Peer Review File

Reviewer A

I found the manuscript very interesting and informative. I have the following considerations:
Great analysis and evaluation of the cytokine expression, this study helps to shine some light on a very unknown fascinating process. However, if clinical pregnancy is considered an endpoint, then more clinical information is needed.

Reply: We are gratefully for your positive. We have revised each of your comments as follows.

1. Table comparing the age of the patients in both groups.

Reply: We appreciate your comments a lot. In Figure 2, we include a comparison between patients' ages in both groups. For the reader's convenience, we have elaborated on patients' mean age in the *Patients* part, *Materials and Methods* section, following your suggestion. As "The mean ages of patients in the amnion and control groups were 30.2 and 29.7, respectively." (see Page 8, Line 139-140)

2. Technique used to perform the lysis of adhesions

Reply: Thanks for the constructive suggestion. Based on your suggestion, we have added *Hysteroscopic adhesiolysis* part to the *Materials and Methods* section. It is shown as "Hysteroscopic adhesiolysis was performed by an experienced hysteroscopist (H.D.) in all patients under general anaesthesia. Two misoprostol tablets (200 μg each), were vaginally administered for cervical priming the night before the procedure. A bipolar resectoscope with a 8-mm sheath and a 4-mm 12° telescope (Olympus Optical Company, Tokyo, Japan) was used after cervical dilation with a Hegar size 10 cervical dilator. Ultrasonography was routinely performed during the procedures. Laparoscopy was used to inspect the pelvic pathologies, such as endometriosis, and to check tubal patency at the end of the hysteroscopic surgery. Normal saline, delivered by an automatic hysteroscopic dilatation pump at a rate of 280-300 ml/min under an intrauterine pressure of 100 mm Hg, was used as the dilatation medium.
After assessing the location, extent, and severity of the IUAs, they were resected by a

needle or loop diathermy, using an electrosurgical generator voltage set at 320 W for the cutting mode. Then, fluid volume was recorded by a modified automated fluid management system. The operating surgeon determined when complete adhesiolysis had been achieved for all participants, and this was verified by the normal panoramic view of the uterine cavity under direct hysteroscopic visualization. Adhesiolysis was evidenced by an adequate uterine cavity, no signs of IUA, and visible bilateral uterine horn with or without fallopian ostium." (see Page 9-10, Line 166-184).

3. Was a follow up second look in office hysteroscopy performed soon after the first procedure?

Reply: Thanks for your kind reminder. Indeed, we performed hysteroscopy for patients and assess the AFS again at 3 months postoperatively to observe their endometrial recovery. The corresponding methods and results are added in the *Materials and Methods* section and the *Results* section of the manuscript, respectively. "During follow-up, hysteroscopy was performed to assess the American Fertility Society (AFS) again at 3 months postoperatively to observe their endometrial recovery." (*Materials and Methods*). "The decrease in postoperative AFS levels between the two groups was significant (p=0.003), implying better endometrial recovery in the amnion group, as shown in Fig. 3C." (*Results*). and the corresponding results have been added to Fig. 3 and its legend. (see Page 6-7 Line 110-112; Page 15 Line 289-291; Fig. 3 and Fig. 3 legend)

4. Was any fertility enhancing technique used by the couples trying to conceive? (ART?)

Reply: We greatly appreciate your suggestions. We have added data on patients undergoing ART in the *Cytokines expression patterns and clinical outcomes* part of the *Results* section. As "During the follow-up period, a total of seven women in the amnion group achieved pregnancy. Of these, five pregnancies occurred naturally, while two occurred after in vitro fertilization and embryonic transfer. Spontaneous abortion during the first trimester was reported in one of the seven pregnancies of the amnion group. The remaining six pregnancies were viable at the time of the last follow-up. In the control group, three pregnancies were reported, and all had occurred after in vitro fertilization and embryonic transfer. Among these pregnancies, no spontaneous abortion was reported during the first trimester, and all three pregnancies were viable at the last follow-up." (see Page 15 Line 292-300)

