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ORIGINAL ARTICLE

Soluble factors of mesenchimal stem cells (FS-MSC) as a potential tool to reduce inflammation in donor's lungs after hypovolemic shock

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ABSTRACT

Objective: The shortage of viable lungs is still a major obstacle for transplantation. Trauma victims who represent potential lung donors commonly present hypovolemic shock leading to pulmonary inflammation and deterioration and rejection after transplantation. Seeking to improve lung graft, new approaches to donor treatment have been tested. This study focuses on treatment with mesenchymal stem cells (MSCs) or soluble factors produced by MSCs (FS-MSC) using a rat model for lung donors after hemorrhagic shock. **Methods:** Forty-eight rats were divided into four groups**:** Sham (n=12), animals without induction of hypovolemic shock; Shock (n=12), animals submitted to hypovolemic shock (mean arterial pressure 40 mmHg); MSC (n=12), animals submitted to hypovolemic shock and treated with MSCs, and FS (n=12), animals submitted to hypovolemic shock and treated with FS-MSC. The animals were subjected to a 50-minute hypovolemic shock (40 mmHg) procedure. The treated animals were monitored for 115 minutes. We performed histopathology of lung tissue and quantification of inflammatory markers (TNF-α, IL-1β, IL-6, IL-10, iCAM and vCAM) in lung tissue and peripheral blood leukocytes (PBLs). **Results:** Hemorrhagic shock resulted in higher PBLs and neutrophil infiltrate in the lungs. FS animals had lower neutrophil density comparing with Shock and MSC animals ($p<0.001$). No differences in the cytokine levels in lung tissue were observed between the groups. **Conclusions:** The lungs of rats submitted to hemorrhagic shock and treated with FS-MSC showed reduced inflammation indicated in a decrease in lung neutrophil infiltrate.

Keywords: Lung transplantation; Tissue donors; Hypovolemic shock; Mesenchymal cells; Inflammation.

INTRODUCTION

Lung transplantation improves quality of life and survival in patients with end-stage lung disease.[1] Despite all developments achieved in lung transplantation, the number of recipients on waiting list has not decreased, in fact, it now exceeds the number of organs available for donation.[2] Several strategies have been proposed to increase the number of effective donors, including public pro-donor campaigns, use of donors after circulatory arrest, use of live donors, *ex vivo* lung perfusion techniques, and cell therapies.[3-5]

Acute graft rejection is common after lung transplantation and occurs in half of lung transplant recipients after transplantation[6] through mechanisms such as inflammation and pulmonary edema.

Mesenchymal stem cells (MSCs) have been studied as cell therapy for a variety of degenerative, immunological, and inflammatory disorders.[7,8] Animal studies showed that MSCs induce repair of injured organs, decrease inflammation and have an immunomodulatory action with protective effects on the cells by releasing soluble factors (FS-MSC) such as anti-inflammatory cytokines and growth factors.[9] Our hypothesis is that treating lung donors with MSCs (can potentially – can reduce or potentially reduces) reduce graft inflammation. Considering its nature as final product and immediate action, FS-MSC

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could be the alternative to contribute to an increase in the number of viable lungs for transplantation.

This study focuses on the impact of *in vivo* treatment with MSCs and FS-MSC on lung inflammation in a rat model of lung donation after hemorrhagic shock.

METHODS

This study was approved by the ethics committee [Research protocol nº 188/14]. All animals were treated according to ethical principles of the Brazilian College of Animal Experimentation and the Guide for Care and Use of Laboratory Animals, provided by the Laboratory of Animal Research Institute and published by the National Academies Press, 8th Edition, 2011.

