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PROJECT COMPLETION REPORT

1. **Project Number** : P8194

2. **Title of the Project** : Stem Cell Derived Exosome Therapy for Clinical Management of Lung Damage in Critically-ill Corona Viral Pneumonia Patients

3. **Funding Agency Name** : SERB, New Delhi

4. **Project Reference Number provided by the Funding Agency**: CVD/2020/000224

5. **Principal Investigator (Name & Address) :** Dr. Naresh Kasoju, SCTIMST Tvm

6. **Co-Investigators (Name & Address):**
   - i) Dr. Senthilkumar Mutusamy, SCTIMST Tvm
   - ii) Dr. Harikrishnan VS, SCTIMST Tvm
   - iii) Dr. Sabareeswaran A, SCTIMST Tvm
   - iv) Dr. Anil Kumar PR, SCTIMST Tvm
   - v) Dr. Francis B. Fernandez, SCTIMST Tvm

7. **Implementing Institution** : SCTIMST Trivandrum

8. **Collaborating Institutions** : N/A

9. **Date of Commencement** : 26 Aug 2020

10. **Duration** : 1 Year and 4 months of no-cost extension.

11. **Date of Completion** : 30 Dec 2021

12. **Objectives as approved:**
    - i) Isolation and ex vivo expansion of MSCs from Wharton's jelly of umbilical cord
    - ii) Preparation, purification and characterization of WJ-MSCs derived exosomes
    - iii) Development and treatment of acute lung damage in animals with WJ-MSC derived exosomes

13. **Deviation made from original objectives if any, while implementing the project and reasons thereof : N/A**

14. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs : *Kindly refer to the details presented in S. No. 16.*

15. Detailed anaylsis of results : *Kindly refer to the details presented in S. No. 16.*

16. Summary sheet of not more than 2 pages under following heads: (Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)

Title: Stem Cell Derived Exosome Therapy for Clinical Management of Lung Damage in Critically-ill Corona Viral Pneumonia Patients

Introduction and Rationale: Chinese health authorities reported a group of pneumonia cases of unfamiliar aetiology in Wuhan, China in December 2019. Subsequent studies suggested a zoonotic coronavirus which showed 79% sequence similarity to severe acute respiratory syndrome corona virus (SARS-CoV) (Zhu et al. 2020). This novel beta-coronavirus, named as SARS-CoV-2, has been declared as the causative agent of the coronavirus disease 2019 (COVID-19) (Guan et al. 2020). A retrospective analysis of clinical and thoracic computed tomography (CT) of 120 COVID19 patients indicated ground-glass opacities ranging from 87% to 97% as the predominant radiological finding, across peripheral and lower lung (Zhang et al. 2020). Detailed analysis of biochemical parameters revealed a cytokine and chemokine storm behind the pulmonary inflammation and extensive lung damage in COVID19 patients (Huang et al. 2020). As of now, there is no specific recommended treatment for COVID19, other than providing supportive care through oxygenation, ventilation and fluid management. Several anti-viral and anti-inflammatory drug regimens are proposed to be used in management of lung damage (Stebbing et al. 2020; Mehta et al. 2020). However, while some support this strategy, others warn this could be a double edged sword. Alternatively, apart from anti-viral therapeutics, in this study, we propose to use extracellular vesicular fractions obtained from Wharton's jelly of umbilical cord derived mesenchymal stem cells (WJ-MSCs) as the immune-modulatory regime with regenerative potential in the management of lung damage.

Objectives:
i) Isolation and ex vivo expansion of MSCs from Wharton's jelly of umbilical cord
ii) Preparation, purification and characterization of WJ-MSCs derived exosomes
iii) Development and treatment of acute lung damage in animals with WJ-MSC derived exosomes

Methodology:
Objective 1. Isolation and ex vivo expansion of MSCs from Wharton's jelly of umbilical cord
In this study, we established the primary culture of human WJ-MSCs and characterized by their ability to adhere to plastic surfaces and to differentiate into chondrogenic, osteogenic and adipogenic differentiation upon induction, following earlier protocols (Goyal et al. 2018) (HiMedia, Bangalore). The induction of exosomes from WJ-MSCs was carried out by following the gold standard serum starvation approach as described previously (Nikfarjam et al. 2020). Briefly, the WJ-MSCs were cultured routinely in T75 culture flasks till 70-80% confluence. Subsequently, the cells

were then fed with fresh DMEM supplemented with 1% Pen-Strep but without FBS and incubated in a CO_2 incubator. After 24-48 h of incubation, the spent medium was collected for further processing. The stem cell study was approved by Institute Committee for Stem Cell Research, SCTIMST Trivandrum (Ref: SCT/IC-SCR/62/September2020).

