Effect of Freeze-Dried Amnion Graft on Cytokines in Uterine Exudates Following Hysteroscopic Adhesiolysis of Severe Intrauterine Adhesions

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Research

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Abstract

Objective

Our research group previously demonstrated the clinical benefit of freeze-dried amnion graft for intrauterine adhesion patients. However, the mechanism of its action remains unclear. Here, we conducted a prospective randomized controlled study to explore the impact of freeze-dried amnion graft on postoperative wound inflammation.

Methods

30 patients diagnosed with severe Intrauterine adhesion were enrolled and randomly subdivided into the study group (with freeze-dried amnion gift treatment after-surgery, N = 15) and the control group (without the gift, N = 15). Enzyme-linked immunosorbent assay was used to detect adhesion and repair related cytokine concentrations of uterine exudates, including IL-1β, TNF-α, and VEGF at different time intervals after surgery. A second-look hysteroscopy was performed at 3 months post-surgery. American Fertility Society (AFS) was used to evaluate the adhesion during hysteroscopy.

Results

It was found that the volume of uterine exudates in each group changed significantly with time, and the gift significantly reduced the amount of uterine exudate. Notably, the concentration of TNF-α, VEGF, and IL-1β showed a significant increasing pattern after surgery. In the study group, their concentrations were significantly lower than in the control group. Moreover, IL-1β exhibited a long-lasting effect in the study group. AFS and re-adhesion rates were significantly lower in the study group than the control group during postoperative hysteroscopy.

Conclusion

Our findings suggest that amnion graft is beneficial to the postoperative recovery of patients with severe IUA, and might control the re-adhesion rate after hysteroscopic adhesiolysis by adhesion-related cytokines including TNF-α, VEGF, and repair associated factor (IL-1β).

Introduction

Intrauterine adhesion (IUA) is a condition caused by endometrial basal layer injury, which severely affects physiological and reproductive functions including, reduction in menstruation, amenorrhea, secondary infertility, and cessation of embryo development in women. Currently, the standard method of treatment of IUAs is transcervical resection of adhesion (TCRA)[1]. Nonetheless, due to the absence of effective postoperative management measures, the postoperative re-adhesion rate in patients with severe IUA

increases to 62.5%[2], which seriously influences the efficacy of hysteroscopic adhesiolysis. Therefore, postoperative re-adhesion is the primary problem faced by surgeons. Numerous methods are currently used to prevent postoperative re-adhesions. To repair the uterine cavity through residual endometrium regeneration facilitated by a temporary barrier, some substance, like Foley's balloon, is placed in the uterine cavity as a barrier, then estrogen and progesterone therapies are used to promote endometrial growth.

Previous studies found that under electric heating, isolated wounds exude fluid containing several cytokines[3, 4], including interleukin-1 beta (IL-1β), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) after hysteroscopic adhesiolysis. These factors play important regulatory roles in injury repair. Notably, an appropriate amount of cytokines regulate the balance between the degradation and synthesis of the extracellular matrix (ECM) as well as promote repair of the uterine cavity. Nevertheless, excess production of cytokines disrupts this balance, causing excessive accumulation of ECM and the formation of IUA. In recent years, freeze-dried amnion was reported to secrete a variety of biologically active factors that promote cell growth and improve the microenvironment by regulating cell differentiation and function. Moreover, freeze-dried amnion graft exhibits anti-fibrotic, anti-inflammatory, anti-angiogenic, and anti-microbial features[5], which promote tissue repair. As a result, it is widely used in clinical fields including, ophthalmology, dermatology, and orthopedics to prevent the formation of scars[6]. In terms of IUA, there are also case studies where, freeze-dried amnion graft was applied to uterine cavity wounds after hysteroscopic adhesiolysis[7, 8]. Given that the fresh amnion is implicated in many issues such as ethics, quality control, preservation, and transportation, our research group previously selected freeze-dried amnion graft having similar biological activity to that applied in patients diagnosed with severe IUA. Hysteroscopic re-examination and follow-up records showed that freeze-dried amnion graft reduces adhesion scoring and improve menstruation of patients with severe IUA[8]. However, its precise mechanisms and efficacy are still controversial.

Based on the findings from previous clinical research, this study used freeze-dried amnion to cover the uterine cavity wounds after hysteroscopic adhesiolysis and measured the amount of uterine exudates. Further, we monitored changes in the concentration of adhesion-related cytokines i.e., TNF-α, VEGF, and repair associated factors (IL-1β), in the exudates. Subsequently, the effect of freeze-dried amnion graft on the amount of uterine exudate and repair of the endometrium was analyzed. Further, the mechanism of freeze-dried amnion graft in preventing re-adhesion was explored geared towards providing a theoretical foundation for clinical practice. Meanwhile, the duration of Foley's balloon placement has not yet been unified. The clinical treatment ranges from 3–7 days after surgery. There have been no systematic studies on the duration of balloon placement. Therefore, we calculated the amount of uterine cavity exudate after the operation, and initially explored the duration of postoperative uterine cavity inflammation.

Methods

Patients

Based on the inclusion and exclusion criteria, this study selected patients who underwent intrauterine adhesiolysis for severe intrauterine in Beijing Obstetrics and Gynecology Hospital affiliated to Capital Medical University between February 2017 and December 2018. The American Fertility Society (AFS) scoring system was used to evaluate the intrauterine adhesions[9]. The inclusion criteria included, (1) age younger than 40 years, (2) hypomenorrhea or amenorrhea, (3) infertility or spontaneous abortion (≥1), and (4) a previous AFS IUA score of at least eight. On the other hand, the exclusion criteria included, (1) premature menopause, (2) presence of other intrauterine lesions (e.g. polyps, myoma, or septa), and (3) severe intercurrent disease (e.g. systemic disease, coagulative disorders, or severe disease of the kidneys or liver).

