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Reviewer#1

Specific comments to authors

Comment: MSC therapies have not been FDA-approved for treatment yet, but they have been approved for treatment in the European Union, Canada, and Australia, and there are more than 300 major MSC clinical trials underway worldwide focusing on a wide range of medical conditions, including Heart disease. An International Society for Cell and Gene Therapy Mesenchymal Stromal Cells (MSC) Committee perspectives on International Standards Organization/Technical Committee 276 Biobanking Standards for bone marrow-MSCs and umbilical cord-derived MSCs for research purposes. [Sowmya Viswanathan et al., Cytotherapy. 2023 Aug;25(8):803-807. doi: 10.1016/j.jcyt.2023.04.005. Epub 2023 May 6. PMID: 37149800 DOI: 10.1016/j.jcyt.2023.04.005].(They referred to Ref #55 in 2006, not updated; refer to specific comment #14 below). Given the demands (the former) and heterogeneity (the later) of various MSCs, this manuscript attempted to expand a niche: "We found that most studies focused on bone marrow- or adipose-derived MSCs, but umbilical cord-derived MSCs (UC-MSCs) are more suitable for clinical research and large-scale use without ethical problems due to their abundant source and stronger proliferative ability. Therefore, we chose UC-MSCs to study. To our knowledge, this is the first study to pretreat hUC-MSCs with a combination of inflammatory cytokines (IL-1β, TNF-α, IFN-γ) and hypoxia (2% O2). We aimed to simulate the injury-induced environment using a combination of inflammatory cytokines and hypoxia preconditioning in vitro to determine whether this preconditioning could improve the immunomodulatory capacity of human MSCs, enhance their therapeutic capacity after administration in vivo and without affecting cell quality and function." The above statement, however, did not reflect their data sets.

We have revised the language description on page 6 of the manuscript

Neither therapeutic nor in vivo was fully assessed. Nor as in literature, as they cited in

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Discussion: "Cansu Gorgun et al [34]. analysed the effects of hypoxia and inflammatory factor (TNF-α, IL-1α) pretreatment on the angiogenic potential of adipose-derived MSCs." Also, citation #53. They still claimed:" To the best of our knowledge, this is a new combined pretreatment method." Indeed, we can search the PubMed with keywords "hypoxia, MSC preconditioning" below: https://pubmed.ncbi.nlm.nih.gov/?term=hypoxia%2C+MSC+preconditioning and came out with 144 publications, comprehensively conveying what this manuscript attempted to accomplish. Nevertheless, https://pubmed.ncbi.nlm.nih.gov/?term=hypoxia%2C+hUC-MSC+, came in 9. Crossing of hUC-MSC, inflammatory with cytokines, came in 38 articles,https://pubmed.ncbi.nlm.nih.gov/?term=hUC-MSC%2C+inflammatory+cytokines+ Thus, the authors should have navigated the literature to sort out and refine their novel specifics of physiological parameters as some of the specifics mentioned below to enhance clarity. The entire sections of "Materials and Methods" lack specifics and justifications. All of those said, however, gaining a deeper understanding of how hypoxia-induced signaling pathways, oxidative stress, and mitochondrial function interact in a complex manner of MSC transplantation will offer improved comprehension of the fundamental mechanisms driving the development of diseases. Thus, the manuscript is of interest.

The pretreatment methods of MSCs include hypoxia, inflammatory factors, gene modification and other pretreatment methods, which have been mentioned in various articles, but the combination of these pretreatment methods is still worth studying, including the combination of various inflammatory factors and the combination of different pretreatment methods. Although these methods have been mentioned in existing papers, our combinations are rare, and the use of such combinations for umbilical cord derived stem cells is rare. We therefore think that the combination of our preconditioning

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modalities and the selection of sources of mesenchymal stem cells is minimal in the current paper reports, adding to the existing research. So we use this description.

Specific comments:

1) The current version of the Title does NOT reflect the data sets of the manuscript, as all the data sets were in vitro characterizations, nothing in vivo, which is the functional test.

