**Reviews for** “Vitronectin mediates survival of human WJ-MSCs under inflammatory temperature stress via cell cycle arrest”

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**DOI:** 10.3233/STJ-220002

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**Review Round 1 Decision: Revise with major revisions**

**Reviewer 1** has selected to remain anonymous.

Originality, novelty and significance of results: Good

Technical Quality of Work: Good

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: Good

**Reviewer Recommendation Term:** Accepted pending minor revisions

**Narrative (as sent to corresponding author):**

MINOR REVISIONS:

1. The authors should better describe the methodology followed for the performance of the experiments. There are inconsistencies between the text included in the methods section and the results and figure captions. Authors should describe more clearly:

\*\*Isolation, expansion and culture of the WJ-MSCs used:
a) The three culture groups/conditions on which the experiments are performed are not clearly defined: control, exposure to 40ºC and hypoxia.
b) Cell concentration(s) used for expansion and culture of WJ-MSCs.
c) Culture methodology: conditions (temperature, CO2, O2 and humidity), time for medium changes, etc...

For example:
- WJ-MSCs were exposed to 40°C for durations of 48, 72, and 96 hs and VTN protein expression was evaluated.: In the methods section, only a 48h exposure is mentioned.
- WJ-MSCs were plated at different cell seeding density of 2000, 5000 and 8000 cells/cm2: In the methods section, only a seeding concentration of 5.000 cells/cm2 is mentioned.

\*\*Number of umbilical cords used, as well as number of experiments and conditions used for each experiment. It becomes difficult to understand the material with which the experiments are made, the number of samples used in them, etc.

2. Although it may seem obvious, authors are requested to add in the text the meaning of each abbreviation used for a better understanding of the work.

**Reviewer 2** has selected to remain anonymous.

Originality, novelty and significance of results: Adequate

Technical Quality of Work: Adequate

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: Inadequate

**Reviewer Recommendation Term:** Reject

**Narrative (as sent to corresponding author):**

In this study the authors investigated in vitro whether temperature stress induces changes in MSCs that might compromise their future survival when transplanted into individuals with fever. The study has a number of major caveats especially on statistics and conclusions as well as a lack of purity study etc which should be addressed before publication is recommended:

1. The rationale for the study seems a little confusing to this reviewer. Do the authors consider it realistic to inject MSCs into patients with 40 in fever?
2. The introductions is a bit lengthy, but still lacks more general info on other studies besides the authors own on temperature stress in cells. Remove the final part in the introduction which is repetitive or should be part of the abstract. The abstract could also be sharpened.
3. Check for typing errors throughout the manus would be needed.
4. Data presented should be mean, SD and not SEM. Statistical analysis performed require normal distribution of data, but the authors do not include any data on such analysis. Having three samples is not enough for estimating normality and therefore in many cases non-parametric tests should have been used.
5. There is no data on the purity of the MSCs-it seems from M&M that cells are used in P0 passage? At this stage the purity will be heterogenous in most cases. The authors should provide some data on this and clarify in M&M if and when they passaged the cells or not.
6. It is not adequate to state that" an increase in VTN expression both at mRNA (p <0.01) and protein levels (not significant)" if not significant. However, by looking at Fig. 1B-it seems significant? Yet, also in many other places the authors conclude something not in agreement with statistics. As an example : Moreover, assessment of proliferation using MTT assay showed a reduction in proliferation of WJ-MSCs at 40°C, though not significant. Another two examples: "This resulted in a reduction in viable population of WJ-MSCs from 80.20 ± 3.27 to 69.82 ± 3.85, though not significant, as compared to NC siRNA transfected WJ-MSCs. Also, a corresponding increase in early and late apoptotic populations from 13.83 ± 3.03 to 17.97 ± 6.5 and 5.26 ± 2.46 to 11.29 ± 3.73, respectively, again not significant, was noted from apoptosis assays (Fig. 3B, C). The authors should change such incorrect statements and summarize if this changes the overall conclusions.
7. The authors should list statistical analysis used in figure legends.
8. In fig. 2D,E. Are the gates correct-they do not have a positive control and seem to cut through the main negative population. How was the gating strategy-do we see all cells or did they pregate on FSS/SSC beforehand? Same goes in figure 3? Do the authors in general observe 10-15% of cells being (pre)apoptotic-then it seems as the culture in general is compromised.
9. Left part of figure 3 is missing in the PDF?
10. The authors should show stainings or flow cytometry of their VTN knockdown instead of just qPCR. It is not easy to interpret the apoptosis and cell cycle data without, as knockdown may have occurred in only a subpopulation? Also since no assay of purity of the MSCs have been presented-one may not know in which cell type knockdown occurred.
11. Why did the authors use E2F1, CCNA2, AURKA, and CDKN3 as markers for cell cycling-although they are associated with the cell cycling CyclinD1 is the major initiation factor on G1 to proceed to S? All qPCR data are normalized to only one gene control-whereas most studies now represent multiple controls used at the same time.
12. Results to Figure 4/5-As above in many cases data are not significant-one may ask do they matter then? The Authors conclude, but is this appropriate?The review by Boopalan et al., provides a thorough overview of ongoing clinical trial activities with stem cells in India. The manuscript introduces the field with a balanced description of different types of stem cells and their potential applications for treatments as well as provides a useful summary of the cell therapies which have been granted market approval so far. The review provides a highly useful and impressively comprehensive overview of ongoing trial activities in India in Fig. 2 and Table 1 - such compiled resources are highly valuable to researchers in the field, since clinical trial information is otherwise scattered over many separate sites. The manuscript further describes also relevant problem with stem cell therapies in India, such as trial results which are not published and the availability of unproven and illegal treatments with cell therapies at private clinics. Overall, I think the manuscript is well-written, balanced and thorough in its overview, and only have a few minor comments:

1) Please insert relevant references to support the statement on page 2: "Stem cell therapy has shown promising results in preclinical and clinical trials for several diseases including, Parkinson's disease, Diabetes mellitus, Crohn's disease and various haematological disorders."

2) The authors write on page 4: "Multipotent stem cells are also found in other tissues like the brain, the retina, the liver and the gut [4,6]." However, this statement is not entirely correct, since the liver does not contain stem cell, but rather regenerates through expansion of differentiated hepatocytes. Similarly, the retina cannot regenerate and is not believed to contain any stem cells. It should also be mentioned that although the brain contains stem cells, these are very limited in both number and differentiation potential.

3) The authors write on page 4: "In general, stem cells reside in a "stem cell niche", which is a micro-environment that favours self-renewal of such stem cells. Notably, these stem cells remain dormant under normal conditions and are only activated by signals generated by damaged tissue or injury." Again, this statement is not correct, since stem cells in the skin, intestine and blood and constantly active in tissue regeneration, also under normal homeostatic conditions.

4) On page 6, when listing approved MSC-based therapies (Prochyman, Temcell, Cartistem, Cellgram and Stemirac, please mention which diseases they are indicated for.

5) On page 9, it is stated about trial results from MSCs "Multiple reasons are attributed to such moderate outcomes and they include potential immune rejection by monocytes within 24 hrs after injection resulting in poor graft survival." It should be mentioned here that poor trial outcome from MSC trials can also in many cases be attributed to inadequate efficacy data from animal models and unclear hypotheses about the mechanism of action.

6) In the description of limbal stem cells, it should be mentioned that Holoclar was the first stem cell product to be approved by the EMA, in 2014.

7) For Fig. 2C, please specify what is on the Y-axis (i.e. "number of clinical trials")

8) For Fig. 2D, it wold be more intuitive to order the columns on the y-axis according to Phases, i.e. Phase 1 to 4 from left to right. Also, please increase font size on the labels.

