

Dec 25, 2023

Dear Editor,

We submit the revised version of manuscript "Role of anti-TNF therapy in Crohn's perianal fistula closure rate after stem cell transplantation" to be considered for publication in "*World Journal of Stem cells*". I completely agree with reviewers' pertinent comments and sincerely appreciate them. I have done my best to correct and consulted a statistician for accurate statistical analysis. We added in accordance with the comments. Complete corrections are provided in the revised text as highlighted characters in red and here are point-by-point answers with corrections and additions.

Reviewer-1

Q1. Introduction: "Stem cell transplantation is a promising therapeutic option..." You should provide a clear statement on how your study's approach or findings differ from or build upon existing research, as currently, your introduction lacks specific differentiation from previous studies.

Ans-1: As we mentioned in the "Introduction" section, the previous studies are limited to prove whether the combination treatment of biologics such as anti-TNF agents and stem cell transplantation improves the effect of fistula closure. And the specific differentiation of our study is evaluating the long-term outcomes of stem cell transplantation and compare the closure rates of stem cell transplantation with and without anti-TNF therapy. Also, this study evaluated the risk factors for therapeutic failure and CPF recurrence after stem cell transplantation. To acknowledge your positive comment, we added sentences in the Abstract session to emphasize the specific differentiation of this study. (Page 3, line 4)

Q2. Efficacy of Combined Treatment: "Furthermore, the combination of medication and surgery was more effective than either alone, with 52% and 43% complete healing rates, respectively[15]." To clarify the efficacy rates of combined treatments compared to individual treatments, please reference specific studies.

Ans-2: We used a systematic review article as a reference because we thought that synthesizing the results of

various studies rather than relying on a specific study result will help understand the trends in similar research findings to date. The used reference article is a systematic review article about comparing the outcomes between combination of surgical and medical treatment and single therapy of fistulizing perianal Crohn's disease. Total 24 studies were reviewed and the result showed that mean 52% closure rate in the combination therapy group and mean 43% closure rate in the single therapy group. To appropriately incorporate your positive comment, we added a range of healing rates from various studies through the referenced article. (Page 6, line 2)

Q3. Patients and Clinical Variables: There is no concurrent control group for patients undergoing surgery. In your "Methods" section, discuss and acknowledge potential biases in methodology or data analysis, and explain how these biases were mitigated.

Ans-3: This study is in a retrospective manner, and the patients who did not undergo surgery was excluded because the aim of this study is to compare the closure rate after stem cell transplantation between combination therapy group with anti-TNF and non-combination therapy group. We described the potential bias and limitations in the Discussion section. We added about how we tried to minimize the bias. (Page 12)

Q4. Single Stem Cell Transplantation: "All patients included in this study received only one stem cell transplantation." This statement seems to contradict the abstract, which states, "A total of 65 procedures of 64 patients were included." Please verify and correct this to resolve any contradictions.

Ans-4: Sorry for the mistake. There was an error in confirming the number of cases, and the correction has been reflected only in the main text, not in the abstract. We corrected the abstract. (Page3, line 11)

Q5. Management of Anti-TNF Therapy: "Anti-TNF agents: Infliximab was administered two and six weeks after the first dose, and eight weeks after the third dose. Adalimumab was administered every two weeks after the first dose." You should clarify the administration protocol for anti-TNF agents, including specific situations for medication, dosage, and any adjustments based on patient response.

Ans-5: In Korea, the administration and dosage adjustment of biologics are determined by a gastroenterologist. Information regarding this, including dosage details, has been added to the method section. (Page 8)

Q6. Method of Autologous ASC Preparation: I recommend swapping the order of autologous ASC preparation with surgical procedures, anti-TNF agents, and postoperative management in the "Methods" section. Also, add detailed steps of ASC preparation, such as cell culture conditions, culture duration, cell counting, and quality control.

Ans-6: We swapped the order as you recommended. (Page7) Detailed steps of ASC preparation and information about quality control by the Korean Food and Drug Administration were described in Method section. (Page 7) We added the information about cell count in a single-use vial.

