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Extracellular Vesicles Derived from MSCs Carrying Dexamethasone Alleviate Inflammatory Damage in Septic Mice

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Abbreviations

ALT: alanine transaminase; **AST**: aspartate aminotransferase; **BCA**: Bicinchoninic acid assay; **Bil** : Billirubine; **Caspase**: cysteine-dependent, aspartate-specific peptidase; **CLP:** Cecal ligation and puncture; **Cr**: Creatinine; **DAMP**: Damaged-associated molecular patterns; **Dex**: Dexamethasone; **DLS**: Dynamic light scatering; **DMEM-F12**: Dulbecco's modified eagle medium/nutrient mixture F-12; **Exo**: Exosome; **FBS**: Fetal bovine serum; **FESEM**: Field emission scanning electron microscope; **HMGB-1**: high mobility group box-1; **IFN-γ**: Interferon-gamma; **IL**: Interleukin; **L-Dex-EV:** MSC-EVs loaded with Dex; **LPS**: Lipopolysaccharide; **MSC:** Mesenchymal stem cell; **MSC-EVs**: Mesenchymal stem cell-derived extracellular vesicles; **MSS:** Murine sepsis score; **NF-κB:** Nuclear factor kappa B; **NK**: Natural killer cell; **NLR**: Neutrophil to lymphocyte ratio; **NLRP-3**: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing-3; **NO**: Nitrite Oxide; **NOD**: NOD-like receptor; **PAMP**: Pathogen-associated molecular patterns; **PBS**: Phosphate buffered saline; **PI3-K:** Phosphoinositide 3-kinases; **Pre-Dex-EV:** EVs isolated from Dex-preconditioned MSCs; **PRRs**: Pattern recognition receptors; **ROS**: Reactive oxygen species; **SDS-PAGE**: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis; **TEM**: Transmission electron microscopy; **TGF-β**: Transforming growth factor-beta; **Th**: T helper; **TLR**: Toll-like receptor; **TNF-α**: Tumor necrosis factor-alpha; **Treg:** Regulatory T cell.

Abstract

Sepsis is a life-threatening condition resulting from an uncontrol immune response to infection. Current treatment options remain limited and often ineffective, highlighting the need for supportive anti-inflammatory and immunomodulatory strategies . Mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) can be a good supportive candidate because of their immunomodulatory and drug-delivery properties. Dexamethasone (Dex), a commonly used anti-inflammatory agent, was assessed in this study alongside MSC-EVs to evaluate their combined anti-inflammatory, immunomodulatory, and tissue-repairing effects in septic mice. Hence, we designed two combinations of EVs and Dex: a simple mixture of EVs and Dex (Dex+EV), and EVs loaded with Dex (L-Dex-EVs). Then the cecal ligation and puncture CLP-induced sepsis C57BL/6 mice were grouped based on treatment: 1) EVs, 2) Dex, 3) Dex+EV, and 4) L-Dex-EVs, as well as 5) CLP and 6) Sham groups as control. Tissue samples, bronchoalveolar lavage (Molinaro et al.) fluid, and serum were collected following the treatments to investigate inflammation and tissue damage-related factors. The findings indicated that both combined treatments resulted in decreased levels of IL-6 and TNF-α, while increasing IL-10 and TGF-β levels in BAL fluid and serum. Furthermore, the expression of HMGB1, NLRP3, caspase-1, and caspase-3 was downregulated in lung tissue, whereas iNOS expression was decreased and Arg-1 expression was upregulated in the spleen. Histological analysis (H&E staining) and serum biomarkers' assessments revealed diminished inflammation and improved tissue repair in the groups receiving combined treatments. These findings suggest that the combination of MSC-EVs and Dex,exerting synergistic effects that attenuate inflammatory responses and promote tissue regeneration in septic mice.

Keywords: Mesenchymal stem cell; Dexamethasone; Extracellular Vesicles; Inflammation; Sepsis.