We included forty-eight adult male Sprague Dawley rats weighing 250-350 g in this study, which were randomly attributed to the following experimental groups: Sham (n=12) – animals subjected to vascular catheterization without shock induction or treatment; Shock (n=12) – animals subjected to vascular catheterization, induction of hemorrhagic shock and treated with replacement of 25% of the blood volume previously withdrawn; MSC (n=12) – animals subjected to vascular catheterization, induction of hemorrhagic shock through blood withdrawal, and treatment with replacement of 25% of the blood volume previously withdrawn, followed by infusion of MSCs via femoral vein; FS (n=12) – animals subjected to vascular catheterization, induction of hemorrhagic shock, and treatment with replacement of 25% of the blood volume withdrawn, followed by FS-MSC infusion via femoral vein.

Experimental protocol

The animals were anesthetized with 5% isoflurane in an acrylic chamber, weighed, and immobilized on a preparation board. We performed an orotracheal intubation using a tube adequate for small animals and initiated mechanical ventilation using a ventilator, also for small animals (Harvard Apparatus, Model 683), with a tidal volume of 10 ml/kg and a frequency of 80 cycles per minute. Anesthesia was maintained by the same isoflurane.

Each anesthetized animal was placed on the operating board. After shaving the right femoral region, we made an incision followed by vessels dissection and cannulation of right femoral artery and vein using tygon indwelling catheters (Critchley, Australia) filled with saline solution and heparin. For catheters insertion into the femoral artery and vein, we used a binocular stereoscopic microscope with a magnification of 4.5X (Olympus, model SZ6145). Right femoral artery catheter was connected to a monitor (Dixtal, DX2021, Brazil) for mean blood pressure (MBP) recordings. Right femoral vein catheter was used to induce and maintain hemorrhagic shock through blood withdrawal, in addition to blood replacement and administration of MSCs and FS-MSC.

Hemorrhagic shock was induced by withdrawing blood from right femoral vein with successive aliquots until MBP reached 40 mmHg within the first 10 minutes of the experiment, as described by Nepomuceno et al.[10] Rats remained in hemorrhagic shock for 50 minutes either by withdrawing or reinfusing the blood in case of MBP change (±5 mmHg).

We monitored the Sham group throughout the experimental period. MSC, FS and Shock groups were subjected to treatment with reinfusion of 25% of the blood withdrawn after hemorrhagic shock. The MSC group received infusion of MSCs at a concentration of 1×10^7 cells in 1 ml of culture medium over a 5-minute period. The FS group received infusion of soluble factors in medium at a volume of 1 ml over a period of 5 minutes. MBP was observed during a 115-minute period (Figure 1).

Lung extraction and preservation

In the end of the experiment, we carried out pulmonary extraction through a laparotomy extended upwards with resection of the sternum and radial opening of the diaphragm. Heart was exposed and a right ventriculotomy was performed adjacent to the pulmonary artery. Inferior vena cava was severed and left ventricle was sectioned longitudinally at the tip. Antegrade pulmonary perfusion was performed with preservation solution (Perfadex®, Vitrolife Göteborg, Sweden) using a cannula introduced into the pulmonary artery through the right ventriculotomy. Preservation solution was administered by gravity from a reservoir positioned 20 cm above the heart, with spontaneous drainage of the effluent through the left ventriculotomy.

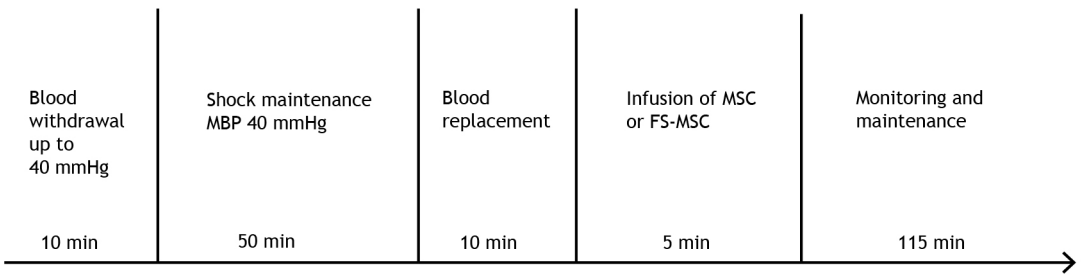

Figure 1. Timeline of the experimental procedure.