Q7. Result Statement on Fistula Closure Rate: "All patients who received anti-TNF treatment experienced fistula closure within two years. The 1- and 2-year closure rate for anti-TNF-treated patients was 63.0%, and 66.7%, respectively." Reconfirm and rephrase these results to avoid confusion due to the apparent contradiction between sentences.

Ans-7: We rephrase the sentence to avoid confusion and clarify the meaning. (Page 10, line 16)

Q8. Follow-up Period in Recurrence Rate: "During the follow-up period of approximately 5 years, 14.0% of patients with fistula closure experienced recurrence." Since Figure 2 only shows up to 3 years, and the result is around 14%, clarify the exact follow-up duration.

Ans-8: According to the Table 2, all recurrences occurred within 3 years, and Figure 2 illustrates the cumulative closure rate for this. We added additional sentence in the Result section. (Page10, line 14)

Q9. Discussion - Future Research: "However, few studies have focused on the long-term outcomes..." Your discussion lacks direction for future research. Suggest areas for future investigation, particularly regarding long-term outcomes.

Ans-9: We added the suggestion about the future study at the conclusion. (Page 13, line 1)

Q10. Conclusion - Summarizing Key Findings: "Anti-TNF therapy did not increase CPF closure rates..." The conclusion is brief and lacks a summary of the study's implications. Provide a concise summary of the main findings and their significance in the field.

Ans-10: We changed the summary more concisely with main findings. (Page 12, line 26)

Q11. Overall Structure - Emphasizing Novelty: The entire manuscript. The novel aspect of your study is not prominently highlighted. Throughout the manuscript, regularly emphasize the unique aspects or contributions of your study.

Ans-11: This study has novelties not only in evaluating the long-term outcomes of stem cell transplantation in patients with CPF but also identified factors associated with fistula closure after stem cell transplantation. Therefore, we emphasized this aspect by adding the information to the conclusion. (Page 12, line 26)

Q12. General - Addressing Biases: The entire manuscript. The manuscript does not address potential biases in study design or data interpretation. Discuss and acknowledge any potential biases in the methodology or data analysis, and how they were mitigated.

Ans-12: We described the potential bias and limitations in the Discussion section. We added about how we tried to minimize the bias. (Page 12)

Again, I appreciate the editorials and reviewers to point out very important issues, enabling our study to be clearly informative. I look forward to your review.

Best wishes.

Yours sincerely,

Yong S. Yoon, MD, PhD

**Yong Sik Yoon, MD, PhD, Professor, Department of Colon and Rectal Surgery,
Asan Medical Center, University of College of Medicine, 88, Olympic-ro 43-gil,
Songpa-gu, Seoul 05505, Republic of Korea
Tel. 82-2-3010-5318; FAX. 82-2-3010-6701; E-mail. yoonys@amc.seoul.kr**

Feb 4, 2024

Dear Editor,

We submit the revised version of manuscript "Role of anti-TNF therapy in Crohn's perianal fistula closure rate after stem cell transplantation" to be considered for publication in "*World Journal of Stem cells*". I completely agree with reviewers' pertinent comments and sincerely appreciate them. I have done my best to correct and consulted a statistician for accurate statistical analysis. We added in accordance with the comments. Complete corrections are provided in the revised text as highlighted characters in red and here are point-by-point answers with corrections and additions.

- 1) The title did not capture the content. A more concrete title should be conveyed.

A: We modified the title of this study.

- 2) The abstract did not reflect the schemes.

A: We modified the Aim more accurately in the abstract.

- 3) Page 6: Autologous adipose tissue-derived mesenchymal stem cells (hASC) (Cupistem®, Antrogen, South Korea) were used in this study. How did they standardize "Autologous hASC procedures and quality controls of the MSCs? What were their MSC dosing schemes? How did they assess the efficacy for each patient?