Introduction

Cytokine storm (CS) was first reported after the significant increase in the host inflammatory cytokines following the allogeneic hematopoietic stem cells (HSCs) transplantation (Fajgenbaum & June, 2020). A similar phenomenon was also observed after treatment with T lymphocytes expressing a chimeric antigen receptor (CAR)(Coley, 1893; R. A. Morgan et al., 2010). During the COVID-19 pandemic (2019-2020), severe inflammation with a significant increase in inflammatory cytokines was reported as one of the serious complications of the virus (Motallebnezhad et al., 2023; Soy et al., 2020). Moreover, various conditions can trigger immune responses thatlead to CS. Among these, sepsis (viral or bacterial) (Cecconi, Evans, Levy, & Rhodes, 2018), immunotherapies such as blinatumomab, and conditions such as malignancies and autoimmune disorders—both congenital and acquired, including Epstein-Barr virus (EBV)-associated hemophagocytic lymphohistiocytosis (HLH), are notable contributors to CS (Fajgenbaum & June, 2020). CS is a general term used to describe a clinical syndrome marked by structural problems, elevated circulating cytokines, acute systemic inflammatory responses, and multi-organ dysfunction, most commonly involving the kidneys, liver, and lungs(H. Wang & Ma, 2008). Failure to receive proper treatment may lead to organ failure and eventually death. Cytokines such as IFN-γ, IL-6, IL-10, and the soluble IL-2 receptor alpha chain (signaling the activation of T lymphocytes) are the main biomarkers elevated in CS (Grupp et al., 2013). The function of these cytokines is to facilitate the response of the inflammatory phase, which appears in the host in the form of fever, an increase in leukocytes, changes in glucose metabolism, and activation of the complement system and coagulation cascade (Moshage, 1997).

So far, various methods have been used or suggested for the treatment or prevention of CS, including the administration of antibodies that targeting inflammatory cytokines or their receptors (e.g., IL-6, IL-18, TNF-α), antibodies that inhibit transcription factors (e.g., NF-κB, mTOR, JAK-STAT), IVIG therapy (Nnaji, Iwu, Ndwandwe, Jordan, & Wiysonge, 2020). Nevertheless, corticosteroids remain the most commonly used agents to treat inflammation. Among them is dexamethasone (Dex), which inhibits inflammatory responses (Cohen, 1973). Studies have shown that using Dex shortens patients' hospitalization periods and reduces the mortality of critically ill patients (Lammers et al., 2020). Due to its

long-term effectiveness (36-48 hours), high anti-inflammatory effects, and minimal mineralocorticoid effects, Dex has been considered an appropriate treatment (Moustafa et al., 2020). However, long-term use of Dex causes some side effects, such as increased incidence of infectious diseases, hyperglycemia , increased appetite and weight, osteoporosis, sleep disorders, etc. (Polderman et al., 2018). Therefore, it is warranted to find an approach that can reduce the side effects while enhancing the efficacy of the treatment.

Mesenchymal stem cells (MSCs) have demonstrated substantial clinical potential for the treatment of different disorders. (Malekpour et al., 2022). MSCs are widely used in regenerative medicine because of their immunomodulatory properties, production of different growth factors, and the ability to differentiate into different types of cells (Abbasi-Kenarsari et al., 2020; Brooke et al., 2007; M. Heidari et al., 2018; N. Heidari et al., 2021). Also, MSCs produce a high number of extracellular vesicles (EVs), which play critical roles in intercellular communication (Wiklander, Brennan, Lötvall, Breakefield, & El Andaloussi, 2019). The inner space of EVs is rich in bioactive biomolecules, such as enzymes, cytokines, growth factors, and microRNAs (Hazrati, Soudi, Malekpour, et al., 2022; Taghavi-Farahabadi et al., 2020). EVs alter the functions of target cells by transferring various contents related to the originating cell upon reaching and fusing with them (N. Heidari et al., 2022; Mahmoudi, Taghavi Farahabadi, & Hashemi, 2019). Due to some limitations associated with direct MSC therapy—such as potential tumorigenicity, poor homing efficiency, and risks of inappropriate differentiation—MSC-derived EVs (MSC-EVs) are suggested as a safer and more practical alternative MSC-EVs(Hazrati, Malekpour, Soudi, & Hashemi, 2022; Khosrojerdi, Soudi, Hosseini, Eshghi, et al., 2021; Timaner, Tsai, & Shaked, 2020). Moreover, various studies show that EVs have a higher potential for use in drug delivery, and their use is preferable to liposomes (Hazrati et al., 2023; Sadeghi, Tehrani, Tahmasebi, Shafiee, & Hashemi, 2023). MSC-EVs are proficiently laden with drugs through various methods, including freeze-thaw, sonication, and electroporation (Kimiz-Gebologlu & Oncel, 2022; Mirsanei et al., 2024). Studies have shown that EV-based drug loading can enhance drug delivery efficacy and bioavailability (Niknam et al., 2023; van der Koog, Gandek, & Nagelkerke, 2022).