**Author’s reply to reviewers:**

Reviewer #1: MINOR REVISIONS:

1. The authors should better describe the methodology followed for the performance of the experiments. There are inconsistencies between the text included in the methods section and the results and figure captions. Authors should describe more clearly:

\*\*Isolation, expansion and culture of the WJ-MSCs used:
a) The three culture groups/conditions on which the experiments are performed are not clearly defined: control, exposure to 40ºC and hypoxia.
b) Cell concentration(s) used for expansion and culture of WJ-MSCs.
c) Culture methodology: conditions (temperature, CO2, O2 and humidity), time for medium changes, etc...
For example:
- WJ-MSCs were exposed to 40°C for durations of 48, 72, and 96 hs and VTN protein expression was evaluated.: In the methods section, only a 48h exposure is mentioned.
- WJ-MSCs were plated at different cell seeding density of 2000, 5000 and 8000 cells/cm2: In the methods section, only a seeding concentration of 5000 cells/cm2 is mentioned.

\*\*Number of umbilical cords used, as well as number of experiments and conditions used for each experiment. It becomes difficult to understand the material with which the experiments are made, the number of samples used in them, etc.

Answer: We thank the reviewer for carefully going through our manuscript. We have now included all the experimental details as per the reviewer’s suggestion.
In the figure legends we have included all details of the number of biological samples (umbilical cord samples) used, the statistical tests done etc.

2. Although it may seem obvious, authors are requested to add in the text the meaning of each abbreviation used for a better understanding of the work.

Answer: As per the reviewer’s suggestion, abbreviations have been expanded in the text.

Reviewer #2: In this study the authors investigated in vitro whether temperature stress induces changes in MSCs that might compromise their future survival when transplanted into individuals with fever. The study has a number of major caveats especially on statistics and conclusions as well as a lack of purity study etc which should be addressed before publication is recommended:

1. The rationale for the study seems a little confusing to this reviewer. Do the authors consider it realistic to inject MSCs into patients with 40 in fever?
Answer: MSCs are known to have potent anti-inflammatory and immunomodulatory properties, with the capability of treating many immune-mediated disorders. Inflammation is often accompanied by increase in body temperature and fever is one of the four cardinal signs of inflammation. The impact of elevated temperature on stem cells has usually not been taken into account or studied very extensively. We felt that this was an important parameter to investigate.
In our previously published work, we had tested 38.5°C as well, as milder febrile condition, and found the changes in cell cycle status to be in between 37 and 40°C (ref). Hence, this study was conducted at 40°C.
Reference:
Goyal U, Ta M. p53-NF-κB Crosstalk in Febrile Temperature-Treated Human Umbilical Cord-Derived Mesenchymal Stem Cells. Stem Cells Dev. 2019 Jan 1;28(1):56-68.

2. The introductions is a bit lengthy, but still lacks more general info on other studies besides the authors own on temperature stress in cells. Remove the final part in the introduction which is repetitive or should be part of the abstract. The abstract could also be sharpened.
Answer: As per the reviewer’s suggestion we have cut down unnecessary details from the introduction and tried to sharpen it.
The final part of introduction has been removed.
Other general information with references have been included on temperature stress in MSCs.

3. Check for typing errors throughout the manus would be needed.
Answer: We thank the reviewer, and have corrected the manuscript at several places.

4. Data presented should be mean, SD and not SEM. Statistical analysis performed require normal distribution of data, but the authors do not include any data on such analysis. Having three samples is not enough for estimating normality and therefore in many cases non-parametric tests should have been used.
Answer: We have noted the point of the reviewer. However, wherever we have n = 3-5 and bars represent mean ± SEM, we have shown the individual data points as well on the bars. This is to show spread of the data points and the variability of data. As SD is mainly used to denote the dispersion of the data, this point has been taken care of.
In cases, where we have n = 50-80, and individual data points could not be shown on the bars, there we have re-plotted the data as mean ± SD now.
For certain key experiments where we had n=3, we have now included 2 more biological sample sets and made n equal to 5.