A: Cupistem®, an autologous adipose tissue-derived mesenchymal stem cell (ASC) therapy, was approved by the Korea Ministry of Food and Drug Safety (MFDS) in 2012 (advanced therapy medicinal product). Cupistem® manufacturing process was validated and standardized during product development and critical process parameter has been established to ensure product quality. Prior to release, Cupistem® is tested for cell appearance, cell contents, cell viability, cell surface marker, impurity in addition to adventitious agents including mycoplasma and bacteria, fungi, and endotoxin. We added about it in the Method section. (Page6-7)

Dosing schemes are described on page 8 as follows; The ASC dose was determined based on fistula length and diameter, which were measured using a probe before injection. When the fistula diameter was <1 cm,

approximately 3×10^7 cells were injected per cm. When the fistula diameter was between 1 and 2 cm, approximately 6×10^7 cells were injected per cm. The dosage and administration method of Cupistem® were determined during the phase I clinical trial (Cell Transplantation 22:279–285;2013). As described in Efficacy assess method is described on page 9. Efficacy outcomes of each patient were evaluated as a complete closure. Fistula tract closure was defined as no discharge, swelling, or pain. This method of assessing fistula closure is widely used and accepted in the clinical field (N Engl J Med 340:1398-1405;1999, Gut 58(7): 940–948; 2009)

- 4) Page 6: anti-TNF agents used in this study were infliximab (Remicade®, Janssen Biotech, Inc., Horsham, PA, United States) and adalimumab (Humira®, AbbVie, Inc., North Chicago, IL, United States). The agents should be clearly labeled in both Fig 1 and 2.

A: We added information about the agents in figure legends of Fig 1 and 2.

- 5) A treatment timeline should be provided, including Autologous adipose tissue-derived mesenchymal stem cells, infliximab, and adalimumab. For example, page 5 says, "However, few studies have focused on the long-term outcomes of stem cell transplantation and the risk factors affecting them." How did they call it "long-term?" What was the outcome? For clarity, thus, a timeline schematic diagram of diagnostic and treatment courses should be provided, including the duration of the time of treatment and outcomes. Separated timelines should be drawn for those patients corresponding to Table 1 and all the other figures for specific mentions in the text.

A: The previous phase I and II trials reported the outcome between 1 and 3 years after stem cell transplantation. Studies reported the outcome up to 5 years have been published as "long-term" outcome, which is commonly accepted in clinical fields after stem cell transplantation in patients with Crohn's perianal fistula.

As we mentioned in Method section, the dosage for Infliximab was 5mg/kg per infusion. Infliximab was administered two and six weeks after the first dose, and every eight weeks after the third dose. Adalimumab was administered every two weeks after the first dose. Adalimumab was administered at 100mg for the first dose, 80mg at the second week, and subsequently 40mg every two weeks.

Patients treated with anti-TNF agents at least once from three months before surgery to three months after surgery were defined as the "anti-TNF group" regardless of the interval between the initiation of anti-TNF treatment and stem cell transplantation. We added about it with a more accurate phrase. (Page 9)

Postoperative follow-up was done at an outpatient clinic every one or two months until the fistula closure was confirmed. Fistula closure was checked through physical examination at the outpatient clinic.

Anti-TNF treatment and stem cell transplantation are different treatment options and are not on one timeline in this study. Therefore, we couldn't provide a timeline schematic diagram, but we mentioned about the treatment timeline including dosage and follow up schedule separately in Method section.

- 6) All the Figures (1-2): Figure titles must carry self-explanatory information. An ideal figure title should give complete information to the reader even without reading the text. The figure should have a governing title followed by the descriptive interpretation of panel contents. All the figure legend descriptions were not written in keeping this point in mind in the current manuscript version. For example, all the abbreviations should be spelled out so that the readers not in the field do not need to search around. Precisely, "Figure 1 Closure rate with and without anti-tumor necrosis factor agents. TNF: Tumor necrosis factor." This figure legend did not give a complete picture of the data without searching for corresponding text.
A: We modified the Figure title and figure legends.

- 7) Another example was that the authors needed to elaborate on concrete descriptions of "advantages and disadvantages" and specific examples of "various samples and provide biomolecular information," which should be illustrated within the Figure and the table, as the current version manifested too simple (common sense without reading this manuscript) to convey any expert literature review.
A: We specified the conclusions resulted from this study and reflected them in the conclusions to elaborate the advantages of this study. Unfortunately, this study was not analyzed for biomolecular change due to this is the clinical study, so the figure for this could not be added.
However, if necessary, an illustration regarding the surgical process of stem cell transplantation can be added.