In this study, the synergistic impact of MSC-EVs and Dex was assessed using an in vivo sepsis model. The effectiveness of these combined treatments was then compared to single-treatment approaches in terms of inflammatory markers, tissue damage, and the histopathological changes of different tissues.

2. Material & Methods

2.1. Study design

30 male C57BL/6 mice, aged 6-8 weeks and weighing 18-22 grams, were procured from Ibn Sina Research Institute, Shahid Beheshti University. Animal handling followed the guidelines for laboratory animal care and use approved by the National Ethics Committee in Research, Shahid Beheshti University of Medical Sciences, with ethics code IR.SBMU.MSP.REC.1400.831. The mice were allowed to acclimatize for two weeks before the start of the study. The mice were kept in optimal conditions with a light rhythm of 12 hours day and 12 hours night with full access to water and food and in proper ventilation conditions. Five mice served as untreated controls (Group 1), receiving neither sepsis induction nor therapeutic intervention. To model sepsis, the cecal ligation and puncture (CLP) technique was performed on an additional 25 mice, following a previously described method (Khosrojerdi, Soudi, Hosseini, Khaligh, & Hashemi, 2021). One hour after induction, these septic mice were randomly assigned into five experimental groups (Groups 2–6), with five mice in each group. They received the following respective treatments (Figure 1): Group 2: Injected intravenously with PBS, Group 3: Injected intravenously with 300 μg extracellular vesicles (EVs), Group 4: Injected intravenously with 75 μg dexamethasone (Dex), Group 5: Injected intravenously with a combination of 300 μg EVs and 75 μg Dex (Dex+EV), Group 6: Injected intravenously with Dex-loaded EVs (containing an equivalent Dex concentration).

2.2. MSCs isolation and characterization

The umbilical cord (purchased from Royan Institute, Iran) was divided into small pieces (3-4 mm) and transferred to a T25 culture flask containing DMEM-F12 medium supplemented with 10% FBS and 1%

penicillin-streptomycin. The flask was then placed in a humidified incubator at 37°C with 5% CO_2 atmosphere. The cells migrated out of the umbilical cord tissue within 7–14 days of culture and adhered to the bottom of the flask. After a good number of adherent cells appeared, the tissue pieces were removed and replaced by new culture medium. When the cells reached 80% confluence, they were washed twice with cold PBS and then trypsinized to detach them from the bottom of the flask. The trypsin was then neutralized by the addition of a DMEM-F12 culture medium containing FBS. After centrifugation (5 min, 1000 g, 4°C), the cell pellet was resuspended and transferred to new culture flasks (Gibco). Following this, the morphology of the cells was examined, and their differentiation potential and surface marker expression were characterized. To evaluate the MSC's phenotype, the cells were tested by flow cytometry using cell surface negative markers (CD14, CD34, and CD45) as well as positive markers (CD105, CD90, and CD73). For MSCs differentiation capacity, the cells were cultured in osteogenic and adipogenic medium (Alizarin Red & Oil Red stainings, respectively) for 21 days with regular medium changes. After 21 days, the cells are evaluated to check fatty droplets and calcium accumulations.