Objective 2. Preparation, purification and characterization of WJ-MSCs derived exosomes

The exosomal fraction was isolated following the ultra-centrifugation approach as previously described (Nikfarjam et al. 2020). Briefly, the spent media was subjected to ultracentrifugation at 1,00,000g for 90 min at 4°C using Beckman Optima L-90K Ultracentrifuge (type 45 Ti fixed angle rotor). The supernatant was carefully removed and the pellet was re-suspended in 500 µl of PBS. The resultant exosomal fraction was subjected to total protein content using protein quantification kit, total nucleic acid content using Nanodrop, morphological analysis using transmission electron microscopy, size analysis by dynamic light scattering, protein profile by SDS-PAGE and surface marker analysis (CD63, CD81 and TSG101) by western blotting. Further, exosomal uptake by A549 cells was also assessed in vitro.

Objective 3. Development and treatment of acute lung damage in animals with WJ-MSC derived exosomes

In the current study, we have chosen mice as model animal (C57BL/6J strain, aged between 8-10 weeks old, weighing between 20-30 g, male and female both). The animal studies have been initiated and are ongoing. Model development groups includes: *Group-1* was subjected to intra-tracheal (IT) instillation of bleomycin (BLM) (2 mg/kg body weight) and *Group-2* was subjected to IT instillation of saline; both housed for 14 days. Experimental groups include: Group-3 was BLM-treated and infused with hWJ-MSCs intravenously, Group-4 was BML-treated and infused with hWJ-MSC-EVs intravenously, and Group-5 was BLM-treated and without any therapeutic regime; all housed for 21 days. Subsequently, the lung tissues were excised and subjected to histopathological analysis, and the data was evaluated following standard protocols at our laboratory. The animal study was approved by Institute Animal Ethics Committee, SCTIMST Trivandrum (Ref: SCT/ABS/IAEC-107/14, dated 09/12/2020).

Results:

Objective 1. Isolation and ex vivo expansion of MSCs from Wharton's jelly of umbilical cord

In this study, we established the primary culture of human WJ-MSCs (HiMedia, Bangalore), wherein the cells were plastic adherent and were showing their characteristic spindle shaped fibroblast-like morphology. The WJ-MSCs were then subjected to tri-lineage differentiation over a 21-days culture period in presence of osteogenic, adipogenic and chondrogenic differentiation media. Subsequently, the cells were positively stained for alizarin red staining, oil red O staining and alcian blue staining, indicating successful differentiation on WJ-MSCs into osteogenic, adipogenic and chondrogenic lineages respectively.

Objective 2. Preparation, purification and characterization of WJ-MSCs derived exosomes

In the current study, the induction of exosomes from WJ-MSCs was carried out by following the gold standard serum starvation approach. Briefly, the WJ-MSCs, cultured in T75 culture flasks till 70-80% confluence, were starved in serum-free DMEM medium for 24-48 h. The cell morphology before and after serum-starvation was observed using a light microscope, and it was found that there were no significant changes in the cell morphology. The conditioned medium was then collected aseptically, subjected to ultra-centrifugation process and resultant pellet was resuspended in PBS.

One T75 flask containing about 5×10^6 cells of MSCs were starved in 10 ml serum-free medium and the resultant pellet was resuspended in 500 µl of 1x-PBS. The total protein content was found to be 126 ± 24 µg/ml as determined by BCA assay. The total nucleic acid content was found to be about 10 ng/ml as determined by NanoDrop. The TEM analysis revealed intact spherical shaped vesicles and the DLS analysis indicated a dimeter ranging from 150-200 nm. The exosomal fraction, when subjected to SDS-PAGE followed by western blotting revealed characteristic peaks for exosomal markers CD63, TSG101 and CD81 (however, CD63 was the most prominent band). Subsequently, DiO labelled exosomal fraction was fed to A549 cells and after 24 h, the cells were seen with green fluorescence indicating successful uptake of the exosomes by the lung epithelial cells in vitro.

Objective 3. Development and treatment of acute lung damage in animals with WJ-MSC derived exosomes

In the current study, we have chosen mice as model animal (C57BL/6J strain, aged between 8-10 weeks old, weighing between 20-30 g) and bleomycin as drug molecule to induce lung injury. The animals were given anesthesia, an incision was made to expose the trachea, 50 ul of Bleomycin solution was instilled intratracheally (final BLM dose: 2 mg/kg of body weight). The wound was closed and animal was observed till it regains consciousness. Subsequently, they were housed and the health was monitored on regular basis.

The histopathological analysis of lung tissues from experimental animals after 21 days of housing was performed and the results are presented in Figure 6. In the saline control group, all lobes were noted and no abnormality could be detected. Single walled alveoli with occasional mononuclear cells noted. In the BLM-treated group, all lobes noted and consolidation of lung parenchyma noted in all the lung lobes, where moderate to severe inflammation was observed. The experimental treatment groups i.e. BLM-treated with hWJ-MSCs and hWJ-MSC-EXO intravenously were found to show similar results compared to the saline control group. Here, all lobes were noted and no abnormality could be detected. Single walled alveoli with occasional mononuclear cells noted. The results indicated that the MSC as well as MSC-EXO infusions have aided in lung repair and regeneration in BLM-induced lung injury in small animal models. However, detailed molecular investigations should be undertaken for confirming the mechanism and potential insights in this regard.