- 8) Neither the abstract nor the conclusion gave a clear picture of what the cohort was designed about (e.g., not precise inclusivity and exclusivity), confusing the reader with how to reference their treatment schemes and outcome measurements.

A: The indication of stem cell transplantation was patients with Crohns' perianal fistula without active inflammation. We added the sentence in Method section. (Page 8)

- 9) Peer-reviewer 1 asked: "1. Introduction: "Stem cell transplantation is a promising therapeutic option..."You should provide a clear statement on how your study's approach or findings differ from or build upon existing research, as currently, your introduction lacks specific differentiation from previous

studies." On page 5, they did not fully address this requirement: Neither did they specifically narrate any single treatment outcomes, nor did they specifically differentiate from previous studies.

A: This study investigates whether combining the surgical option of stem cell transplantation with the medical option of anti-TNF therapy is more effective in increasing the fistula closure rate after stem cell transplantation compared to results reported in previous studies. Unfortunately, this study found that adding anti-TNF did not have a significant impact on increasing the closure rate of stem cell transplantation. However, since most patients enrolled in this study were taking anti-TNF agents for the treatment of bowel inflammation rather than perianal fistula, further research is considered helpful to determine whether combining stem cell transplantation and anti-TNF solely for perianal fistula treatment is effective. We modified the conclusion and added about the needs of further study. (Page 13)

- 10) Page 5: "2. Efficacy of Combined Treatment: "Furthermore, the combination of medication and surgery was more effective than either alone, with 52% and 43% complete healing rates, respectively [15]." Peer-reviewed 1 specifically requested, "To clarify the efficacy rates of combined treatments compared to individual treatments, please reference specific studies." The authors did not clarify it.

A: We clarify the rates by using the specific reference studies. And we also clarify the novelty of this study. The contents has been included in the Introduction session. (Page 6)

- 11) Page 7: "The stromal vascular fraction isolated from the fat tissue was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1 ng/mL human basic fibroblast growth factor to obtain the required number of ASCs for injection." How did they remove residue trace animal products from clinically-used hASCs? How did you manage the immunity rejections if they did not clear it? How did they know if in vitro culture procedures introduce detrimental factors? How did the cultured ASCs deviate from the original biomarker profiles? It is well known that fetal bovine serum cultured modifies stem cell genome profiles. How did they ensure that change? How did they elaborate on the severity of inflammation in the fistula caused by the animal products (page 12)? Note: "Examining the influence of various experimental and culture-related factors on hASC immunoregulatory functions in vitro is essential, including interaction mode (contact vs. contactless), and oxygen tension on hASCs; Conditioning methods before hASC interaction, culture medium type (xenogenic or xenofree)(Here, they used FBS), dimensionality (two-dimensional vs. three-dimensional with biomaterials), and passage number (Mahmoud, M., Abdel-Rasheed, M., Galal, E.R. et al. Factors Defining Human Adipose Stem/Stromal Cell Immunomodulation in Vitro. Stem Cell Rev and Rep 20, 175–205 (2024)).

<https://doi.org/10.1007/s12015-023-10654-7>). All these parameters affect the efficiency of MSCs due to the cultured subclonal evolution [<https://doi.org/10.3389/fcell.2022.699144>].

A: Residual animal products are removed through a washing process of ASCs. During the development of manufacturing method of Cupistem®, washing validation and risk assessment for residuals were conducted to ensure product quality and safety. The risk of residual substances such as FBS was evaluated based on "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993)" and 21CFR610.15. Further, safety assessment during clinical trial and post market surveillance after market approval demonstrate that Cupistem® is a safe treatment for patients with Crohn's fistula. We added about it in Method section (Page 7-8)

As you commented, the stemness of the stem cells can be influenced during in vitro culture with various culturing conditions. In this regard, the biomarker profiles were analyzed with cultured ASCs which were produced with the same process as Cupistem®; 1) Stem cell marker (SOX-2, NANOG, OCT4), 2) ASC marker (representative positive CD markers CD73, CD90, CD105 and negative CD markers CD34, CD45, MHC class II), and 3) single nucleotide polymorphism analysis using Illumina 550K Chip. During cultivation from passage 1 to passage 5, no significant changes in biomarker profile were observed. In addition, biological properties of ASCs including immunomodulation potential, paracrine factor secretion, and differentiation properties did not change until cultivation to passage 5. Based on the biomarker analysis and biological activity evaluation, the manufacturing method of Cupistem® was established.