2.3. MSC-EVs isolation and characterization

MSCs were cultured in T75 flasks in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin-streptomycin. To harvest the conditioned medium (CM) for EV isolation, the cells were gradually adapted to a culture medium without FBS. The FBS concentration in the MSC culture medium was decreased gradually every 48 hours, from 10% to 5%, 2.5%, 1%, and finally 0%. The FBS-free CM isolated from MSCs (MSCs-CM) was collected every 72 hours and centrifuged and passed through a 0.22 µm filter to remove the cell debris. Total protein concentration was determined using the BCA kit (ThermoFisher Scientific). Size analysis was conducted using dynamic light scattering (DLS) with a Zetasizer NANO from Malvern, while scanning electron microscopy (SEM) and transmission electron microscopy (Agrawal, Pandey, Pandey, & research) from JEOL Ltd., Tokyo, Japan, was utilized to assess EVs' shape. Phenotyping was performed using western blot analysis to detect CD9 and CD63 as extracellular vesicle markers, and

184 calnexin as a housekeeping protein. These markers were also assessed in the corresponding cell line, which
185 served as a negative control for EVs.

186 **2.4. Dex loading in MSC-EVs (L-Dex-EVs)**

187 50µg of dexamethasone and 100µg of MSC-EV were gently mixed in 0.5 mL of electroporation buffer (125
188 mM NaCl, 1.5 mM CaCl2, 5 mM KCl, 10 mM glucose, and 20 mM HEPES; pH 7.4) at room temperature
189 (RT) (W. Zhang et al., 2022). Using an Eppendorf electroporation device, two 300V pulses were given at
190 a one-minute interval in 0.5 cm electroporation cuvettes containing EVs and Dex solution. The mixture was
191 kept at 37°C for 30 minutes to restore the membrane integrity of the vesicles. To remove the unloaded Dex
192 and the electroporation buffer, three washes were done using an electrofiltration Falcon tube with 10 kDa
193 molecular weight cutoff (MWCO) pores. Liquid chromatography-mass spectrometry (LC-MS) was applied
194 to calculate the concentration of Dex captured by EVs, as described in section 2.5. After generating L-Dex-
195 EVs, the size distribution and morphology of dexamethasone-loaded EVs were re-examined using dynamic
196 light scattering (DLS) and electron microscopy imaging techniques (SEM and TEM) to verify the integrity
197 of MSC-EVs post-electroporation

198 **2.5. Quantitative analysis of dexamethasone loading efficiency using LC–MS/Q-TOF**

199 Liquid chromatography-mass spectrometry (LC-MS) analyses were conducted using an Agilent
200 Technologies 6530 Accurate-Mass Q-TOF LC-MS System to quantify dexamethasone levels in the
201 supernatant. A HPLC Column (Agilent, ZORBAX Eclipse Plus, C18, 95Å, 1,8 µm, 3 x 100 mm) was
202 employed and maintained at 40 °C. A gradient elution system was utilized with a binary mobile phase
203 comprising solvent A (0.1% formic acid and 10 mM ammonium formate in milli-Q water) and solvent B
204 (0.1% formic acid and 10 mM ammonium formate in 100% acetonitrile). The elution profile was initiated
205 with 5% A and 95% B for 5.0 minutes, followed by a return to initial conditions until 6.1 minutes,
206 maintained until 50 minutes. The flow rate was 0.3 mL/min. Column and autosampler temperatures were
207 maintained at 40°C and 0.8 °C, respectively. A sample injection volume of 10.00 µL was used, with

dexamethasone retention time determined as 1.820 min under these conditions. The mass spectrometer operated in positive ion mode with a nebulizer pressure of 40 psi, a drying gas flow rate of 8 L/min, and a drying gas temperature of 300 °C. Tuning parameters were optimized using direct infusion of dexamethasone standard solutions, setting the capillary voltage to –3500 V, skimmer voltage to 65 V, Oct 1 DC voltage to 4 V, fragmentor voltage to 175 V, and Oct RF voltage to 750 V.