In the end of infusion, the trachea was ligated with suture below the cannula, and pulmonary extraction was performed.

Isolation and culture of mesenchymal stem cells

We performed the isolation and culture of MSCs from the adipose tissue according to protocols described in the literature by the Human Genome Group of the School of Medicine of the University of São Paulo (FMUSP).[11] The cells were stored at a concentration of $1x10^7$ cells in 1 ml of the culture medium to be applied as a single dose bolus. This concentration was determined in previous studies of the Human Genome Group of the School of Medicine of the University of São Paulo.[12] We cultured the MSCs in serum-free medium (T225 at 80% confluence) to obtain the FS-MSC . Subsequently, they were rinsed three times with 36 ml of PBS and cultured in 36 ml of DMEM/F12 culture medium without serum or antibiotics. Subsequently, we incubated the cells for eight hours followed by three rinses with 36 ml of PBS and subsequent addition of 36 ml of DMEM/F12 without serum or antibiotics. Another incubation was performed for 20 hours. Afterward, we performed the medium collection, cell count, and centrifugation at 300 × g for 5 minutes to remove debris. The supernatant was collected and the conditioned medium was stored for use. FS-MSC were stored in the medium at a volume of 1 ml to be used as a single dose bolus in each animal of the indicated group. Initial cell density, protein concentration and particle size distribution were not assessed because previous studies of the Human Genome Group of the School of Medicine of the University of São Paulo had already described these characteristics.[13]

Histology analysis

The upper right lobes of lungs were immersed in 10% buffered formalin for fixation. After 24 hours, paraffin blocks were prepared with the samples, sliced into 5 µm sections and stained with hematoxylin-eosin. Analyses were performed in duplicate by two trained researchers who were blinded for the study groups. The slides were analyzed using an Olympus CX22LED microscope with a lattice of dots and lines. For each slide, we assessed 15 fields containing greater lattice increase by counting the dots that touched the areas of pulmonary parenchyma, as well as the total neutrophil count. It was possible to estimate the parenchymal area of each animal examined through the predetermined lattice area, and the ratio of neutrophil counts to the parenchyma area provided the density of neutrophils per squared micrometer (μm^2) of pulmonary tissue. Values were then converted to squared centimeters (cm^2) to facilitate calculations.

Quantification of leukocytes in peripheral blood

Peripheral blood samples (20 µl) were collected from the tail of rats at the beginning of the protocol immediately before induction of hemorrhagic shock, as well as in the end, just before lung extraction. These samples were analyzed through total neutrophil count in Neubauer chamber.

Analysis of cytokines in lung tissue

We prepared the total protein from the animals' tissues by adding RIPA buffer (RIPA Lysis Buffer, 10x – MERK#20-188) at a ratio of 0.4 ml to each 0.1 g of tissue. A total of 10 µl of protease inhibitor cocktail (Protease Inhibitor Cocktail Set I - MERK#53131) was added to each 1 ml of the diluted RIPA buffer. The mixture was then homogenized on ice using a homogenizer (Fisherbrand™ Pellet Pestles™) and the homogenate transferred to a 2 ml Eppendorf tube. We incubated the material on ice for 10 minutes and centrifuged it at 10,000 × g at 4°C for 10 minutes. The supernatant was collected and transferred to a new Eppendorf tube. Total proteins in each sample were quantified and normalized to the value found in the assay (Kit BCA protein assay MERK #71285).

After that, we sent the material for multiplex analysis of the inflammatory cytokines TNF-α, IL-6, IL-1β, and IL-10 according to the xMap methodology (Luminex™ xMAP - Kit Cat. #RECYTMAG-65K-04 (IL-1β, IL-6, IL-10 and TNF- α), Millipore, St. Charles, MO, USA).

Statistical analysis

We performed descriptive analyses for quantitative data, means, and respective standard deviations (SD). Data without a normal distribution are presented using medians and interquartile ranges IQ (25-75%). The assumptions of the normal distribution in each group and the homogeneity of the variances between groups were assessed using the Shapiro-Wilk test and the Levene test, respectively.

