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Inhalation of Mesenchymal Stem Cell- Derived Small Extracellular Vesicles as a Possible Approach to Ameliorating Acute Lung Injury Caused by Cytokine Storm

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ABSTRACT

Lung is one of the vital organs that get severely affected by cytokine storm and sepsis, leading to the development of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). Mesenchymal stem cell- derived small extracellular vesicles (MSC- derived sEVs) are one of the therapeutic approaches for ARDS/ ALI caused by sepsis. Apart from sEVs' ability to carry medication, MSC- derived sEVs also possess anti- inflammatory, tissue repair, and regeneration properties. Targeted drug delivery has been a crucial area of concern in the medical field, specifically for treating lung diseases. To treat lung diseases locally, inhalation of drug products has been developed. Drug delivery by inhalation has emerged as an effective method for local administration of therapeutic agents, with numerous benefits including better efficacy at lower doses and decreased toxicity. Additionally, inhalation administration is a viable option for the systemic distribution of medications due to the lungs' considerable absorption surface and their ability to bypass initial metabolism. Therefore, our hypothesis proposes the inhalation of MSC- derived sEVs as a potential strategy for alleviating acute lung injury induced by sepsis-related cytokine storm. Following the isolation and characterization of these MSC- derived sEVs, they will be administered to an animal model of sepsis via a nebulizer, either in their pure form or loaded with drugs. Several approaches will be employed to evaluate lung functionality, including histological analysis and the measurement of inflammatory and regulatory cytokine levels to assess changes in both the humoral and cellular immune systems.

INTRODUCTION

Cytokine storm is a complex pathophysiological phenomenon characterized by uncontrolled release of cytokines; it can lead to life-threatening systemic hyperinflammation (1). Cytokine storm is commonly caused by various triggers such as viral and bacterial pathogens. Other factors such as immunotherapy, cancers, autoimmune conditions, and monogenic disorders may also cause cytokine storm (1). The recent pandemic, COVID-19, was a global concern that affected the immune system, induced cytokine storm, and resulted in a high morbidity and mortality rate. Growing evidence suggests that COVID-19 infection is

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associated with sepsis and lung damage (2, 3). One in five deaths worldwide is caused by sepsis (4). Sepsis- induced acute lung injury is one of the first symptoms of the disease, which is categorized as acute lung injuries (ALI)[1] or acute respiratory distress syndrome (ARDS) (5-7). Severe acute inflammation plays a critical role in septic ALI/ARDS.

Innate immunity is triggered by pathogen-associated molecular patterns (PAMPs) (8), damage-associated molecular patterns (DAMPs) (9), and chromatin-associated molecular patterns (CAMPs) (10). This activation triggers the infiltration of neutrophils and monocytes, and overproduction of inflammatory cytokines such as interleukin (IL)-1β, IL-6, and Tumor necrosis factor alpha (TNF-α). The disruption of the alveolar-capillary barrier and heightened permeability worsen the severity of sepsis-associated ALI / ARDS.

The current treatment regimen for septic patients remains supportive care including early administration of antibiotics directed at the virulent pathogen, with no role for immunomodulators, while sepsis has catastrophic effects due to the overstimulated- immune response, followed by the inactivity of immune cells, resulting in secondary immunosuppression and immune paralysis (11). In an effort to alleviate sepsis-induced injury, recent research has focused on reducing the overstimulated inflammatory response through the administration of immunomodulating agents, including cells and their products. Orally Inhaled and Nasal Drug Products (OINDPs) are important for treating lung diseases locally and for the systemic distribution of medications because the lungs offer a significant absorption region and can bypass first- pass metabolism. Moreover, OINDPs are still beneficial for treating systemic illnesses as well as lung illnesses since no needles are required, and they have higher compliance rates.

Since mesenchymal stem cells (MSCs) have the ability to regulate inflammation, induce angiogenesis, repair tissues, and enhance bacterial clearance they are the most prominent candidates for immune homeostasis and tissue healing (12, 13), MSC- derived extracellular vesicles represent superior functionality and causing less side effects compared to MSCs themselves (14-16). Several reports have shown that MSC-derived sEVs, alone or enriched with anti-inflammatory agents, alleviate inflammation, and administering extracellular vesicles through inhalation would be the most effective way to relieve septic ALI / ARDS (17-21).