5. Outcomes of the pregnancies of those patients who conceived.

Reply: We appreciate your constructive suggestions. We have added patients' pregnancy outcomes in the *Cytokines expression patterns and clinical outcomes* part of the *Results* section. As "During the follow-up period, a total of seven women in the amnion group achieved pregnancy. Of these, five pregnancies occurred naturally, while two occurred after in vitro fertilization and embryonic transfer. Spontaneous abortion during the first trimester was reported in one of the seven pregnancies of the amnion group. The remaining six pregnancies were viable at the time of the last follow-up. In the control group, three pregnancies were reported, and all had occurred after in vitro fertilization and embryonic transfer. Among these pregnancies, no spontaneous abortion was reported during the first trimester, and all three pregnancies were viable at the last follow-up." (see Page 15 Line 292-300)

Reviewer B

English minor changes e. g. (30) "improvement"
- (39) not "by" but "changes" as sign of...

Reply: Thank you very much for your patient guidance. As suggested, after making further changes where needed, we had the paper checked by a very experienced language editor and uploaded the relevant certificate in the form of attachments (Supplementary file). In the resubmitted version, we have thoroughly revised the language and presentation to make data presentation comprehensible according to your suggestions. We have corrected all the errors you pointed out. As follow "Compared to the control group, cytokine expression patterns of the amnion group revealed significant stratifications, which were highly correlated with the expression levels of IL1B on the sixth to seventh day after surgery, **improving the pregnant rate**." and Amnion promotes endometrial repair and receptivity **by altering the expression levels and patterns of IL1B**." (See Page 2-3 Line 34-35; Page 3 Line 41-42)

- Throughout the text, IL should be a "sign" of how the mechanisms work and not the deciding parameter. Please implement this message in the abstact and the discussion.

Reply: We appreciate your correction. As you suggested, we have revised the abstract as "Furthermore, by affecting lysosomal, cell cycle, and calcium signaling pathways, IL1B may be one of the factors involved in endometrial repair and receptivity recovery."In the *Discussion* section we have added the statement as follow " Therefore, we conclude that *IL1B* is a "sign" of the recovery of endometrial

receptivity after hysteroscopic adhesiolysis. However, further studies using *in vitro* cell cultures and animal models should be performed to explore the effects of *IL1B* and its related pathways on the recovery of endometrial receptivity. Thus, it cannot be concluded that *IL1B* is the deciding parameter." (See Page 3 Line 42-44; Page 24 Line 477-481)

Reviewer C

The paper describes a thorough work to explore the immunological effects of amnion grafts on improving results after uterine adhesiolysis. This is done by exploring the cytokine patterns in uterine exudate postoperatively and relate the findings to gene analysis of different biological processes. The focus of mechanistic processes behind the observed differences and especially the elaboration of this in the discussion adds important value to the paper. The study provides interesting results, but the paper suffers from an unstructured presentation of methods and results, inadequate figure legends and somewhat imprecise language. Several improvements are needed to strengthen this manuscript.

Reply: We are gratefully for your positive. The manuscript was corrected according to each of your suggestions.

• Title: The focus on the hierarchical clustering analyses in the title is perhaps not mirrored in the paper, which includes several different statistical analyses. If the authors wish is to keep the focus on the HCA, this should maybe be mirrored in at least the background part of the abstract with a description of why this method is the best to answer the study aim.

Reply: We appreciate your comments greatly. We have added in the background section of the abstract about the importance of selecting HCA for this study, as "In this study, hierarchical cluster analysis enabled the evaluation of cytokine expression changes after amnion treatment to be explored by cluster patterns. The role of IL1B on endometrial repair and receptivity was revealed." Also, in the introduction section, we have described HCA in detail and illustrated the importance of applying HCA in this study. (See Page 2 Line 17-19)

• Abstract: Methods section: The materials and methods of the gene analyses should be included here (and not only in the results section of the abstract). Conclusion section: [line 40] The sentence implies that the study found differences in other cytokines than IL-1b, but as far as this reviewer understand that is imprecise as there

was no differences in expression of TNFa and VEGF?