Translational potential:

The current project was a proof of concept study looking into feasibility of exploring the exosomal fractions obtained from Wharton's jelly of umbilical cord derived mesenchymal stem cells as the regenerative therapeutic regime for the management of acute lung injury. Based on the available data, the concept can be further taken up to clinical trials stage in association with any stem cell company. Commercial interest

lies mainly in the formulation of a stable and off-the-shelf of exosomes which requires further studies that are outside the scope of the current project.

Key references

(i) Goyal, U., et al. (2018). Isolation and Establishment of Mesenchymal Stem Cells from Wharton's Jelly of Human Umbilical Cord. *Bio-protocol* 8(4): e2735. (ii) Guan WJ, et al. Clinical Characteristics of Coronavirus Disease 2019 in China [2020 Feb 28]. N Engl J Med. 2020. (iii) Mehta P, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. Lancet. 2020;395(10229):1033–1034. (iv) Nikfarjam, S., et al. Mesenchymal stem cell derived-exosomes: a modern approach in translational medicine. J Transl Med 18, 449 (2020). (v) Stebbing J, et al. COVID-19: combining antiviral and anti-inflammatory treatments. Lancet Infect Dis. 2020;20(4):400–402.

17. Contributions made towards increasing the state of knowledge in the subject :

The current project successfully provided proof of concept data in support of the feasibility of exploring the exosomal fractions obtained from Wharton's jelly of umbilical cord derived mesenchymal stem cells as the regenerative therapeutic regime for the management of acute lung injury. Based on the available data, the concept can be further taken up to clinical trials stage in association with any stem cell company.

18. Conclusions summarising the achievements and indication of scope for future work : *Kindly refer to the details presented in S. No. 16.*

19. Science and Technology benefits accrued :

a. List of publications with complete details : 02

(i) Anand et al. Mesenchymal stem cell derived extracellular vesicles in COVID-19 management: a review on publications, clinical trials and patent landscape. Tissue Engineering and Regenerative Medicine 19 (4), 659-673.

(ii) Anand et al. Human Wharton's jelly mesenchymal stem cells and their extracellular vesicles in management of bleomycin-induced lung injury in animal models. *Under revision.*

b. Manpower trained on the project :

i. Research Scientists or Research Fellows	**:**	01 Project Staff
ii. No. of PhD's produced	**:**	None
iii. Other Technical Personnel trained	**:**	N/A
c. Patents taken, if any	**:**	N/A
d. Products developed, if any	**:**	N/A

20. Abstract: (In 300 words for possible publication in ..... Bulletin)

The 2019 novel corona virus (2019-nCoV) that was originated in Wuhan City of Hubei Province of China has rapidly spread to the rest of the world. This virus, subsequently renamed as the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), is causing several pathological conditions including pneumonia, acute respiratory distress syndrome (ARDS), extreme rise in inflammatory cytokines, etc., and eventually leading to respiratory failure and death. Apart from controlling the viral load through anti-viral regimes, there is a serious need to regenerate the damaged lung tissue. In many regenerative studies, mesenchymal stem cells (MSCs) have shown to be great therapeutic alternatives; however,

treatment using MSCs has ethical, technical and regulatory concerns in India. Therefore, in the current study, we hypothesized that the MSC-derived extracellular vesicles, otherwise called exosomes, could aid in regeneration of lung regeneration in ARDS mice models. To this end, here we have explored the use of human Wharton's jelly mesenchymal stem cell (WJ-MSC) derived extracellular vesicular fraction for the repair of lung injury in the bleomycin treated C57BL/6J mice models. Overall, the results demonstrated that the bleomycin induced lung injury can be managed by the extracellular vesicular fraction derived from WJ-MSCs, and therefore, indicates a promising alternative for potential applications in COVID19 and beyond.

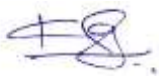
21. Procurement/Usage of Equipment:

a. Details of Equipment:

Sl. No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
1	Mini magnetic stirrer	Remi Mini Stirrer	Rs. 8593/-	23.02. 2021	100%	-
2	Bottle roller	Neuation IRoll	Rs. 20790/-	30.06. 2021	100%	-
3	Accessories for flow cytometer: Flow cell	Guava EasyCyte	USD 550	10.11. 2021	100%	-

b. Suggestions for disposal of equipment(s):

The items are currently working in good condition.



Dr. Naresh Kasoju
Scientist-C (Applied Biology)
29.11.2023

Routing: *Signed copy of "Project completion Report" by PI →* root@sctimst.ac.in, rpc@sctimst.ac.in