This was a randomized controlled clinical trial (Trial Registration Number NCT02496052). A total of 30 cases of patients were randomly assigned to either the amnion group or the control group in a 1:1 ratio using a computer-generated randomization sheet. There was no statistical difference between the two groups in age, gravidity and parity history, and preoperative AFS scoring (Table 1). This study obtained approval from the Clinical Research Ethics Committee of Beijing Obstetrics and Gynecology Hospital. All enrolled patients signed an informed consent before surgery.

Ethical approval

The Informed Consent Form was obtained from each patient enrolled in the study. The study was approved by the Research Ethics Committee of Beijing Obstetrics and Gynecology Hospital in which the experiments were performed (2017-KY-082-02) and were in accord with the Helsinki Declaration of 1975, as revised in 2008.

Sterilized freeze-dried amnion grafts

Jiangxi Rui Ji Biotechnology (Jiangxi, China) provided the sterilized freeze-dried amnion grafts used in this study, and its production processes were as follows. Fresh amniotic tissues were collected and sterilized by low-temperature freeze-drying as well as cobalt-60 radiation. The above steps allow the amniotic cells to remain in a "dormant" state, thereby guaranteeing their ability to be stored and transported at room temperature in a sealed package.

Sample collection

Under the ultrasound guidance, continuous perfusion hysteroscopy using a resectoscope was used to perform hysteroscopic adhesiolysis. In the study group, two pieces of 30 mm × 20 mm freeze-dried amnion were wrapped in the top of Foley's balloon and placed into the uterine cavity. The specific processing flow is shown in Figure 1. In the control group, only Foley's balloon was placed. Uterine exudates were collected at intervals of 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, and 72 h and 4 d, 5 d, 6 d, and 7 d

after surgery, and the total amount was measured at every time interval. Less than 4ml of exudate were transferred into a vacuum centrifuge tube, immediately centrifuged at 3000r room temperature for 15 minutes, after which 300μl supernatant were taken and transferred to a cryotube at -80°C.

Detection of concentration of adhesion-related factors

The concentration of IL-1β、TNF-α and VEGF in uterine exudate collected at different time points after hysteroscopic adhesiolysis was detected using human IL-1β、TNF-α and VEGF enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc, Wuhan, China) following the manufacturer's instructions. Then, 50 μL of sample or standard separately was added to each well, and 50 μL of detection reagent was applied and incubated at 37 °C for 30 min. Subsequently, 50 μL color development reagent A and 50 μL reagent B were added and incubated at 37 °C for 15 min. Finally, 50 μL of stop solution was added to each well, and optical density (OD) was recorded at 450 nm. During the procedure, washing the plate was according to the ELISA routine method. According to the Yolken RH standard, the linear regression equation of the standard curve was calculated using the concentration and OD value of the standard substance. The OD values of the samples were substituted into the equation to calculate the sample concentration in ng/L, which was then multiplied by the dilution factor to obtain the actual cytokine concentration.

Statistical analyses

Statistical analysis was performed by SPSS statistical software version 22.0 (IBM, NY). Quantitative values were expressed as mean ± standard deviation (SD). Moreover, One-way analysis of t-tests were performed to compare the differences within and between the groups. The comparison of different time points within the group was obtained by the SNK test. The measurement data were expressed by the median (minimum value ~ maximum value), using a rank-sum test, p value<.05 was considered statistically significant.

Results

Volume change of uterine exudates

The total volume of the uterine exudates is shown in the Fig.1. First, an analysis of the total exudation volume of the two groups after surgery was performed. It was found that the total uterine exudates in the study group were significantly lower than that in the control group (P=0.003).

Then, the daily amounts of exudate were measured, and it was found that the volume of uterine exudates in the study group began to decrease 48 hours after surgery while reduced 4 days after surgery in the control group. The peak values between the two groups were significantly different (P=0.00).

In contrast with the peak value, the volume of exudates in both groups decreased significantly after seven days, P=0.000 and P=0.002, respectively. The amount of uterine exudate in the study group was significantly lower than that in the control group at seven days postoperative (P=0.000).

Cytokine concentrations in uterine exudates

After surgery, the average concentration of TNF-α in the study group was significantly lower than that in the control group (P = 0.014).

As shown in Fig.3, the concentration of uterine exudate in the control group was significantly higher than that in the study group at any time (P<0.05). The level of TNF-α in the uterine cavity exudate of the two groups of patients increased first then decreased. The peak of TNF-α concentration in the exudates of the two groups appeared on the second day after surgery, then gradually reduced. In the study group, there was a significant difference in the concentration of TNF-α between the seventh day after surgery and the peak value (P<0.05) compared to the control group (P>0.05).

After surgery, the average concentration of VEGF in the study group was significantly lower than that in the control group (P = 0.004). As shown in Fig. 4, the VEGF concentration in the control group increased rapidly and reached a peak one day after surgery, while it took 3 days in the study group. Except for the third and fourth days after surgery, the concentration of VEGF in the study group was significantly lower than that in the control group (P<0.05). On the 7th day after surgery, the levels of VEGF in the two groups were significantly lower than the peak value (P<0.05).

Within seven days after surgery, the average concentration of IL-1β in the study group was significantly lower than the control group (P=0.005).

Unlike in the aforementioned cytokines, the concentration of IL-1β reached its peak on the 6th day and 5th day in the study group and the control group after surgery respectively. Still, the downward trend was not significant afterward (P>0.05). Moreover, although the IL-1β expression level between the study group and the control group was significantly different at each time point (P<0.05), the expression levels of the two were seemingly similar on the 7th day after surgery (P>0.05). The specific details are shown in Fig. 5.