HYPOTHESIS

With the limitation of intraperitoneal (I.P) and intravenous (I.V) injections, we hypothesized that the inhalation of sEVs, alone or as an anti-inflammatory cargo carrier would have better curative and tissue regenerative effects in sepsis-induced acute lung injury (figure 1). The beneficial administration of extracellular vesicles through inhalation will be elucidated to prove its most protective remedial way for septic ALI/ARDS treatment. In 2015, Monsel *et al.* reported that MSC-derived sEVs could enhance alveolar type II cells' survival by increasing intracellular ATP levels (22). MSC- derived sEVs, which have the ability to carry biological molecules such as RNAs, proteins, and hydrophobic and hydrophilic drugs, have promising therapeutic potentials (23). In addition, long-term stability, specific tissue targets, low immunogenicity, low risk of developing teratoma, and immunomodulatory characteristics make sEVs superior to mesenchymal stem cells (14, 21). Moreover, the amount of medicine delivered to the lungs via inhalation is more than via intravenous or intraperitoneal methods, despite the lower administered doses. This prevents systemic medication distribution and minimizes medicine-related side effects especially if they are utilized as nanocarriers (24).

[1] **Abbreviations** Acute Lung Injury (ALI), Acute Respiratory Distress Syndrome (ARDS), Mesenchymal stem cell (MSC), Small extracellular vesicles (sEVs), Interleukin (IL), Tumor necrosis factor alpha (TNF-α), Orally Inhaled and Nasal Drug Products (OINDPs), Intraperitoneal (I.P) , Intravenous (I.V) , Dynamic light scattering (DLS), Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Lipopolysaccharide (LPS), Cecal ligation and puncture (CLP), Immunohistochemistry (IHC), Enzyme-linked immunoassay (ELISA), Bronchoalveolar lavage (BAL), Prostaglandin E2 (PGE2), Indoleamine 2,3-dioxygenase (IDO), Nitric oxide (NO), T helper (Th), Keratinocyte growth factor (KGF), Vascular endothelial growth factor (VEGF), Platelet-Derived Growth Factor (PDGF), Hepatocyte growth factor (HGF).