For analysis of two factors (Group and Time), we applied two-way repeated measures ANOVA for a single factor (Time), while ANOVA was used to assess normally distributed variables. In case it is necessary to perform multiple comparisons of means, we used the Bonferroni test.

We applied Kruskal-Wallis test for non-normally distributed variablesand Dunn's test when multiple comparisons were required. All inferential analyses considered an α of 0.05. Descriptive and inferential statistical analyses were performed on SPSS software version 21 (SPSS 21.0 for Windows).

RESULTS

The Shock, FS and MSC groups were submitted to hemorrhagic shock from 0 to 10 minutes, showing a decrease in MBP, as predicted ($p<0.001$). During the period of hemorrhagic shock maintenance (10 to 60 minutes), these groups remained with lower MBP than the Sham group, and their means were close to that of the study design. After blood replacement (70 minutes), blood pressure of the Shock, FS and MSC groups increased. However, pressure values

remained lower comparing with those in the Sham group ($p<0.05$) and no significant differences were found ($p>0.10$) (Figure 2).

The groups subjected to hemorrhagic shock, with or without treatment, had more peripheral leukocytes in the end of the experiment in relation to the baseline data ($p<0.001$). The same increase was not found between baseline and final data in the Sham group ($p=0.805$), as observed in Figure 3a.

There was no statistically significant difference in the initial leukocyte count between the distinct experimental groups and Sham group, as expected ($p=0.33$). However, significant increase in circulating leukocytes after hemorrhagic shock was found in the

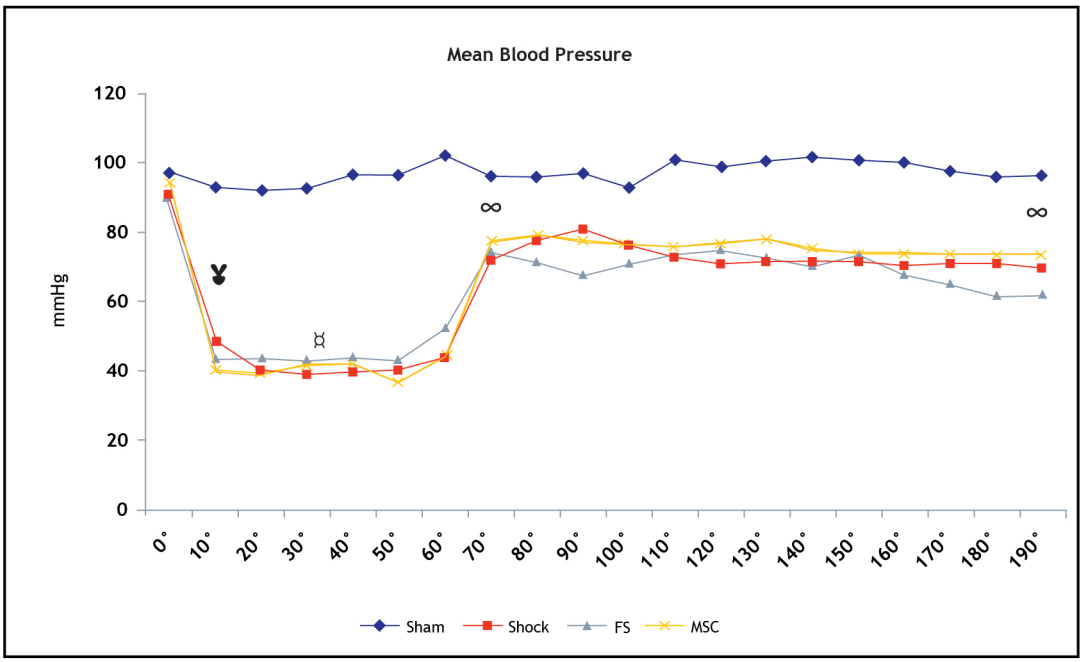

Figure 2. Mean in vivo arterial blood pressure. A statistically significant difference is found in the groups subjected to hemorrhagic shock (Shock, FS and MSC) compared to baseline (¥ $p<0.001$). During the maintenance of hemorrhagic shock, there was a significant difference between the Shock, FS and MSC groups compared to the Sham group (¤ $p<0.001$). At the end of 70 minutes and at 190 minutes, the Shock, FS and MSC groups showed a statistically significant difference compared to the Sham group (∞$p<0.05$).