Postoperative AFS and adhesion reformation rate

We performed hysteroscopy for patients and assess the AFS again at 3 months postoperatively to observe their endometrial recovery. According to the results, we found a significant decrease in postoperative AFS for both groups, while the postoperative decrease level was significantly different when compared between the two groups, as shown in Figure 6A. Based on the scoring results, we defined postoperative adhesion reformation as an AFS > 5 and calculated the postoperative adhesion reformation rate for both groups separately. Likewise there was a significant difference in the rate of postoperative adhesion re-formation between the two groups, as shown in Figure 6B.

Discussion

hysteroscopic adhesiolysis is a type of plastic surgery performed on the uterine cavity under direct hysteroscopic visualization to resect scar tissue and restore anatomical morphology. Notably, endometrial damage is extensive in severe cases of IUA, therefore, surgery emphasizes effective protection of the residual endometrium. However, the residual endometrium area is less than 1/3 the area of the uterine cavity, causing a large wound after surgical resection[1]. Besides, due to electrical intervention during the operation, the high-frequency electric action of the electrode inevitably produces a particular electrical heating effect on healthy endometrial tissue and uterine muscle wall tissue, simultaneously separating and removing scar tissue in the uterine cavity[4]. Nevertheless, the role of high-frequency electric hysteroscopy in separation and correction surgery for severe IUA is still significantly important. Moreover, the unique anatomical morphology of the uterine cavity is a factor in wound exudation after hysteroscopic adhesiolysis surgery and in the action of cytokines on surgical wounds. It is currently agreed that wound exudates contain many repair and adhesion-related factors. Partial cytokines might activate the proliferation of mesenchymal cells (mainly fibroblasts), thereby causing large amounts of fibrinogen, fibronectin, and other substances deposited to form fibrin scaffold-covered wounds resulting in the accumulation of excess ECM. Inflammatory granulation tissue and fibrous scars would be formed, which cause re-adhesion. This is in agreement with previous clinical results, which showed a re-adhesion rate of 62.5% after the implementation of hysteroscopic adhesiolysis for severe IUA[2]. Meanwhile, some other factors play a vital role in the repair of the endometrium and incision thereby restoring the normal function and receptivity of the endometrium.

Like other surgical wounds, uterine wounds after hysteroscopic adhesiolysis exude high levels of cytokines due to electrothermal effects. Among these cytokines, IL-1β, TNF-α, and VEGF regulate tissue damage and repair[10]. Notably, TNF-α and VEGF are the central cytokines secreted in the early stages of inflammation. Their expression is closely related to the formation of new capillaries and the prevention of wound infections. In contrast, excessive cytokine exudation might induce excessive accumulation of ECM and promote granulation tissue and fibrous scar formation. The high expression of TNF-α in wound exudates release high levels of VEGF, thereby triggering fibroblast aggregation and an increase in ECM synthesis[11]. Moreover, TNF-α causes unbalanced ECM synthesis and degradation by inhibiting the secretion of matrix metalloproteinase-9 (MMP-9) resulting in the accumulation of ECM and promotion of fibrous tissue hyperplasia as well as scar formation[12]. In this study, the two aforementioned cytokines reached their peaks on the second day and decreased significantly on the seventh day after surgery. However, compared to the control group, the concentration of postoperative cytokines in the study group was significantly lower.

Macrophages secrete IL-1β facilitated by TNF-α or itself activating TNFR and IL-1R1, respectively. Here, the duration taken by IL-1β concentration to reach its peak was longer than the above two cytokines[13]. Besides promoting repair of endometrial tissue and maintaining a balance of immune activity in the uterine cavity, it was found that IL-1β promotes a healthy intrauterine environment and normal receptivity of the endometrium[14]. Although its concentration in the study group was lower than the control group

due to the inhibition of TNF-α and VEGF in the early stage, IL-1β promotes self-secretion through the IL-1β/IL-1R1 pathway. Moreover, its level in the study group was similar to that of the control group during the endometrial repair period 7 days after operation. According to statistical trends, IL-1β in the study group can be maintained for a prolonged duration to promote the endometrial repair process. Therefore, effective postoperative inhibition of adhesion-related factor exudation after hysteroscopic adhesiolysis is essential for reducing the re-adhesion rate and improving surgical outcomes.

Being widely used in the medical fields, the freeze-dried amnion contains a high level of cytokines and cytokine receptors, which promote the repair of damaged tissues. In animal studies, freeze-dried amnion graft repaired eyelid corneal injury in rabbits by inhibiting the expression of TNF-□ and VEGF in the cornea. Further studies found that the freeze-dried amnion inhibits the expression of TNF and VEGF in injured tissues, accelerates apoptosis of inflammatory cells, and reduces the inflammatory response. At the same time, freeze-dried amnion graft can promote the migration and adhesion of corneal epithelial cells and prevent their apoptosis. Also, it has been used in the treatment of IUA due to its unique features including its ability to reduce the release of adhesion factors and promote damage repair. The advantage of freeze-dried amnion graft on intimalization after hysteroscopic adhesiolysis might be the reason it improves the reproductive prognosis. Our clinical study, which involved postoperative hysteroscopic re-examination, improvements in menstrual flow and postoperative follow-up for an average of 14.6 months during pregnancy, found that the increase in the postoperative re-adhesion score and menstrual flow was significantly different than the score and menstrual flow before treatment, and the pregnancy rate also increased[15]. This indicates that freeze-dried amnion graft can effectively improve the endometrial status by preventing the occurrence of re-adhesions[16].