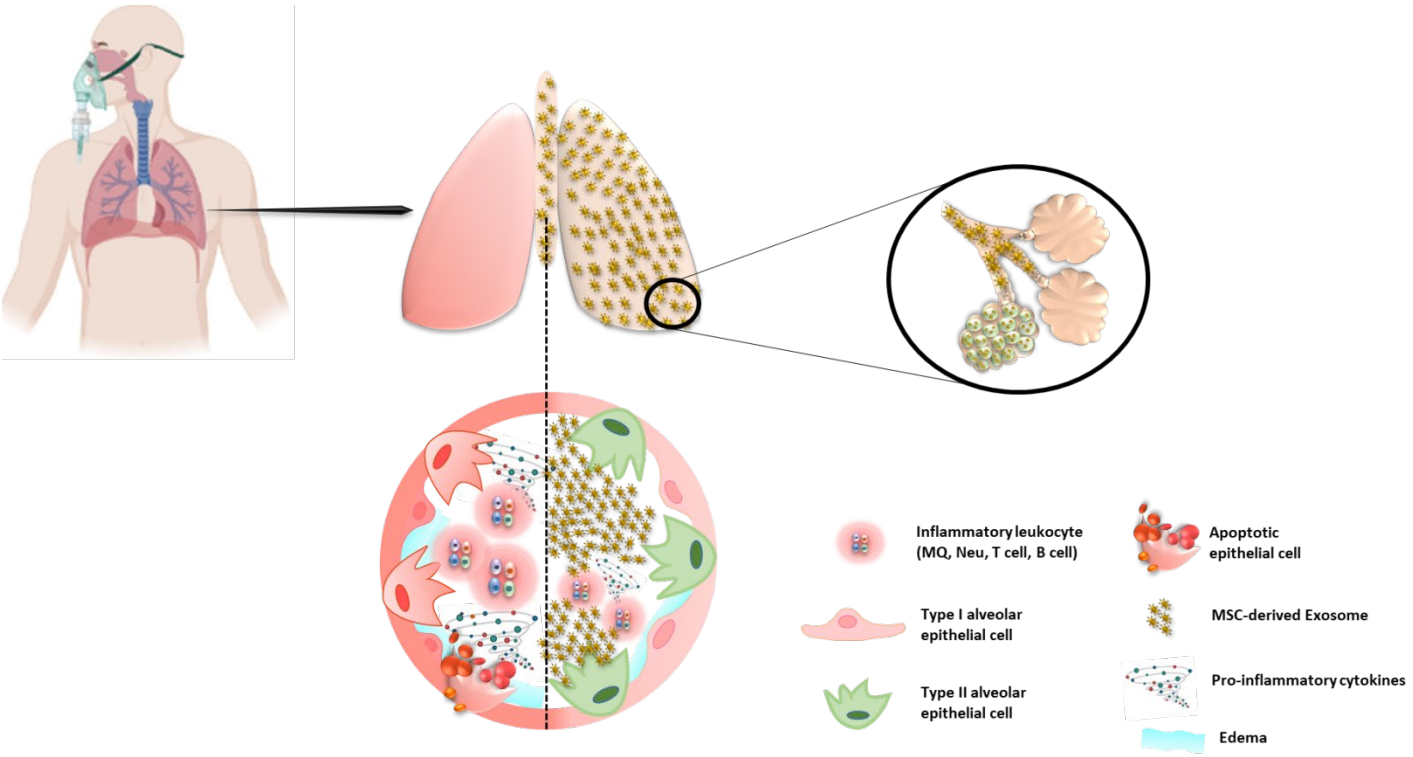

FIGURE 1. **Cytokine storm- induced lung injury and the therapeutic potential of MSC- derived sEVs.** Various stimuli, such as viral infection (Covid-19) or bacterial infection (sepsis), lead to the uncontrolled production of inflammatory cytokines, such as IL1, IL6, and TNF-a, followed by immune inflammatory cells, such as macrophages, monocytes, and Neutrophils that are activated and intensify the defective cycle of cytokine overproduction. The immigration of neutrophils, monocytes, and T cells is induced by cytokines and chemokines. Cytokine storm is the main cause of lung injury (ALI / ARD in sepsis and covid-19 and causes apoptosis of alveolar cells, lung fibrosis, edema, and lung failure. Immunomodulatory properties of MSC-derived sEVs reduce the severity of ALI / ARDS lung injuries. Because of the PGE2 generated by these cells, macrophages undergo phenotypic changes from M1 to M2. MSC-derived sEVs also decrease IL-6 and increase IL-10. By producing IDO, NO, and IL 10, Th2 and Treg lymphocytes are raised, while Th1 and Th17 lymphocytes are attenuated. KGF, VEGF, PDGF, and HGF are also produced by MSC- derived sEVs, which have the potential to restore tissue.

The small size of extracellular vesicles facilitates their entry into the alveoli at the end of lung lobes through inhalation, specifically as nanocarriers (25). In addition, the extracellular vesicles also remain in place for a long time due to their long-term stability and gradual absorption by the tissue. In this way, extracellular vesicles can change the inflammatory state of the area and improves and regenerates lung tissue due to their anti-inflammatory and regenerative properties (26, 27). Inhalation is also easier to administer, easier to access, and less painful for patients.

TESTING THE HYPOTHESIS

The following steps can be followed to study the effects of inhalation of extracellular vesicles derived from MSCs: First, MSCs will be isolated from known sources including adipose tissue, bone marrow, or Wharton jelly. The MSCs will be characterized by their spindle- shaped morphology, differentiation potential into osteoclasts and adipocytes, and immunophenotyping of CD markers. sEVs will be isolated from the MSC culture supernatants by either an isolation kit or ultracentrifugation. As part of sEV characterization, dynamic light scattering (DLS) will be used to evaluate the size distribution. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) will be used to observe the sEV morphology and the flow cytometry will be used to verify the membrane-specific CD markers.

The *in-vivo* part of the study will involve preparing animal models and administering extracellular vesicles. There are two standard protocols to establish a sepsis-induced ALI / ARDS model: lipopolysaccharide (LPS) administration to mimic bacterial infection, and cecal ligation and puncture (CLP) procedure to induce peritonitis and mimic sepsis. After sepsis induction, mice will be divided into 5 groups based on the treatment and route of administration: inhalation of extracellular vesicles, inhalation of drug-loaded extracellular vesicles, inhalation of a placebo, intravenous administration of extracellular vesicles (alone or loaded with a drug) and intraperitoneal administration of extracellular vesicles (alone or loaded with a drug).

The effects of extracellular vesicle inhalation will be measured in different groups of mice. Similar to clinical investigation, arterial oxygenation, and static lung compliance will be measured to assess lung function. At the endpoint time, the animal will be sacrificed and serum (for assessing biochemical factor changes), spleen, and lung samples will be collected for assessing tissue injury via Immunohistochemistry (IHC) and H&E staining. For immunological investigation, the gene expression profile of T lymphocytes, macrophages, and neutrophils will be assessed in the lung and spleen (by microarray), and protein production by enzyme- linked immunoassay (ELISA) in serum and bronchoalveolar lavage. Leukocyte count and function will be done for the lung and spleen. Moreover, immunofluorescent staining will exert whether extracellular vesicles reach alveoli and epithelial cells (figure 2).