Reply: Thanks for your constructive suggestions. According to your suggestions, we have added the analysis methods of genes and the corresponding databases to the methods part of abstract, as follow "Single-gene gene set enrichment analysis and differentially expressed genes enrichment analysis of *IL1B* were performed using NCBI GEO (N=151) to evaluate its potential mechanisms and impact on endometrial receptivity.". Also, as you understand, the change of IL1B expression pattern is more meaningful in our analysis. Therefore, we have revised here to "Amnion promotes endometrial repair and receptivity by altering the expression levels and patterns of *IL1B*."(See Page 2 Line 28-31; Page 3 Line 41-42)

• Introduction.
○ The description of intrauterine adhesion is comprehensive and well-written. The introduction could benefit from including a short description of amnion grafts and more details on the immunoregulatory effects of amnion grafts (even if this is described in the references).

Reply: Thanks a lot for your kind advice. Based on your suggestion, we have added a short description of amnion grafts and more details on the immunoregulatory effects of amnion grafts in the *Introduction* section. The details are as follows. "In our previous study, we reported that human amniotic mesenchymal stromal cells promote endometrial cell proliferation by regulating cytokines (10). Amnion gifts exhibit a large number of cytokines and receptors that can enhance injury repair. It accelerates tissue repair by inhibiting the expression levels of TNF-α and VEGF in the wounded area and prevents epithelial cell apoptosis by stimulating cell migration and adhesion. Moreover, in a previous clinical study, we showed that sterilized freeze-dried amnion grafts promote endometrial repair and receptivity after surgery (1). These findings show that amnion graft activity is exerted through cytokine regulation." (See Page 5 Line 72-79)

○ Different variations of term "stages of inflammation" is used several places, exactly what this refers to should be stated in the introduction. E.g. does it refer to any change in cytokine patterns, a shift from pro-inflammatory profile to anti-inflammatory, or other? Or does it refer to the stages in [line 376-385]? If so, this should be clarified and included earlier in the manuscript.

Reply: We greatly appreciate your patient guidance. We explain the "stages of inflammation" in more detail in the *Introduction* section. The specific modifications

are as follows. "However, postoperative inflammation is a dynamic process. The early stages of traumatic inflammation and repair include two phases, the first being inflammatory exudation and neovascularization (inflammatory phase), and the second being fibrosis as well as tissue proliferation and migration (repair phase) (8). Orhue et al. (9) reported that wound healing begins approximately seven days after hysteroscopic adhesiolysis, while Xin et al. (7) showed that the two aforementioned phases have a high correlation with inflammatory responses, which are dominated by macrophage transformations." (See Page 4-5 Line 63-70)

• Materials and methods:
○ It should be stated why comparison of clinical characteristics was done with Odds ratios and not Student t-tests (normal distribution) or Mann-Whitney U (non-normal distribution).

Reply: Thank you for the kindly reminder. We referred to the method of PMID30695035 and used generalized linear regression analysis for the baseline characteristics of the patients. At the same time, in the text, we added a description of the method as follows. "Statistical analyses of the basic demographic properties before grouping were performed by generalized linear regression, and results were corrected for individual characteristics based on the incorporated factors (14)." (See Page 8 Line 140-143)
We also validated the clinical characteristics comparison by using Student t-tests (normal distribution) or Mann-Whitney U (non-normal distribution). As shown in the table below, the results were also without statistical differences.

Parameter	amnion group N=15	Control group N=15	P
Age, mean (SD)	30.2 (1.7)	29.7(2.1)	0.480
Gravidity, median (range)	3 (1,5)	3 (1,4)	0.814
Parity, median (range)	0 (0, 1)	0 (0, 1)	0.626
PBAC, mean (SD)	34.3 (5.2)	33.8(8.7)	0.850

BMI, mean (SD)	20.4 (4.3)	21.8 (5.7)	0.454
AFS, median (range)	10 (9, 12)	10 (9, 12)	0.394

○ Preferably include a section about how the amnion grafts were collected and stored, or refer to a reference where this can be found.