Therefore, freeze-dried amnion graft can be better attached to wounds in the uterine cavity to exert their functions. While the balloon supports the anterior and posterior walls of the uterus in the uterine cavity, being a foreign body in the wound, it also stimulates the production of inflammatory cytokines. The freeze-dried amnion blocks the direct contact between the rubber balloon and the wound, thereby exerting their functions, which include promoting anti-inflammatory and anti-fibrotic responses as well as scar suppression. A study by Orhue *et al.* [17] argued that after hysteroscopic adhesiolysis, the anterior and posterior walls of the uterus separate for at least 7 days before the wound can heal, whereas the function of biological barrier in the freeze-dried amnion persists for at least 21 days to maintain a continuous effect in the uterine cavity. Furthermore, Amer *et al.*[18] used a balloon combined with freeze-dried amnion graft to treat severe IUA. Then, hysteroscopic exploration was performed 2 months after the surgery and the original scar tissue was obtained for examination. The basal cells of the endometrium were observed under an electron microscope and were also found on the degenerated freeze-dried amnion graft, implying that the freeze-dried amnion graft continue to function even with the removal of the balloon 7 days after surgery. Moreover, epithelial and mesenchymal cells derived from freeze-dried amnion have been demonstrated to survive for several weeks, a period sufficient to prevent a new cycle of fibrosis and scar healing.

Whilst acknowledging the present corresponding clinical studies on the application of freeze-dried amnion after hysteroscopic adhesiolysis, there is no consistent theoretical foundation. In this study, we found that freeze-dried amnion graft can effectively reduce wound exudation at the uterine cavity, regulate the increase of acute inflammation-related factors (TNF-α and VEGF), and maintain a relative concentration of IL-1β in the endometrial repair phase. Also, according to our follow-up results, we found a significant decrease in postoperative AFS and postoperative adhesion reformation rate in patients using freeze-dried amnion graft compared to the control group. Thus, we believe that the freeze-dried amnion graft may improve patient outcomes by altering intrauterine cytokine concentrations.

However, it is worth mentioning that the freeze-dried amnion currently used clinically has limitations including, their morphological dimensions which do not adapt to the morphology of the uterine cavity thus limit their clinical application. Nevertheless, this study lays a foundation for the use of freeze-dried amnion graft in the prevention and intervention of re-adhesion in severe cases of IUA. As a consequence, this provides novel strategies for the prevention of postoperative uterine wound re-adhesion after hysteroscopic adhesiolysis surgery. However, the efficacy of freeze-dried amnion in surgeries for severe IUA merits further validation by prospective, multicentre, and large sample size studies.

According to our clinical outcome, freeze-dried amnion graft can effectively reduce the postoperative AFS and reduce the postoperative re-adhesion rate. The duration of Folly's balloon placement is an important clinical consideration. Meanwhile, balloon placement for too long can lead to compression and necrosis of the endometrial tissues, while too short a period of time is not effective in preventing reattachment formation. And according to our study, removal of the balloon 7 days after surgery was found to be an optimal time point to effectively prevent postoperative adhesions. The time point for removal of the balloon after placement of the freeze-dried amnion graft could be considered to be appropriately advanced, thus reducing the necrosis of the surrounding normal endometrial tissue that may result from compression.

In conclusion, our findings show that freeze-dried amnion graft reduces the amount of uterine exudates after hysteroscopic adhesiolysis and shortens the duration of exudation. Furthermore, we discovered that the freeze-dried amnion regulates the concentrations of the adhesion-associated factors including TNF-α, VEGF, and repair associated factors (IL-1β) in uterine exudates thereby restoring relatively low levels of these factors within a limited time.

Abbreviations

AFS	American Fertility Society
IUA	Intrauterine adhesion
TCRA	transcervical resection of adhesion
IL-1β	interleukin-1 beta
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
OD	optical density
SD	standard deviation
MMP-9	matrix metalloproteinase-9

Declarations

Ethical Approval and Consent to participate

The Informed Consent Form was obtained from each patient enrolled in the study. The study was approved by the Research Ethics Committee of Beijing Obstetrics and Gynecology Hospital in which the experiments were performed (2017-KY-082-02) and were in accord with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from the patient.

Consent for publication

All authors consent to the publication of the article.

Availability of supporting data

All analysis results are displayed on the results. For specific experimental data, please contact the corresponding author.

Competing interests

The authors report no conflicts of interest.

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Authors' contributions

SW performed the laboratory experiments, analysed all the data and wrote the manuscript, HD developed the project and edited the manuscript, BL, YW, ZG, XZ edited the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Baseline characteristics of the two groups of patients

Parameter	Study group N=15	Control group N=15	*P*
Age, mean (SD)	28.9 (1.7)	29.8 (2.1)	0.208
Gravidity, mean (SD)	3.1 (1.1)	3.0 (1.2)	0.814
Parity, median (range)	0 (0, 1)	0 (0, 1)	0.426
Dilation and curettage related to pregnancy, media (range)	2 (0,4)	2 (1,4)	0.99
Other intrauterine surgery (e.g. myomectomy; septum or polyp transection), median (range)	0 (0,1)	0 (0,1)	0.99
AFS, mean (SD)	10.4 (0.8)	10.2 (0.4)	0.394

Abbreviations: AFS, American Fertility Society; PBAC, pictorial blood loss assessment chart.