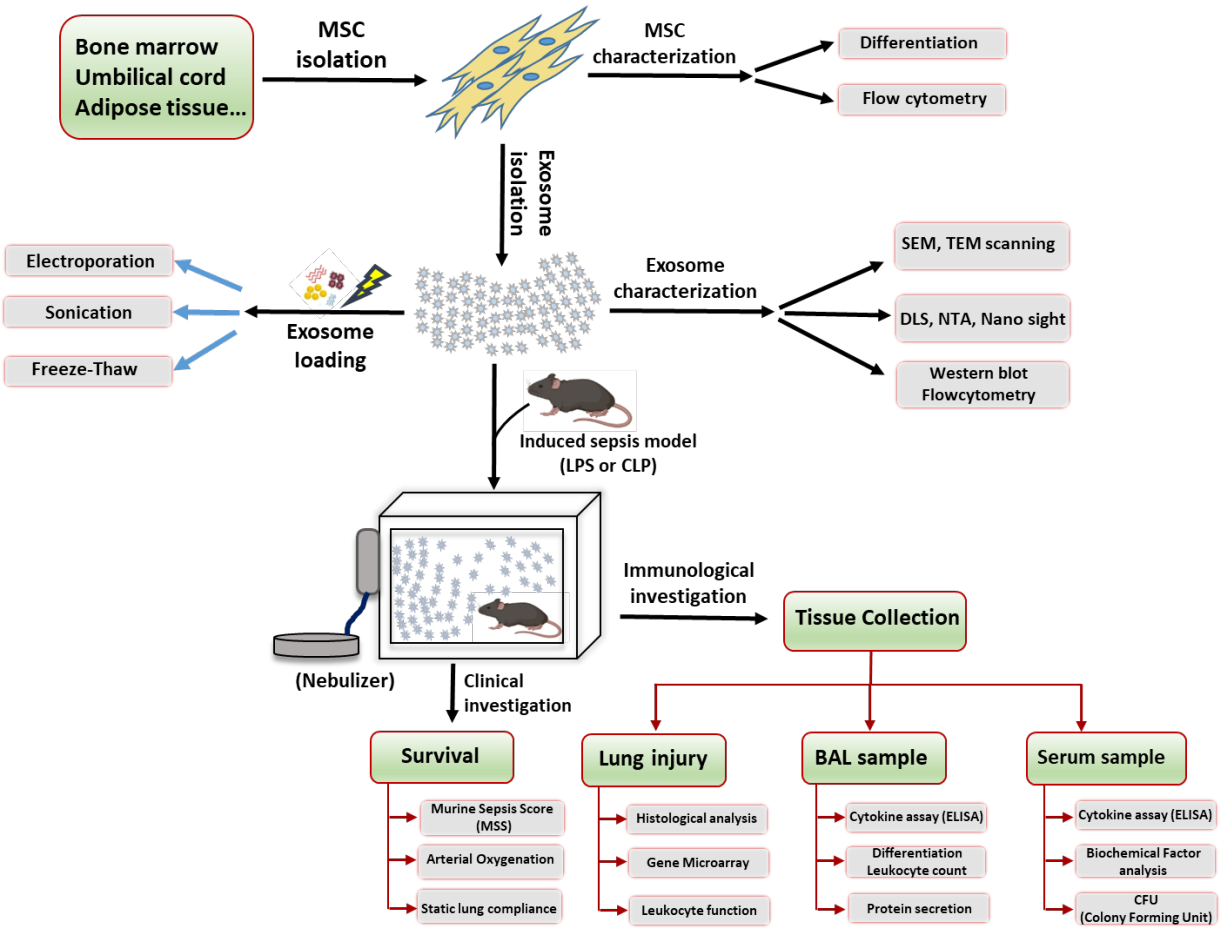

FIGURE 2. Hypothesis examination. MSC, mesenchymal stem cell; SEM, scanning electron microscopy; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; DLS, dynamic light scattering; LPS, Lipopolysaccharide; CLP, Cecal Ligation and Puncture; BAL, bronchoalveolar lavage; ELISA, enzyme- linked immunosorbent assay.

DISCUSSION

A cytokine storm is a severe immune response that occurs according to different factors including infections such as COVID-19 or sepsis after an infection enters the bloodstream. As a result of sepsis, lung damage is one of the most common causes of death in septic patients. In recent years, researchers have investigated the use of immunomodulators with tissue repair properties, such as sEVs derived from mesenchymal stem cells, in conjunction with antibiotic therapy to improve tissue healing and immune homeostasis. Additionally, choosing the appropriate way to administer drugs is crucial to maximizing treatment outcomes. As we mentioned earlier, our hypothesis suggests that the inhalation of sEVs derived from mesenchymal cells has promising potential for sepsis treatment. To achieve the optimum result, dose titration, the optimal exposure time, and the number of times to inhale the sEVs will be checked. The inhalation test will be evaluated and compared to other administrative procedures.

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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ETHICAL APPROVAL

Not required.

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