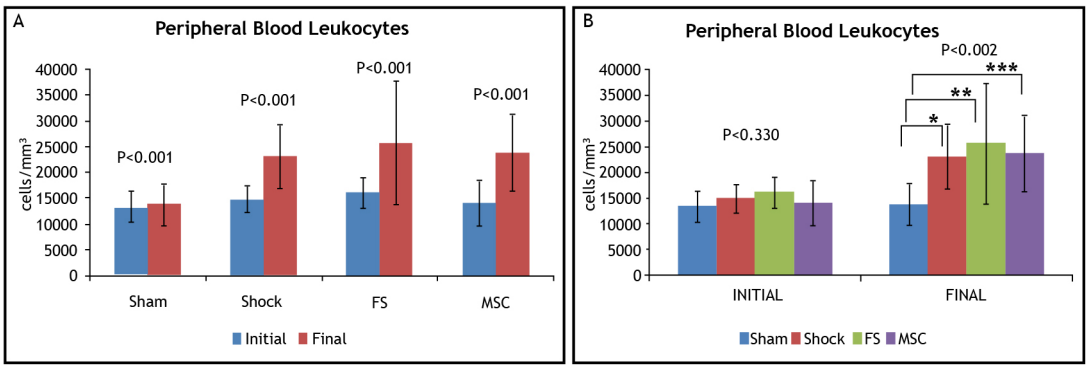

Figure 3. (a) Number of peripheral blood leukocytes collected at the bigining and at the end of the experimental protocol in each group showing a statistically significant difference between the Shock, FS and MSC groups ($p<0.001$). The Sham group showed no difference between dosages ($p=0.805$); **(b)** Figure comparing the quantification of peripheral blood leukocytes in the study groups for the initial moment and end of the experiments. There was no significant difference at baseline between groups ($p=0.33$) as opposed to the end of the experiments between the groups ($p=0.002$). A difference was found in the multiple comparisons between Sham vs Shock (*$p=0.02$), Sham vs FS (**$p=0.006$), and Sham vs MSC (***$p=0.01$).

Shock, FS and MSC groups comparing with the Sham group (p=0.002) (Figure 3b).

Lung histology showed a statistically significant difference in the neutrophil density between groups (p<0.001). MSC group had the highest neutrophil density (180.77 neutrophils/cm^2) of all groups, and the FS group had the lowest cell density (40.38 neutrophils/cm^2) (Figure 4).

By comparing the Shock and FS groups, we found a significant difference in their neutrophil densities (172.52 neutrophils/cm^2 and 40.38 neutrophils/cm^2, respectively, p<0.001); however, no significant differences appeared between the Shock and MSC groups.

Analyses of cytokines in lung tissue did not show significant difference in group comparisons in TNF-α (p=0.21), IL-6 (p=0.21), IL-1β (p=0.58).

DISCUSSION

In the scope of lung transplantation, the shortage of lung donors is one of the primary obstacles leading to a higher number of patients on waiting lists.

Because of their anti-inflammatory and antifibrotic properties, cellular therapies now represent a new therapeutic approach in this context[14,15] Preclinical studies demonstrate improvements following the administration of MSCs in various pulmonary diseases, including chronic obstructive pulmonary disease, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis.[16] In this study, animals were subjected to lung injury by induction of hemorrhagic shock and treated with venous infusion of human MSCs. Lung protective effects may be related to angiogenesis and anti-inflammatory effects.[17-22]

Our study used a model of controlled hemorrhagic shock previously described in the literature.[10,23,24] Our results demonstrated the effectiveness of the reduction and maintenance of pressure levels during shock period in the Shock, MSC, and FS groups. Circulating leukocyte levels quantified prior to shock were lower than those quantified after the onset of hemorrhagic shock. In addition, this shock model showed an increase in neutrophilic infiltrate in the lung in relation to the Sham group. As expected, our results demonstrated that shock acts as a trigger of the inflammation process of. In contrast, levels of TNF-α, IL-6, IL-1β and IL-10 in lung tissue were not significantly different from those found in the Sham group.