Figures

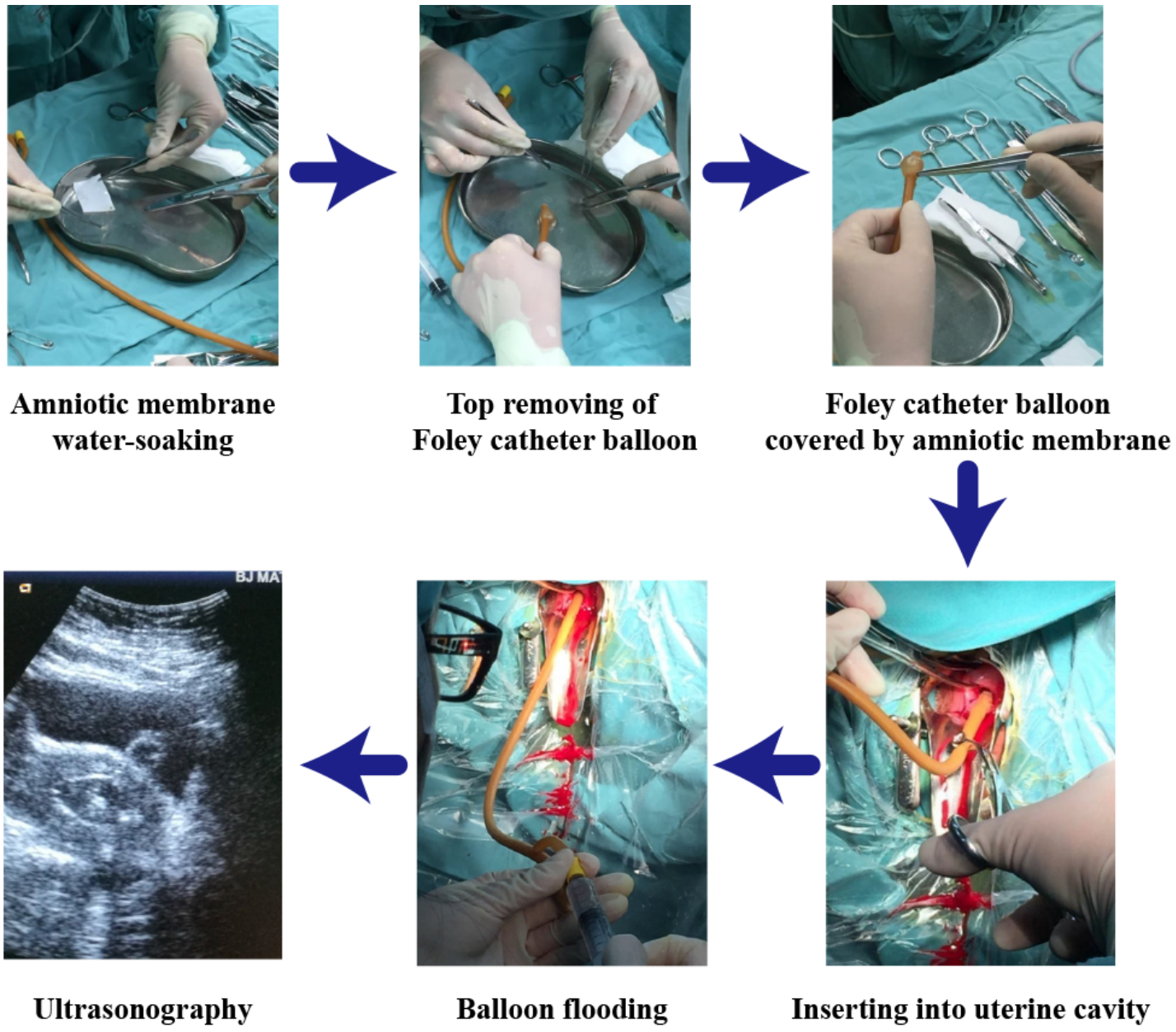

Figure 1

Processing flow of freeze-dried amnion graft

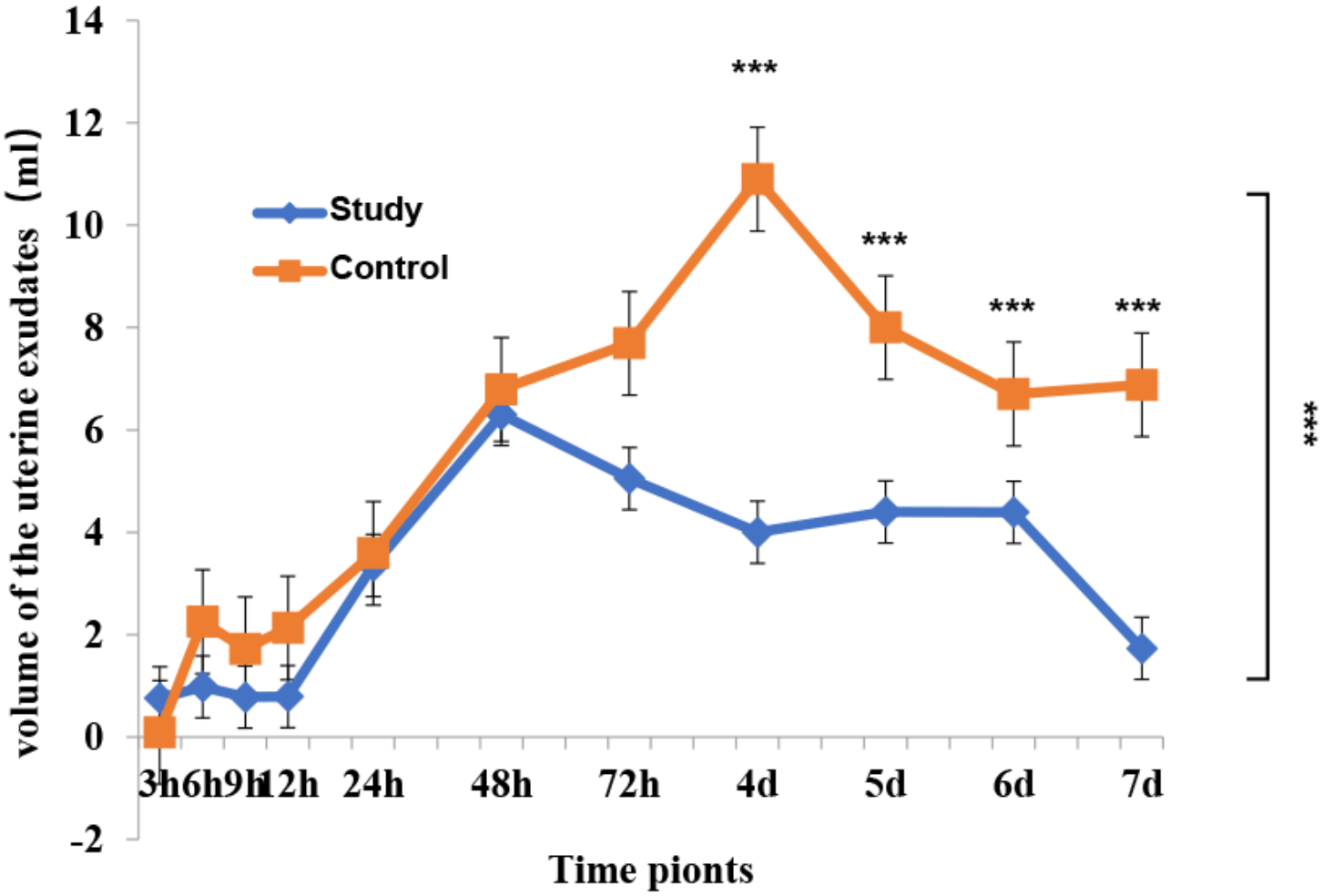

Figure 2

The change of volume of the uterine exudate with time. The volume of daily exudate and total volume were compared, ***P<0.001.