We have changed the title

2) The integration of the abstract, the intro, the results, and the Discussion should be tied up for better logical flow and coherence: "In this context, the main challenge of MSC-based therapy is to find an in vitro culture pretreatment method that can help obtain better immunotherapy function and improve the transplantation efficacy of MSCs to cope with the environment in vivo. In this study, a combination of hypoxia (2% O2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) was used to pretreat umbilical cord-derived MSCs to simulate the injury environment and the characteristics and functions of pretreated MSCs were comprehensively evaluated to study their effects on immunomodulatory ability." The Pretreatment had "no effect on cell vitality, proliferation or size" – All of these statements contridict the published data sets. How could the authors justify both hypoxia (2% O2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) that are relevant to in vivo physiological conditions? Citations? Why did they use the combination of what parameters relevant to physiology? It is well established that a hypoxic microenvironment increases cell proliferation (PMID: 36329893) (PMID: 26151812), changes cell communication mediators (PMID: 35039054), morphology and behaviors (PMID: 35362539). In fact, hypoxia can alter the genome by activating hypoxia-inducible transcription factors (HIFs), which are essential to cellular adaptation to low oxygen levels (PMID: 34155378).

We have revised the abstract, introduction, results and discussion sections for better

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coherence. (page3/4/6/17/21/23)

hypoxia (2% O2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) and correlations in vivo are further explained in the introduction, on page 5 of the manuscript

Hypoxia promotes cell proliferation, but the effects on cell proliferation, viability and size in the combination of inflammatory environment and hypoxia are still worth exploring. Because the inflammatory environment itself has certain damage to cells, the combination of hypoxia and inflammatory factors is real and reliable.

(PMID: 31096722IF: 6.0 Q2) In BM-MSCs, viability was similar under normoxic (94.75%) and hypoxic (95.3%) conditions. Cytomix treatment slightly decreased MSC viability with concomitant increase in cell apoptosis and necrosis. Similarly, the proliferative capacity for BM-MSCS was significantly ($p < 0.05$) diminished under hypoxic conditions (hypoxia and Cytomix-hypoxia), whereas AD-MSC proliferation was unaffected by any of the treatment conditions (PMID: 24682451) Exposure to Inflammatory and Hypoxic Environment Did Not Alter MSC Viability and Specific MSC Marker Expression .

Therefore, our conclusion is true and reliable. The proliferation, viability and size of MSC cells are different only under hypoxia treatment and under hypoxia and inflammatory factors treatment, and there are changes in the source of MSCs. Our study adds a theoretical basis for umbilical cord derived MSCs.

3) "Our result shows that mesenchymal stem cells (MSCs) morphology became elongated after pretreatment, and there was no effect on cell vitality, proliferation or size." Why did MSC morphology became elongated after pretreatment without size changes?" – explanation? “Fig 1D. Cell size was detected by a cell imaging analyser, and the sizes of UC-MSCs and PUC-MSCs were basically the same.”Note that these size distributions were massive. Did they perform such size distributions side-by-side with preconditioning and no-treatment controls? Where were their data to support the point?

Although the shape of the cells became slender after pretreatment, what we measured

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was not the length of the cells, but the conclusion of Figure 1D, which was obtained by using the cell image analyzer to analyze the average size of the cells in suspension state after the cells were digested by trypsin and stained with AOPI. Moreover, this conclusion was the result of automatic analysis by the instrument, reflecting the average size of the number of cells. For example, cell size is measured in Figure 1C. Because our pretreated cells are ultimately delivered intravenously back into the human body, we measure the size of the mesenchymal stem cells in suspension rather than in adherent growth. We also found that in other papers, they mostly measured cell length PMID: 31096722. Finally, we also changed our expression in the materials and methods of the manuscript (page8-9).

4) "In addition, pretreatment did not alter common MSC surface markers, but it significantly reduced the expression of clotting promoters." How did they choose "common MSC surface markers?" What is the definition of common biomarkers?

We have made a change to the way we describe the language in the results on page 3 of the manuscript.