5. There is no data on the purity of the MSCs-it seems from M&M that cells are used in P0 passage? At this stage the purity will be heterogenous in most cases. The authors should provide some data on this and clarify in M&M if and when they passaged the cells or not.
Answer: The WJ-MSCs at passage 4-6 were used for the different experiments.
MSCs are known to be a heterogenous population. However, they are defined by cell surface antigens.
We routinely characterize the WJ-MSCs at passage 4 by assessing cell surface marker expression by flow cytometry. A representative data has been added to the supplementary section. The MSCs demonstrated high degree of homogeneity with respect to cell surface markers.

6. It is not adequate to state that" an increase in VTN expression both at mRNA (p <0.01) and protein levels (not significant)" if not significant. However, by looking at Fig. 1B-it seems significant? Yet, also in many other places the authors conclude something not in agreement with statistics. As an example: Moreover, assessment of proliferation using MTT assay showed a reduction in proliferation of WJ-MSCs at 40°C, though not significant. Another two examples: "This resulted in a reduction in viable population of WJ-MSCs from 80.20 ± 3.27 to 69.82 ± 3.85, though not significant, as compared to NC siRNA transfected WJ-MSCs. Also, a corresponding increase in early and late apoptotic populations from 13.83 ± 3.03 to 17.97 ± 6.5 and 5.26 ± 2.46 to 11.29 ± 3.73, respectively, again not significant, was noted from apoptosis assays (Fig. 3B, C). The authors should change such incorrect statements and summarize if this changes the overall conclusions.

Answer: For Fig. 1B, we increased the sample size to n=5 and the difference is significant now. The new plot has been included in the revised manuscript.
These are not really incorrect statements. It being human samples, sample-to-sample variations are noted in the data. However, the trend remained the same across all the samples tested, whether increase or decrease. Hence, they have been reported in the manuscript. Changed now to (not significant).

7. The authors should list statistical analysis used in figure legends.
Answer: We do appreciate the reviewer pointing this out. We have now included the statistical analysis details in every figure legend.

8. In fig. 2D, E. Are the gates correct-they do not have a positive control and seem to cut through the main negative population. How was the gating strategy-do we see all cells or did they pregate on FSS/SSC beforehand? Same goes in figure 3? Do the authors in general observe 10-15% of cells being (pre)apoptotic-then it seems as the culture in general is compromised.
We thank the reviewer for raising a pertinent question regarding gating in the apoptosis analysis in Fig 2D, E and Fig 3. During apoptosis analysis, we initally performed a pre-gating on the population for FSC-H/SSC-H, which encompassed major population while excluding the debris (Supplementary fig. 1D). Next, gating was applied to the only annexin-V and only PI treated control samples independently, to discriminate viable and apoptotic population. Subsequently, after completion of the gating with only annexin-V and only PI control samples no further changes were made, and both annexin-V and PI treated samples were analysed for control and all the experimental treatments.

9. Left part of figure 3 is missing in the PDF?
Answer: We apologize for this PDF-related problem, although the individual figures uploaded were alright. We will ensure full figure 3 is visible this time in the PDF.

10. The authors should show stainings or flow cytometry of their VTN knockdown instead of just qPCR. It is not easy to interpret the apoptosis and cell cycle data without, as knockdown may have occurred in only a subpopulation? Also since no assay of purity of the MSCs have been presented-one may not know in which cell type knockdown occurred.

Answer: As per the reviewer’s suggestion, we performed immunofluorescence staining with VTN esi-RNA treated WJ-MSC samples. The data has been included in Figure 3.