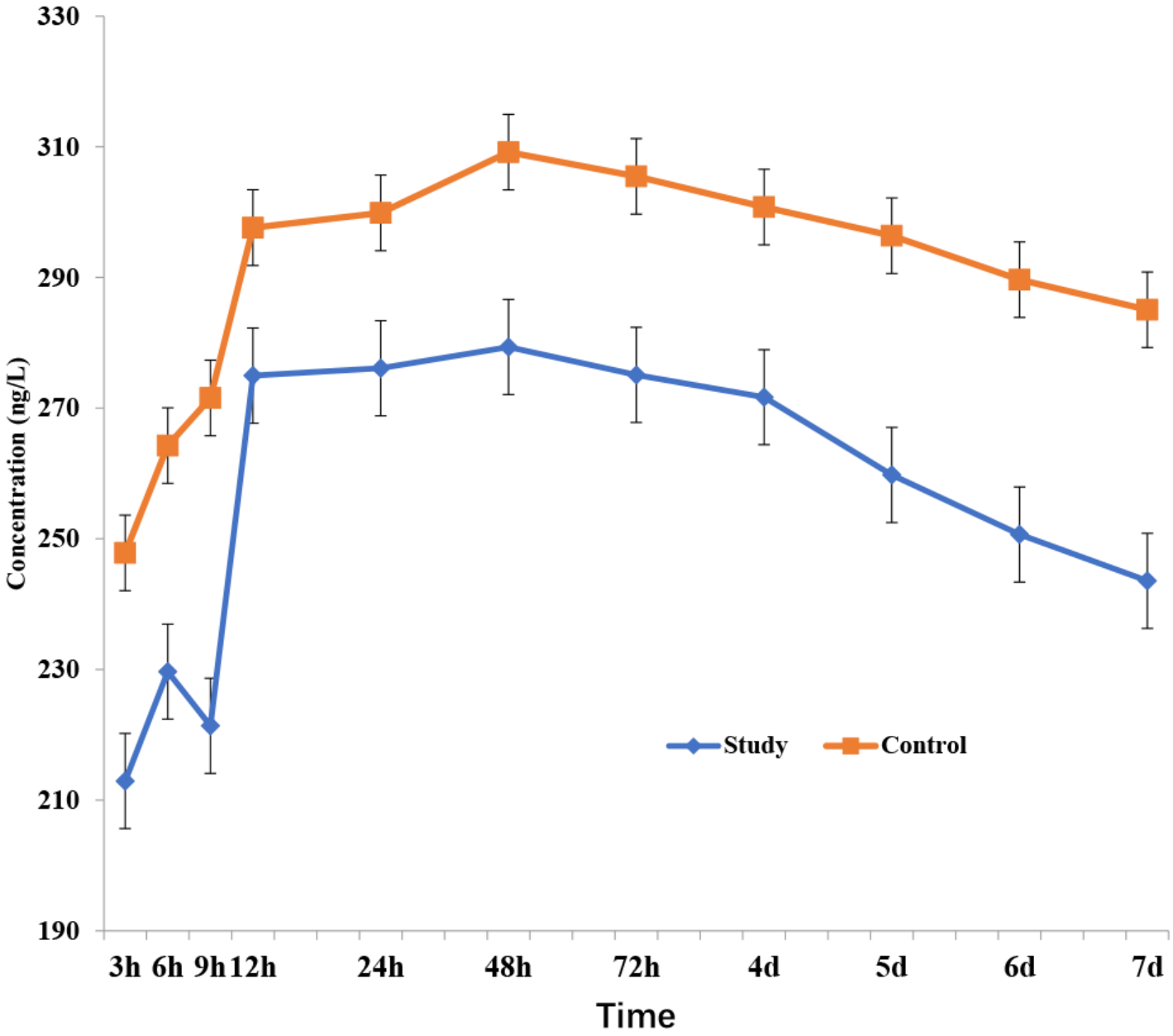

Figure 3

The change of TNF-α of the uterine exudate with time. The concentration of each time point between study and control groups were compared, so did for the concentration between the peak and the 7th day after the operation. ns P>0.05, * P<0.05, ** P<0.01

