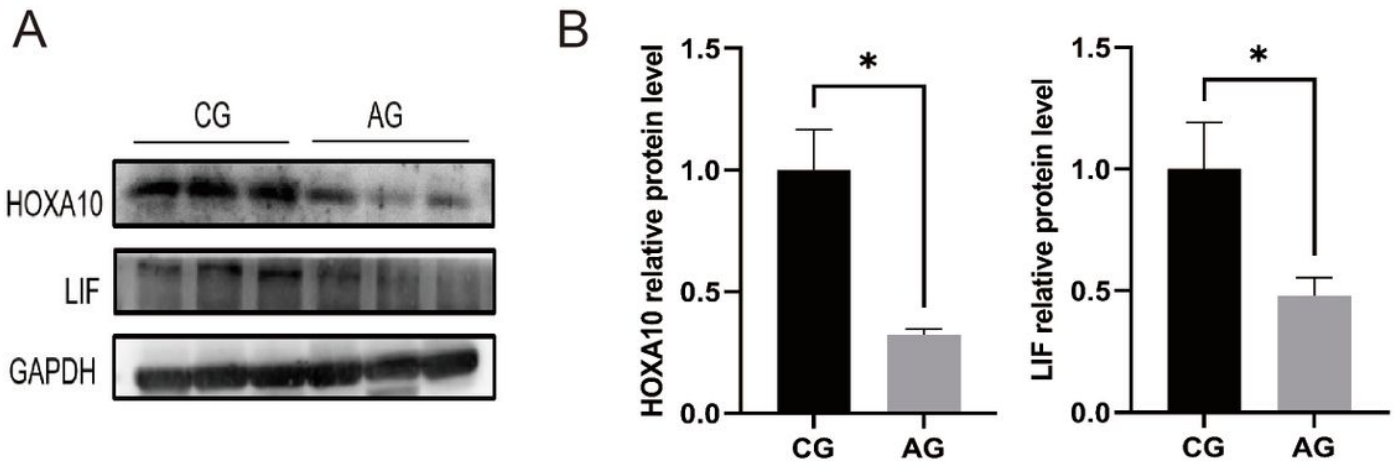


Figure 2

Western blotting and gray value analysis of HOXA10 and LIF in eutopic endometrial biopsies from the mid-stage secretory phase of patients in the CG and AG groups. A: Western blot showing separated protein bands. B: Gray value analysis of protein bands. CG, control group; AG, adenomyosis infertility group (compared with the CG, $^{*}P<0.05$).