5) Fig 3A, Left-panel: Positive contral? Why did they not plot all the 4 sets of cells in the right panel? What was the nature of contral? What was the scale value of the Y-axis in the right panel? "The fluorescence intensity of DCFH-DA in PUC-MSCs was increased by 3-fold compared with that of UC-MSCs" -- given the nature of these two types of MSCs were similar as shown in Fig 1, and Fig 2, it was unlikely a physiological result, but the potential toxicity concern lies more with the possible alteration of cellular processes due to the measurement technique rather than inherent toxicity of DCFH-DA. Note that Fig 3 represents the essential data set. The compound 2'-7'dichlorofluorescin diacetate (DCFH-DA) is a fluorogenic dye that is capable of permeating cell membranes. It is commonly used to assess the activity of hydroxyl, peroxyl, and other reactive oxygen species (ROS) within cells. Following cellular absorption, the compound DCFH-DA undergoes deacetylation by esterases present inside the cell. This enzymatic process

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results in the formation of a non-fluorescent compound. Subsequently, reactive oxygen species (ROS) within the cell oxidize the non-fluorescent compound, producing 2'-7'dichlorofluorescein (DCF). Thus, did the authors consider the following?

a) Concentration: Using very high concentrations of DCFH-DA may lead to non-specific effects and potentially affect cell viability. Therefore, researchers typically use a range of concentrations to optimize experimental conditions. How did they titrate out the non-specific effects? [Page 4: "The cells were collected and suspended in 10 μmol/L DCFH-DA at a concentration of 1 to 20 million/ml and incubated in a 37°C infrared carbon dioxide incubator (Thermo Fisher) for 20 min." – which is not clear, what conditions did they pick the data in Fig 3.

Our results are correct, our description in the material and method is 10 μmol/L, not 10 μmol/ml, so its experimental concentration is normal. Our experimental process was carried out accurately under the guidance of the instruction manual. In addition, we redescribe the ROS experimental process. On page 9 of the manuscript.

b) ROS Production: The conversion of DCFH to DCF, which produces the fluorescent signal, is driven by the presence of reactive oxygen species (ROS). While this is the intended purpose of the dye, it's worth noting that the presence of ROS itself can have various effects on cellular processes, which could indirectly impact cell health.

We supplemented the experiment in FIG3A, which was divided into blank group, control group, experimental group and positive control group to make the experiment reasonable

c) Experimental Design: Proper controls and validation are important when using DCFH-DA to measure ROS production. Without appropriate controls, it can be challenging to distinguish between changes in ROS levels due to experimental treatments and changes due to the dye itself. [Why did the authors omit to plot both blank and positive control?]

We added blank and positive controls, UC-MSC in the picture is our control group, PUC-MSC is Experimental group which is further explained in the experimental method.

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d) Cellular Uptake: DCFH-DA is taken up by cells and can accumulate in cellular compartments. While this is often advantageous for visualizing ROS production, it's important to consider potential effects on cellular compartments and organelles. Given those concerns, why did they not plot the blank and the positive control in 3C and 3D (right panel)?

In FIG. 3C and FIG. 3D, we supplement the experimental results.

6) Fig 4 and Fig 5: Where were their controls of non-treatment?

UC-MSCs were the untreated control group and PU-MSCs were the experimental group. We further modified and supplemented the description of the first experimental method on page 7 of the paper.

7) Fig 5: "After hypoxia and inflammatory factor pretreatment, there was no significant difference except for IL-1ra."

We have modified the FIG5 Footnote

8) Page 3: ". When UC-MSCs were 70-80% confluent, a mixture of IFN-γ (R&D), TNF-α (R&D) and IL-1β (PeproTech) was added to the medium." What were the concentrations that they used? Why? Citations? [page 2: "When MSCs are injected into the body and migrate to damaged tissues or organs, the activation of innate immune cells leads to the enhanced release of chemokines and cytokines(such as TNF-α, IL-1α, and IL-1β) [27]"] – Did this citation provide relevant concentrations?