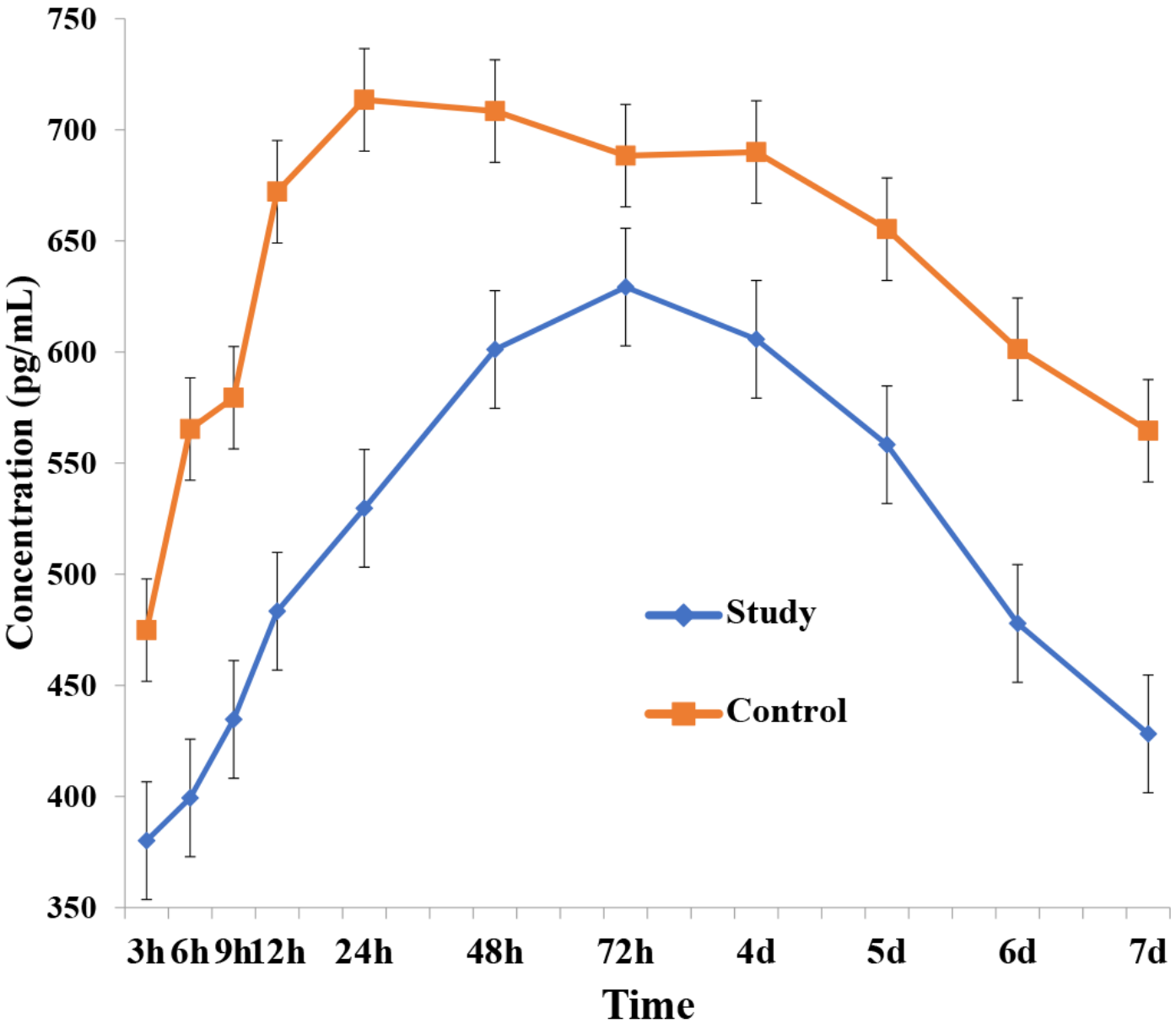

Figure 4

The change of VEGF of the uterine exudate with time. The concentration of each time point between study and control groups were compared, so did for the concentration between the peak and the 7th day after the operation. ns P>0.05, * P<0.05, ** P<0.01, ***P<0.001.