Following the determination of the amount of dexamethasone in the supernatant, the loading efficiency was calculated using Equation 1:

$$\text{Loading efficiency (\%)} = \frac{(\text{Mi} - \text{Ms})}{\text{Ms}} \times 100 \quad (1)$$

Where Mi represents the initial mass of dexamethasone, and Ms represents the mass of dexamethasone in the supernatant.

2.5. Induction of sepsis using the CLP model

Intraperitoneal injections of a cocktail of xylazine (10 mg/kg) and ketamine (90 mg/kg) were used to anesthetize mice. After shaving the mouse's body hair at the surgical site and sterilizing it, a small para midline incision of 1 cm length was made on the skin and then the abdominal wall. After exposing the cecum, using a 4-0 silk suture, a knot was tied to close the distal third (~1 cm). Using a hollow needle, the closed part of the cecum was pierced twice from the basolateral side into the lumen. A small amount of cecal material was removed from the hole. The number and size of perforations will determine the severity of sepsis, inflammatory response, and mortality. The closed cecum was returned to the abdomen. The peritoneum was closed using two or three clips.

2.6. Validity of CLP-induced Sepsis

After operating the CLP surgery and induction of the sepsis in mice, the validity of CLP-induced sepsis was confirmed by examining the clinical and laboratory index of sepsis. For this, 24 hours after CLP

surgery, the sepsis index according to MSS (murine sepsis score) (Mai et al., 2018), Table 1, liver enzyme changes in serum, and histological changes in lung, liver, and kidney were investigated (Table 2).

2.7. Liver enzymes and kidney damage markers measurement in serum

All mice were sacrificed Twenty-four hours after sepsis induction. Blood was then collected directly from their hearts using an insulin syringe and transferred into 1.5 ml microcentrifuge tubes. The tubes were then centrifuged at 3000 g for 20 minutes, and the serum was carefully separated and transferred to 0.2 ml microtubes. Serum samples were used to measure aspartate transferase (Molinaro et al.), alanine transaminase (ALT), and bilirubin levels to evaluate liver function and creatinine level to evaluate kidney function. The biochemical autoanalyzer was analyzed.

2.8. Histopathology of liver, kidney, and lung

After the mice were sacrificed, the liver, kidney, and lung were harvested. The tissues were then cut into small pieces (~0.5 cm) and placed in 10 ml of 10% formalin solution. The fixed tissues were embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E). The encoded samples were then submitted to an expert pathologist for histological analysis in a blinded manner. The liver damage was assessed according to the level of hepatocyte necrosis, degeneration, capillary congestion, and infiltration of inflammatory cells into the parenchyma and sinusoid space. The necrosis of tubular cells, capillary congestion, and inflammation evaluated the kidney damage. The lung damage was examined by its capillary congestion of alveoli, the thickness of the alveolar wall, and the penetration of inflammatory cells into the interstitial space and the airways. Each criterion was scored on a scale from 0 to 4, according to the definitions provided in Table 2. The individual scores for each factor were summed to obtain the overall tissue damage score. These scores were compiled and statistically analyzed to compare the extent of tissue injury among the experimental groups. One representative image from each group was selected and

included in the figures solely for illustrative purposes. These images serve to visually support the quantitative data obtained from the blinded histopathological evaluations.

2.9. Bronchoalveolar lavage (Molinaro et al.) extraction from lung tissue

After euthanizing the mice, the skin and chest were removed gently, and the lung lobes were exposed. 1 ml of PBS was injected slowly into the lung space and alveoli from the epiglottis using a yellow catheter through the trachea. The prominence and enlargement of the lung lobes indicate the correct entry of PBS into the alveoli and lung space. One minute after the PBS injection into the lung, the liquid containing proteins, cytokines, and cells from the alveoli of the lung was slowly aspirated. We repeated the injection and collection of 1 ml of the PBS buffer for the second time and centrifuged the collected volume of BAL at 1200g for 3 minutes. The supernatant is collected and stored in a -70°C freezer for the cytokines measurement using the ELISA technique.