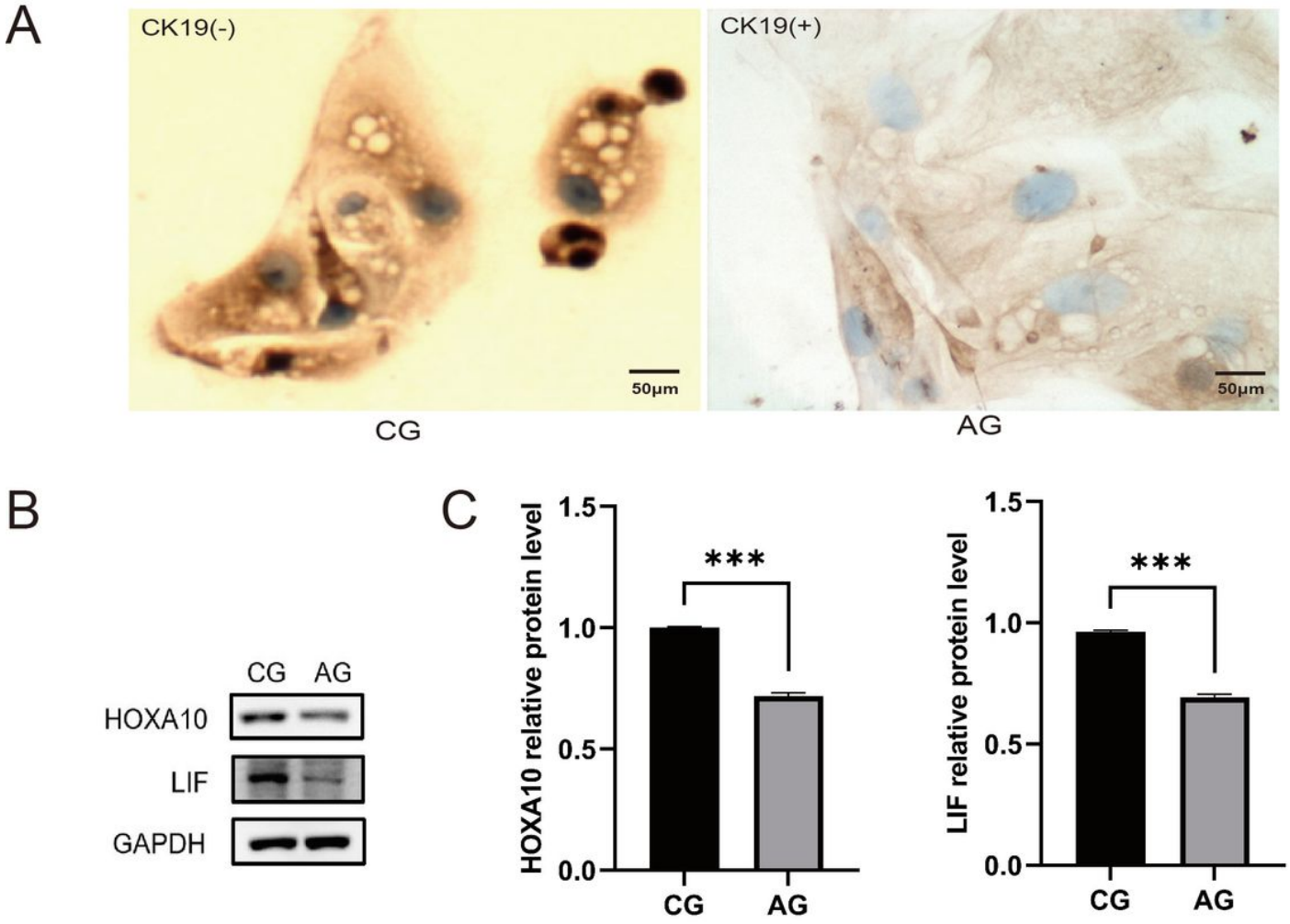


Figure 3

Immunohistochemistry (IHC), western blotting, and gray value analysis of HOXA10 and LIF proteins in cultured cells from the CG and AG groups. A: IHC of CK19 in endometrial epithelial cells from the CG and AG groups. B: Western blots showing separated proteins. C: Gray value analysis of protein bands. CG, control group; AG, adenomyosis infertility group ($^{***}P<0.001$).

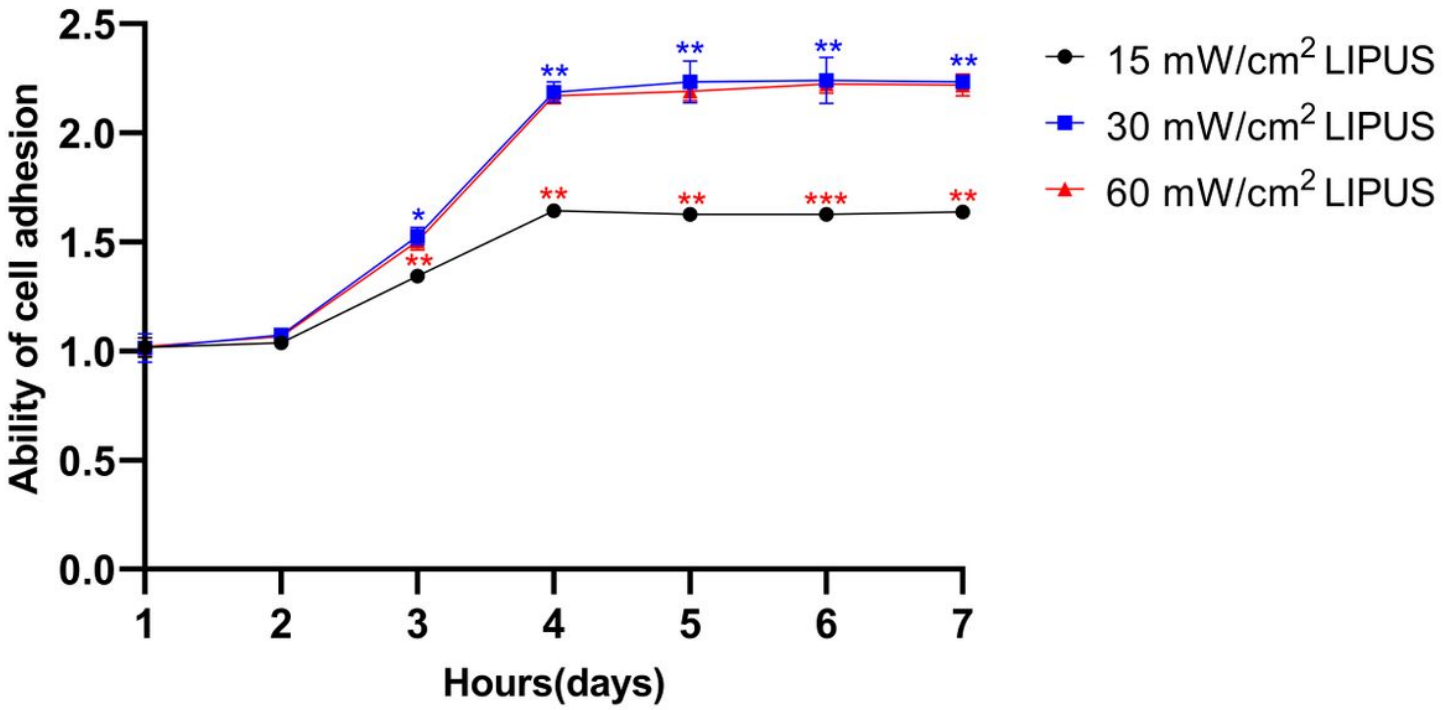


Figure 4

Effect of LIPUS at multiple intensities and for different periods on adhesion in EEECs from patients with AM and infertility (*P<0.05,**P<0.01,***P<0.001)