Reply: This is an excellent advice. We have added the corresponding collection and storage methods in the text. The details are shown below. "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." (See Page 8 Line 144-150)

○ Please elaborate on how the cytokine levels were normalized prior to the hierarchical clustering analysis [line 158].

Reply: Thanks a lot for your comments. We used the Min-max normalisation approach to have the data interval between 0 and 1 for various cytokines. The specific formula we have added in the text is shown below. "The Min-max normalization approach was performed to have the data interval between 0 and 1 for various cytokines. The specific formula used was: $x' = \frac{x - x_{min}}{x_{max}}$." (See Page 11 Line 204-206)

○ Please include reference to all specialized R packages (e.g "Clusterprofiler" [line 193-194]) and also the main R software [line 216].

Reply: Thank you for your kindly reminder. We have added references for " Clusterprofiler" and "Limma" packages in the text. (See Page 13 Line 241; Page 14 Line 259)

○ The paragraph "statistical analyses" should specify which data the analyses were performed on.

Reply: Thanks for the correction. We have revised the *Statistical analyses* paragraph to specify which data the analyses were performed on. As "The statistical test used for the comparison of *IL1B* levels between the normal group and the reduced endometrial receptivity group is the *t* test or paired *t* test. Prognostic curves were generated through the Kaplan-Meier method while log-rank tests were utilized to identify significant differences in pregnancy outcomes. Fisher's exact test was used for enrichment analysis of DEGs. Pearson's correlation coefficient analysis was performed for the single gene GSEA of *IL1B*." (See Page 13-14 Line 254-259)

• Results:
○ Figure 3A, 5B, 6 and 7 is difficult to read, please enlarge, improve DPI or change format to ease interpretation. As currently presented, this reviewer cannot compare the written results to the results presented in the mentioned figures.

Reply: We appreciate your kind reminder. We previously provided vector images in PDF format. We have changed all figures to tif format images at 1200 DPI. If they are still difficult to identify, we will contact the editor to send you original figures. (See Figure 3, 5, 6, 7)

○ Please include denomination of endometrial thickness in figure 3B, and specify what is visualized in the second figure of 3C.

Reply: Thanks for the reminder. We added the dimensional units of endometrial thickness in Fig. 3B. We have changed Fig.3C to Fig.3D and detailed the second part of the figure in the legend based on your suggestion. (See Fig. 3 and Fig. 3 legend)

○ [Line 238-240] describes methods and should be included in the methods section.

Reply: Thanks for the correction. We have moved this portion to the *Methods* section. (See Page 6 Line 107-110)

• Discussion:
○ The first part of the discussion [line 303-312] introduces new background information and should as currently written be included in the introduction or rewritten to more closely relate to the results.

Reply: We are grateful for your corrections. Based on your suggestions, we have revised the first paragraph of the *Discussion* section as follows. "Hysteroscopic lysis of adhesions is an effective surgical treatment approach for uterine scars. However,

there are no effective therapeutic approaches that can prevent recurrence (26). Hysteroscopic surgery is the gold standard for the management of severe IUAs. This technique is limited by the application of energy devices, such as electrodes, which produce electrocaloric effects on normal endometrial and myometrium tissues while separating and removing scar tissues in the uterine cavity. These effects result to a high re-adhesion rate (30%-66%) of the uterine cavity and a low pregnancy rate (40%) after surgery (3). Therefore, avoidance of postoperative adhesion reformation and restoration of fertility are of utmost importance after hysteroscopic adhesiolysis." (See Page 18-19 Line 360-369)

○ The discussion could benefit from elaborating more on the characteristics of the "repair phase", for instance explaining "repair factors" [line 334].