After blood replacement and intervention in the treatment groups, we monitored the rats for 115 minutes and extracted the heart-lung block in the end of experiment. This procedure intended to mimic a clinical multiple organ-donor retrieval situation after the notification of brain death. De Oliveira et al.[25] reported a half-life of inflammatory cytokines ranging from 6 minutes to 4 hours. In a study using a hemorrhagic shock model in rats, Pati et al.[17] also collected samples to assess inflammatory cytokines within two hours. In previous studies, the authors identified that major changes in inflammatory cytokines occurred between one and four hours following shock.[26]

The concentration of MSCs used in our study was 1x10^7 cells diluted in standard medium at a single dose. The concentration of cells used in cell therapy with MSCs is still a controversial issue and not unanimous in publications on this matter. Other studies showed a positive effect for concentrations ranging from 2.5x10^5 to 9x10^7, although better results were not necessarily related to a higher concentration of cells.[27-30] Watanabe et al.[19] used a concentration of 5x10^6 cells to treat ischemia and reperfusion injury in a rat model of pulmonary transplantation. Other authors also used the same concentration of cells in respiratory therapies.[31,32] Therefore, the ideal concentration of MSCs is yet to be established.

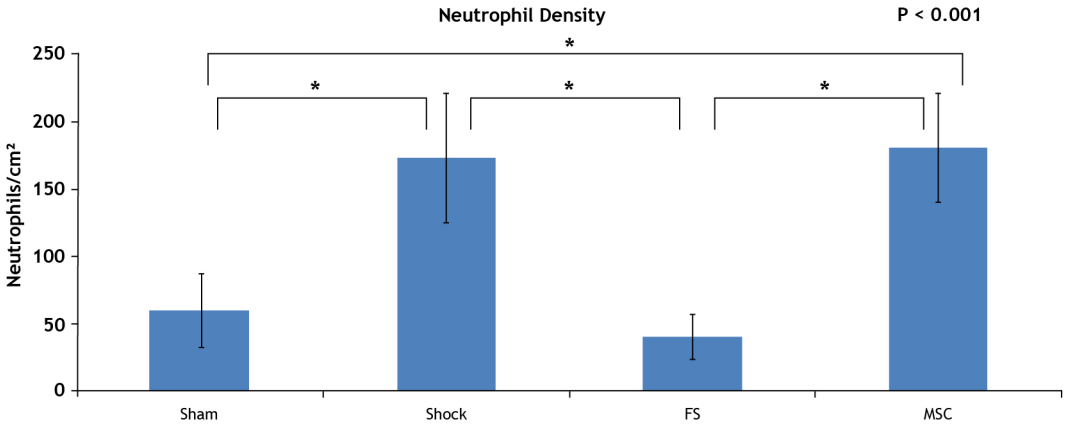

Figure 4. Comparative chart for the neutrophilic infiltrate of the groups, measured as neutrophil density. There was a significant difference between the groups (p<0.001). In the multiple comparisons, there was a significant difference in the comparisons Sham vs Shock (*p<0.001), Sham vs MSC (*p<0.001), Shock vs FS (*p<0.001), and FS vs MSC (*p<0.001).