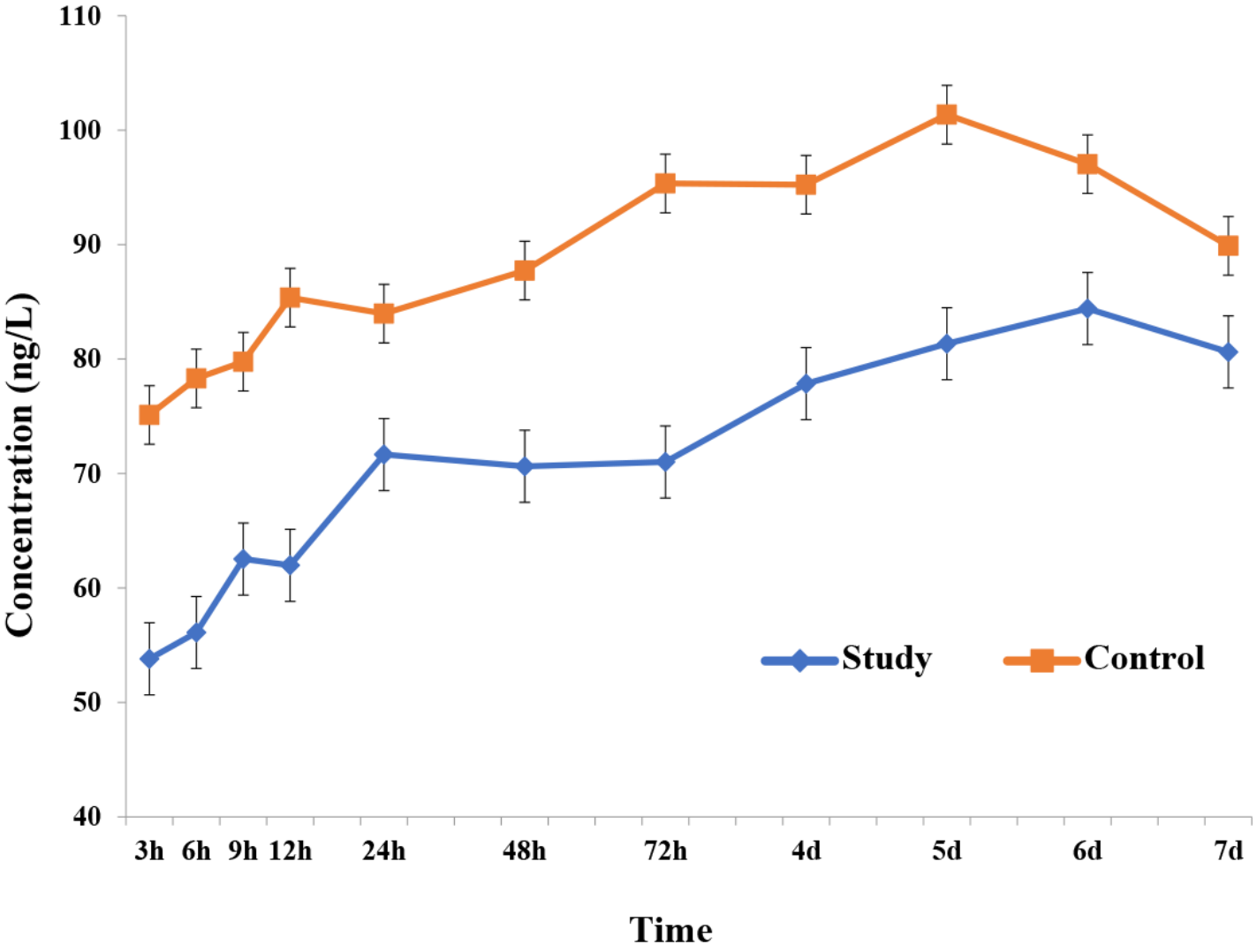

Figure 5

The change of IL-1β of the uterine exudate with time. The concentration of each time point between study and control groups were compared. * P<0.05, ** P<0.01, ***P<0.001.

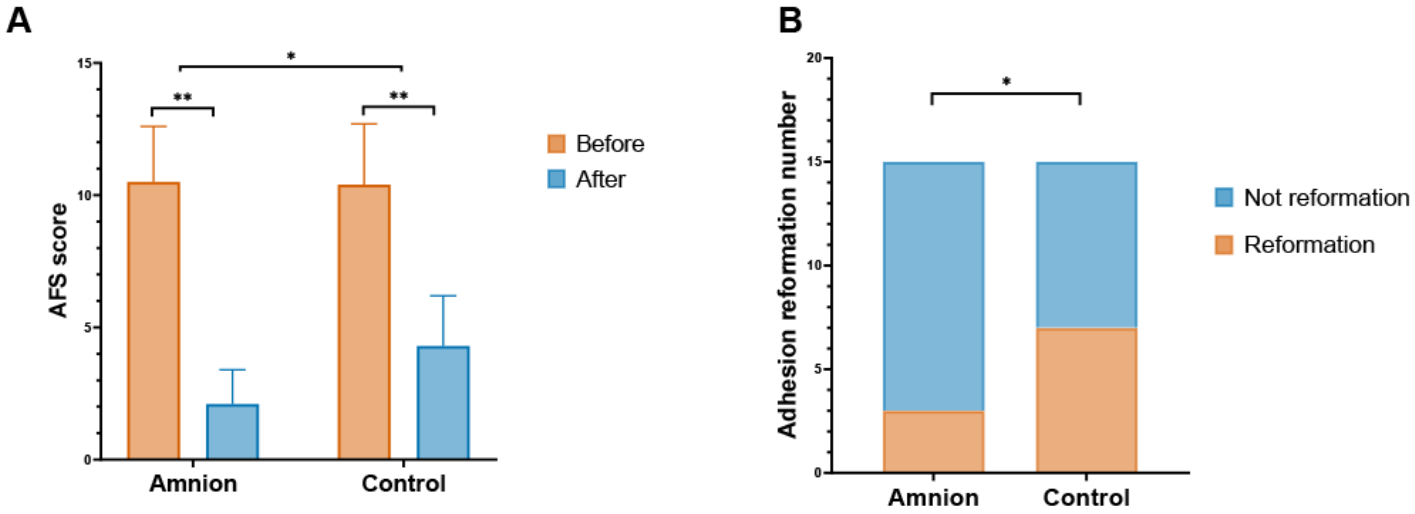

Figure 6

Outcomes of the second-look hysteroscopy. A) Pre- and post-operative AFS between the two groups. B) Postoperative adhesion reformation rate between the two groups. *P<0.05, **P<0.01.