Reply: We appreciate your advice greatly. The description here may confuse the reader, indeed. Thus, we have added content to discuss the characteristics and alterations of macrophages and cytokines in both phases (inflammatory phase and repair phase) in detail. The detail is shown as follow. "Immune pattern stratification may be dominated by macrophage changes. Xin et al. documented that immune regulation of macrophages promotes endometrial regeneration and fertility restoration. Immune responses after tissue trauma lasts for approximately 1-2 weeks. Immune response durations can significantly impact on the healing process. The first stage of immune response is dominated by M1 macrophages, which play a vital role in removing tissues, residual fibers, and tissue debris, as well as synthesizing pro-inflammatory cytokines and growth factors. In the late stage of inflammation, M2 macrophages and T-regs jointly promote repair of the endometrial tissues (7). Elevated expression levels of cytokines such as TNF-α in the early stages promote polarization of M1 macrophages to M2 macrophages, as well as the synthesis and secretion of *IL1B* by macrophages. In addition, *IL1B* in the early stages can act on *IL-1RA* to promote its synthesis and secretion. In this study, the peak of *IL1B* secretion was delayed in the amnion group. This finding implies that the amnion graft prolongs the effect of repair phase by regulating cytokines." (See Page 20 Line 389-403)

○ The term "Combat calculation method" should be explained.

Reply: Thanks for the correction. We have added the instruction of "Combat calculation method" in the revised manuscript, as "Finally, all data in this study were normalized and data deviations generated by different batches and different machines were corrected through the Combat calculation method, **which directly removes known batch effects through the SVA package**." (See Page 24 Line 484-485)

• Figure and tables:

○ The figure legends should be self-explanatory without reading the main manuscript. In addition, all abbreviations must be explained in the figure or table legends. An example of lack of printing out abbreviations is figure 2.

Reply: We appreciate your reminder. As you suggested, we have added the legends of Fig. 2, 3, 5 and 6 and added the abbreviations of Fig. 2 and 3. (See Fig. 2, 3, 5 legends)

○ Figure 4A. Must include an indicator of which samples are amnion and which are control.

Reply: We appreciate your patient review. We are greatly regretful of our carelessness in the Fig.4A legend. Here, we wanted to show by a PCA plot that the batch effect of the integrated data has been eliminated. As per your advice, we have modified the legend and added the supplementary Fig.1 for showing the distribution of the two groups through the PCA plot. (See Page 16 Line 310-312; Fig.4A legend; Supplementary Fig. 1 and its legend)

• The written language could overall benefit from revision.

Reply: We appreciated this reviewer's constructive comments. As suggested, after making further changes where needed, we had the paper checked by a very experienced language editor. In the resubmitted version, we have thoroughly revised the language and presentation to make data presentation comprehensible according to your suggestions.

○ Please ensure to specify which group the described results refers to where this might be unclear from the context, e.g., sentence in [line 333].

Reply: Thanks for your suggestions. Following your advice, we have added group descriptions in some parts of the text to improve the accuracy of the content. "In this study, the peak of *IL1B* secretion was delayed in the **amnion group**." And "This observation implies that the repair phase of endometrial tissues is affected by **amnion gifts**, and it begins from the sixth to seventh day after hysteroscopic adhesiolysis." (See Page 20 Line 402; Page 21 Line 410)

○ The title could benefit from rephrasing, as maybe "Hierarchical cluster analysis in

the study of the effect of cytokine expression patterns on endometrial repair and receptivity after hysteroscopic adhesiolysis" would be more unambiguous.

Reply: Thanks a lot for your guidance. We have revised the title to "Hierarchical cluster analysis in the study of the effect of cytokine expression patterns on endometrial repair and receptivity after hysteroscopic adhesiolysis" as you advised. (See Page 1 Line 2-4)

○ The term "amnion group" should be defined before and not after use.

Reply: We appreciate your comments. As you suggested, we have detailed the amnion group and control group in the *Process flow* part of the *Materials and Methods* section. The details are shown as follow. "For the amnion group, the balloon portion of the Foley catheter was covered with sterilized freeze-dried amnion grafts and hydrated in sterile normal saline for 10 minutes before insertion. The size of each amnion graft was 30 × 20 mm. Two amnion grafts were applied to each Foley catheter with the epithelial amnion membrane surface facing outward. In contrast, for the control group, the Foley catheter was not covered with the gift."(See Page 8-9 Line 153-158)