At the request of the research group, the concentration of each factor will not be provided in order to maintain confidentiality. This is our laboratory unique configuration ratio, will not be published.

9) Page 3: "Then, the cells were immediately placed into a three-gas incubator with 2% O2, 5% CO2, and 93% N2 at 37°C (Panasonic Japan). After 24 h, primed UC-MSCs (PUC-MSCs) were obtained." What was their quality control to tell they had the hypoxia condition?

We have made further explanations in the experimental method to better indicate the state

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of normoxia and hypoxia(page7).

Numerous papers show that MSCs are hypoxic when they are in 2% O2 conditions.

such as：PMCID：PMC6562603 Human MSCs were cultured for 48 h in either normoxia (21% O2) or hypoxia (2% O2) with or without the addition of Cytomix, thus creating 4 groups: (1) normoxia (21%); (2) Cytomix-normoxia (+21%); (3) hypoxia (2%); and (4) Cytomix-hypoxia (+2%).

PMCID： PMC10463845 Thus, relative to their native environment, the normoxic conditions typically found in culture could be considered severely hyperoxic for MSCs. Culturing MSCs at lower oxygen concentrations, referred to as hypoxic conditions (i.e., 1–5% O2 in the headspace), has been shown to enhance their angiogenic potential [19–22],

10) Grammar errors crawl across the pages. E.g., “MSCs do not lead to graft rejection after allotransplantation [9]. therefore, they show great potential, economic value, and social significance and have broad application prospects in the field of cell therapy.”

Grammatical issues we will send to a specialized language touch-up agency for touch-ups.

11) Page 2: “For example, CD142 is the initiator of the clotting process, and under certain conditions, MSCs overexpress CD142, which may increase the risk of thrombosis after intravenous injection [38-42].” Insufficient citations.

We have revised the content of the article and replaced the persuasive quotations(page6).

12) Page 14: “Hypoxia and inflammatory factor pretreatment for 24 h enhances the immunomodulatory activity of MSCs.” Why did they pick 24 h, while other experiments, d5 (Fig 6)?

Hypoxia and inflammatory factor preconditioning we have always chosen 24 hours, when the least damage to the mesenchymal stem cells. In order to study the proliferation inhibition of UC-MSCs and PUC-MSCs on PBMC, the 5d in Figure 6 shows that we co-cultured PBMC and UC-MSC/PUC-MSC for five days, which is different from the pretreatment for 24 hours. At the same time, we have modified the description method

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(page15-16).

13) Pages 15-16: "We plotted the growth curves of PBMCs, observed the changes in cell growth dynamics after direct contact and coculture with UC-MSCs or PUC-MSCs, and found a typical S-shaped proliferation pattern (6A)." What did they mean by "a typical S-shaped proliferation pattern?" any proliferation assays done? Either UC-MSCs alone or PUC-MSCs alone as controls?

We divided the experiment into three groups: PBMC alone culture, PBMC+UC-MSCs, and PBMC+PUC-MSCs co-culture to observe the effects of UC-MSCs and PUC-MSCs on the inhibition of PBMC proliferation. We plotted the growth curve by counting the PBMC of the three groups through 5d culture, which reflected the proliferation inhibition effect of UC-MSCs and PUC-MSCs on PBMCs. We deleted the statement "typical S-shaped proliferation pattern" and emphasized the effect of proliferation inhibition. (page15-16).

14) They cited an out-of-date citation - "[55]Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells, the international society for cellular therapy position statement. Cytotherapy. 2006;8(4):315–317. doi: 10.1080/14653240600855905. They went on to state, "The International Society for Cell and Gene Therapy defines minimum standards for MSC characterization, and most experiments are conducted on this basis. [55]. However, we went on to examine more positive surface markers on MSCs. We found that UC-MSCs and PUC-MSCs from three donors retained high levels of the surface markers CD105, CD90, CD73, CD29, CD166, CD47 and HLA-ABC and were negative for CD31, CD45, CD14, and CD34. This finding indicated that the preconditioning retained the dryness, homing, adhesion, migration, and anti-inflammatory abilities and resistance to NK cell-mediated lysis of these stem cells." This behavior is inappropriate and misleading.