- 12) Page 7: "suspended in DMEM, and packaged into single-use vials containing 3×10^7 cells/mL" – Why in DMEM (compositions complicate local physiology)? How much volume did they use clinically? How many ASC cells did they use for a patient?

A: DMEM is used as a cell suspension solution. DMEM contains nutrients necessary for cell survival, such as amino acids, glucose, mineral salts, and vitamins. The composition of DMEM is not significantly different from that of total parenteral nutrition. DMEM are widely used as a suspension for cell therapy products. DMEM did not cause local complication in preclinical toxicity studies. In addition, Cupistem® has been prescribed for more than 10 years since its approval in 2012, and no complications related to the product have been reported.

As mentioned in Page 8, the ASC dose was determined based on fistula length and diameter, which were measured using a probe before injection. When the fistula diameter was <1 cm, approximately 3×10^7 cells were injected per cm. When the fistula diameter was between 1 and 2 cm, approximately 6×10^7 cells were injected per cm.

- 13) Page 7: "Then, 3×10^7 autologous adipose tissue-derived mesenchymal stem cells/mL (Cupistem®, Antrogen, South Korea) were injected into the submucosa around the internal opening and fistula tract." Did they do any assays or histological assessments to ensure the viability and engraftment of ASCs? Where were their data sets?
A: Primary outcomes of the present study were evaluating the closure rate and the fistula closure was defined as no discharge, swelling, or pain. Therefore, we did not do any assays or histological assessments, but we did digital rectal exam in every follow up at out patient clinic. This method of assessing fistula closure is widely used and accepted in the clinical field (N Engl J Med 340:1398-1405;1999, Gut 58(7): 940-948; 2009).
- 14) Pages 9-10: "During the follow-up, closure rate after stem cell transplantation was 76.9%. The mean duration from stem cell transplantation to fistula closure was 6.94 ± 9.68 months. Moreover, the recurrence rate in patients experiencing fistula closure was 14.0%, with the mean period from fistula closure to recurrence being 16.57 ± 19.38 months. All recurrences were detected in 3 years. The patients who received anti-TNF treatment experienced fistula closure within two years. The closure rates at 1 year and 2 years for the patients who received anti-TNF-treatment were 63.0% and 66.7%, respectively." The above statement is confusing: from 76.9% only with ASCs, went down with combinations of anti-TNF treatment to 63.0% and 66.7%? Why?
A: As we mentioned in Discussion section, the closure rate was slightly lower in patients treated with anti-TNF agents, although it was not statistically significant. This result is possibly because ASCs initiate or enhance tissue regeneration by two different mechanisms: differentiating into skin cells or secreting paracrine factors that initiate the healing process through recruiting endogenous stem cells and endothelial cells or downregulating the inflammatory response. Thus, the anti-inflammatory response of stem cell transplantation, which is a local treatment for CPF, is strong patients and prolonged and minimizes the effect of systemic treatment with anti-TNF. Additionally, more refractory CPF was included in the anti-TNF treatment group as the patients treated with anti-TNF agents had a prolonged disease duration and previous surgeries for CPF might affect the result.
- 15) "Anti-TNF therapy did not increase CPF closure rates..." The conclusion is brief and lacks a summary of the study's implications. Provide a concise summary of the main findings and their significance in the field." What was the molecular mechanism underlined the outcomes?
A: We modified the conclusion more clearly by reflecting the revised title and the aim of this study. (Page 13) Unfortunately, this study results were based on clinical symptoms, and since we did not compare the molecular changes

that occur in the fistulous tract after stem cell transplantation, the contents could not be described in conclusion.