The intravenous route of administration is well-established in literature and several studies demonstrated that lung acts as a "filter" organ responsible for the retention of most infused cells due to the small diameter of the lung network of capillaries in relation to the size of the stem cells. Such endobronchial route is also effective and may be a good option in this type of study.[33-35] A recent study compared the intravenous route to intrabronchial route and showed an advantage associated with significant retention of MSCs by the intravenous route in damaged *ex vivo* perfused lungs.[36] Other studies have also shown that infusion of MSCs is safe and well tolerated and produced no significant adverse events.[37]

Although hemorrhagic shock promotes increased neutrophilic infiltrate in the lungs, MSC treatment was not effective at inhibiting such effect. Nevertheless, we observed that the FS group had no neutrophilic lung infiltrate, which we interpreted as a result of the short observation period, which was insufficient for the mesenchymal cells to settle in the lungs and release the soluble factors.

The mechanisms of action of MSCs and their immunomodulatory actions are not fully understood. MSCs appear to exert their effects through multiple mechanisms – some are dependent on cellular interactions, while others depend on paracrine interactions, which result from either soluble secreted products or from microvesicles or cell-derived exosomes. Pati et al.[17] used a model of hemorrhagic shock similar to that in our study and treated the animals with Ringer's lactate combined with MSCs. However, the posttreatment period was much longer than ours, and the animals were evaluated 96 hours after treatment. The authors observed that MSC treatment reduced CD8+ expression, which is a specific marker for neutrophils. Chimenti et al.[38] also demonstrated the potential of pretreatment with MSCs, followed by high-volume ventilation. Pretreatment with MSCs reduced the fluid content within the lungs and improved the lung histology score. Levels of neutrophils, inflammatory protein of macrophages-2 and IL-1β were also reduced considerably. *Ex vivo* treatment of pigs with MSCs also showed no change in the level of inflammatory cytokines, such as TNF-α and IL-10.[36] Strategies to further enhancing the effectiveness of MSCs, such as overexpression of anti-inflammatory or pro-repair molecules, have also been investigated.[39]

The titration of inflammatory cytokines in the tissue used in our study were not significantly different. Pati et al.[17] obtained similar results and found no significant differences with this type of analysis.

Factors that could justify the presence of significant inflammatory infiltrate in peripheral blood and reduction in neutrophilic infiltrate in lung tissue, as found in our study, can be found in a study by Stone et al.,[40] who found that transendothelial migration of neutrophils into pulmonary endothelial cells can be effectively blocked by FS-MSC, protecting the integrity of endothelial barrier against edema.

Our study has limitations. Factors such as the absence of literature focused on the ideal amount of cells required, as well as the optimum observation time after infusion of MSCs and FS-MSC may have influenced the results. Limited time of animal monitoring and prolonged periods of maintenance of hemorrhagic shock resulted in high mortality.

Based on the neutrophil density value found in the lung, we conclude that infusion of FS-MSC can reduce levels of local inflammation in lungs of rats with hemorrhagic shock. We believe that it could be a better option than MSCs due to the immediate action provided, requiring a shorter treatment time and not allowing the inflammation process growing on.

It is essential to conduct further studies to achieve a better understanding on the action, kinetics, and dynamics of MSCs, as well as on how to use it. Time of exposure to these cells can be a determining factor in this regard. Cell therapy with MSCs and FS-MSC is a promising field in lung transplantation and may potentially contribute to improve outcomes by improving donor status and reducing post-transplant rejection.

AUTHOR CONTRIBUTIONS

VLD is the main author and participated in all phases of the project. KAOB participated in all phases of the project, contributed substantially to the manuscript, and read and approved the final version of the manuscript. NAN participated in all phases of the project and contributed substantially to the manuscript. LMR participated in all phases of the project. JDRP contributed to some phases of the project. ATC was responsible for all statistical analyses and made the Figures for the project. LCCJ was responsible for the culture and supply of the mesenchymal stem cells and their soluble factors. EG was responsible for the culture and supply of mesenchymal stem cells and their soluble factors and wrote the methodology related to the mesenchymal stem cells. MZ was responsible for the culture and supply of mesenchymal stem cells and their soluble factors and wrote the methodology related to mesenchymal stem cells. PMPF was the main advisor of the study and read and approved the final version of the manuscript.

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