We removed this description and made changes(page18)

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15) Page 17: "Our pretreatment method greatly reduced the coagulation-promoting ability of the cells." How did they claim this data?

We have modified this description in the results, discussions, and conclusions, and the mechanism of CD142 is detailed in the introduction. (page 3, 6, 18, 21)

16) Page 17: "The efficacy of MSCs depends on the full function of their mitochondria, which can be damaged in harmful environments. MSC vitality, plasticity, proliferation, differentiation potential, and function are all affected by mitochondrial function and integrity [56]. Therefore, we examined the effects of hypoxia and inflammatory factor pretreatment on mitochondrial function. ROS levels were increased after pretreatment but were within the range of the positive controls." Did they assess "plasticity, proliferation, differentiation potential, and function" in vivo? If not, they overstated their data.

We have removed this language description(page19)

17) Page 19: "In conclusion, we successfully developed a method of in vitro preconditioning to simulate the damaged environment using a combination of hypoxia (2% O2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) to enhance the therapeutic power of UC-MSCs, as indicated by the enhanced functional characteristics and immunosuppressive and immunoregulatory functions. This method does not affect cell function or cell quality." Insufficient to say CD142 is the initiator of the clotting process," speculated in vivo experiments. How did they assess "to enhance the therapeutic power of UC-MSCs" without in vivo testing?

We have modified the verbal description of mesenchymal immunotherapeutic power that appears in the manuscript to highlight the outcome description of the in vitro dataset(page3,21,23). In addition, we have revised the description of CD142 in the introduction, results, discussions and conclusions. We no longer emphasize the expression of the procoagulant ability of our pretreatment reaction in vivo, and fully describe the coagulation initiation of CD142 in the introduction. In addition, Next, our

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research group will conduct in vivo data study on the pre-treated mesenchymal stem cells, which is the next research focus of our research group.

Reviewer#2

Specific comments to authors

The field of the manuscript is important as there are a lot of challenging of the use of MSCs for the treatment. The use of hypoxia and inflammatory factors for conditioning the MSCs before their use for the treatment is tried before. However, it is still important to try different conditioning to get the best outcomes. The authors did good work by using different parameters and techniques to test the quality of MSCs. However, I have two main concerns:

- The authors did not clarify if the cells were divided into two groups where one group was continued as untreated, and the second group was treated with hypoxia and the inflammatory factors. It mentioned at the methodology the following: "Approximately 14 days later, the cells were obtained for passage, and the P4 generation cells were used for experiments. When UC-MSCs were 70-80% confluent, a mixture of IFN-γ (R&D), TNF-α (R&D) and IL-1β (PeproTech) was added to the medium. Then, the cells were immediately placed into a three-gas incubator with 2% O2, 5% CO2, and 93% N2 at 37°C (Panasonic Japan). After 24 h, primed UC-MSCs (PUC-MSCs) were obtained" if there was no untreated group continues in parallel with the treated group than the comparison will not be suitable.

In the first experimental method in Paper 7, we provide a detailed description of the experimental groupings.

- The protentional capacity of the MSCs differentiation to adipocytes, chondrocytes and osteocytes are important to test the functionality of the MSCs. This test need to be done!

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The multilineage differentiation experiment of MSCs is an important index to test the cell function, but the experimental process is time-consuming, and the laboratory does not have the experimental conditions at present, so it is difficult to complete the experiment within 14 days. In addition, we have revised the manuscript not to emphasise its function, but to adopt the term biological characteristics. However, our research group will further carry out research on the in vivo function of MSCs, which is the next focus of our research group. Next, we will further investigate the ability of MSCs to differentiate into adipocytes, chondrocytes and osteocytes. We will also improve the karyotype, exosome secretion experiment and tumourigenicity of MSCs. Therefore, the study of the differentiation ability of MSCs is the next focus of our work, and we will refine this experiment when we conduct in vivo experiments with MSCs.