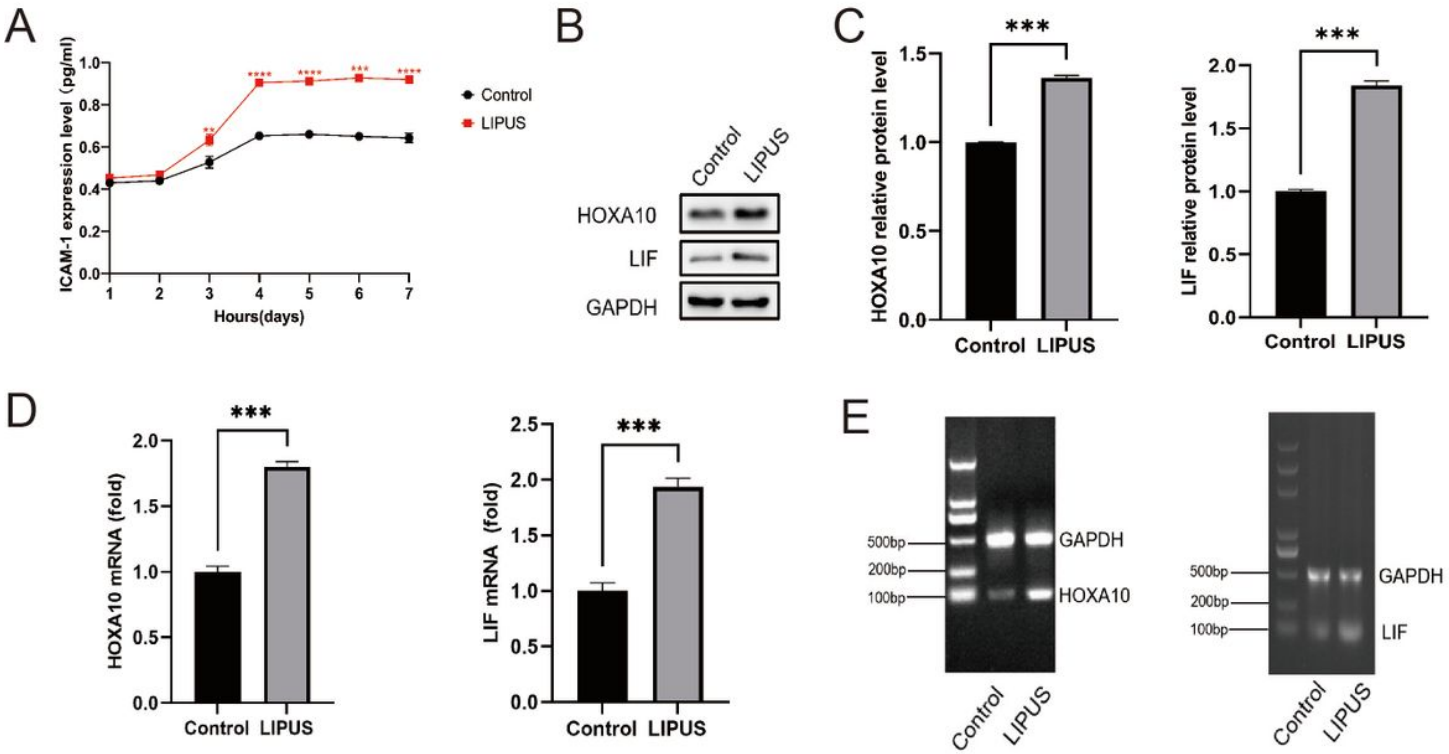


Figure 5

A: The concentration of ICAM-1 in the supern atant treated with 30 mW/cm^2 LIPUS for 7 days in AG cells. B-C: Western blotting and gray value analysis of HOXA10 and LIF proteins in AG cells treated with 30 mW/cm^2 LIPUS for 4 days. D: The mRNA expression levels of HOXA10 and LIF in AG cells treated with 30 mW/cm^2 LIPUS for 4 days. E: An electropherogram of HOXA10 and LIF in AG cells treated with 30mW/cm^2 LIPUS for 4 days. (**P<0.01, ***P<0.001, ****P<0.0001, control, AG cells without LIPUS, LIPUS, AG cells with 30 mW/cm^2 LIPUS).