11. Why did the authors use E2F1, CCNA2, AURKA, and CDKN3 as markers for cell cycling-although they are associated with the cell cycling CyclinD1 is the major initiation factor on G1 to proceed to S? All qPCR data are normalized to only one gene control-whereas most studies now represent multiple controls used at the same time.
Answer: Although the reviewer suggested cyclin D1, we tested cyclin D2 expression level as the primers were readily available in our lab. D-type cyclins are known to drive cells through the G1 restriction point and into the S phase. We have now included the qRT-PCR data for Cyclin D2 from 3 sets of biological samples in Figure 3.
The other genes, E2F1, CCNA2, AURKA, and CDKN3 were selected from different stages of the cell cycle, to confirm that the cells were not entering cell cycling.
The qPCR data have been normalised to GAPDH in our study.
Previously we tested18s rRNA as a housekeeping gene for the normalization of gene expression data and found a strong downregulation of 18s rRNA mRNA at 40°C even when the samples were reverse transcribed starting from equal amount of RNA (ref). GAPDH mRNA levels however remained the same between control and 40°C samples.
In fact, the effect of heat stress on the expression of 18s rRNA had been examined in a few previous reports from other labs too (ref).
Now, based on the suggestion of the reviewer, we tested beta-actin too as an endogenous control. No change was noted in the expression level between the control and 40°C samples, and data has been included in supplementary figure 1E.
References:
a. Goyal U, Ta M. p53-NF-κB Crosstalk in Febrile Temperature-Treated Human Umbilical Cord-Derived Mesenchymal Stem Cells. Stem Cells Dev. 2019 Jan 1;28(1):56-68. doi: 10.1089/scd.2018.0115.
b. Zhao Z, Dammert MA, Hoppe S, Bierhoff H, Grummt I. Heat shock represses rRNA synthesis by inactivation of TIF-IA and lncRNA-dependent changes in nucleosome positioning. Nucleic Acids Res. 2016 Sep 30;44(17):8144-52. doi: 10.1093/nar/gkw496. Epub 2016 Jun 1.

12. Results to Figure 4/5-As above in many cases data are not significant-one may ask do they matter then? The Authors conclude, but is this appropriate?
Answer: Some such data, for instance, hypoxia + PI3K inhibitor have been removed from Figure 5, as per the suggestion of the reviewer.

**Review Round 2 Decision: Revise with minor revisions**

**Reviewer 1** has selected to remain anonymous.

Originality, novelty and significance of results: Good

Technical Quality of Work: Good

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: Good

**Reviewer Recommendation Term:** Accept

**Narrative (as sent to corresponding author):**

The manuscript has been greatly improved with the clarifications provided, including cell characterisation, so as not to lead to confusion with other cell types, e.g. fibroblasts.

**The Associate Editor asked for minor revisions and provided further comments for improvement:**

Reviewer #1: The manuscript has been greatly improved with the clarifications provided, including cell characterisation, so as not to lead to confusion with other cell types, e.g. fibroblasts.

Comments from the editor:
THe manuscript has been greatly improved during revision and can now be accepted for publication. However, as already pointed out by reviewer 2, it is problematic to put conclusion statements and interpretations on non-significant data. These statements below must therefore be modified before proceeding to publication:

o     P15: “Inhibition of ERK and PI3K pathways exhibited a further upregulation in VTN mRNA expression, while a reduction in the expression was noted with (NF-κβ) pathway inhibition (not significant) (Fig. 2A).”

Change to;

o     “Inhibition of ERK and PI3K pathways exhibited a further upregulation in VTN mRNA expression, while (NF-κβ) pathway did not show any significant effect (Fig. 2A).”

o     P16: “Moreover, assessment of proliferation using MTT assay showed a reduction in proliferation of WJ-MSCs at 40°C, though (not significant)”

Change to:

o      “Assessment of proliferation using MTT assay showed no significant reduction in proliferation of WJ-MSCs at 40°C”

o     P17: “This resulted in a reduction in viable population of WJ-MSCs from 81.84 to 72.63 (not significant) as compared to NC siRNA transfected WJ-MSCs. Also, a corresponding increase in eaely and late apoptotic populations from 11.78 to 15.17, respectively (not significant) was noted from apoptosis assay (Fig. 3D)

Change to:

o     “There was no statistically significant change in the viable population or early and late apoptotic populations of WJ-MSCs) as compared to NC siRNA transfected WJ-MSCs (Fig. 3D)

**Reply by the author:**

We thank the reviewers for their suggestions and comments.
In this second round of revision, as per the advice of reviewer 2, all the three statements, on pages 15, 16 and 17, respectively, have been modified accordingly.

**AFTER THE FINAL REVISIONS THE ASSOCIATE EDITOR DECIDED TO ACCEPT.**