2.10. Cytokines concentration measurement

24 hours after the treatments, the mice were sacrificed by CO_2, and their serum and BAL were collected. TNF-α, IL-10, IL-6, and TGF-β cytokines levels were evaluated by ELISA kits (R&D System, USA). According to the manufacturer's protocol, the required materials and solutions were prepared. The standard curve was drawn using the kit standards, and the samples were evaluated accordingly. After coating the capture antibodies in the 96-well plate, 50 µl of blocking solution (1% BSA) was added to the wells, and the plate was incubated for 1 hour at RT. After washing, 100 µl of samples or standard solutions were added to each well. After 2 hours of incubation, 100 µl of detection antibody diluted in PBS was added to the wells. After incubation and washing, 50 microliters of Streptoavidin-HRP conjugate diluted 1 to 40 in PBS buffer, 100 µl of TMB substrate solution (chromogen), and 100 µl of 2N sulfuric acid (stopping solution) were added to each well, respectively. Finally, the optical absorbance (OD) was read at 450 nm (specific absorbance) and 570 nm (reference absorbance).

2.11. Real-Time PCR

Total RNAs were isolated from tissue using triazole reagent following the manufacturer's guidelines. cDNA was synthesized by using 2 µg of total RNA and reverse transcription reagent kit (Thermo Fisher Scientific, Cat no:4368814) with reverse transcriptase and random hexamers. Gene expression analysis was measured by quantitative real-time polymerase chain reaction (qRT-PCR) assay using SYBR Green Master Mix (Thermo Fisher Scientific, Cat no:4472908) on an ABI 7500 detection system (Applied Biosystems). The data were normalized against the β-actin transcript level. The delta-delta CT method was used to calculate the relative gene expression. The primers used in this study are presented in Table 3.

2.12. Statistical analaysis

Analysis of the results and graphs was done using GraphPad Prism software. After proving the normality of the data with the Kolmogorov-Smirnov test, one-way ANOVA tests were used to compare the studied groups. Data are shown as mean ± standard deviation (Mean ± SD). Confidence limits were considered 95% in all experiments, and P less than 0.05 was considered significant. In the graphs, P values less than 0.05 are indicated by one star (*), less than 0.01 by two stars (**), and less than 0.001 by three stars (***).

3. Results

3.1. MSCs characterization

To confirm the MSCs, the cells were cultured in adipogenic and osteogenic differentiation media for 3 weeks and then stained with either Oil-Red O or Alizarin-Red S. The cells were observed under a light microscope. The results showed that the cells had the appearance of adipocyte cells in the adipogenic medium and produced intracellular oil droplets (Figure 2). Red oil droplets and red calcium deposition present in the cells indicate the potential for adipogenesis and osteogenesis of MSCs, respectively (Figure 2B-C). Also, the results of flow cytometry for MSCs-related surface markers show high expression of

CD90, CD73, and CD105, as well as low expression of blood cell markers CD34, CD45, and CD14 (Figure 2D-I).

3.2. MSC-EVs characterization

Given that this study is a continuation of our previous in vitro research, and the detailed characterization of MSC-EVs before and after loading has been reported in our previous study(Mirsanei et al., 2024), we briefly report here the post-loading characterization. For this aim, SEM was used to examine the shape of the Dex-loaded MSC-EVs. The results showed that L-Dex-EVs have a spherical structure, and their size is within the range of MSC-EV size (average ~100 nm) (Figure 3A) (37). TEM scanning illustrated the bilayer round shape of vesicles (Figure 3B). DLS was employed to evaluate the mean diameter of the loaded vesicles. The average diameter of the vesicles was approximately 89.27 nm, which indicates the size distribution of the vesicles after electroporation and loading by Dex, falling within the MSC-EV size range (Figure 3C). The results demonstrated that, in contrast to the cells, CD9 and CD63 were highly expressed in the L-Dex-EVs, whereas calnexin was not detected in these vesicles (Figure 3D).

3.3.Dex loading efficiency in MSC-EVs

Dex's loading efficiency was investigated using the LC-MS/MS method. Dex, being a polar compound, undergoes protonation and forms positive ions under acidic conditions. Consequently, mass detection was conducted in positive ion mode to enhance sensitivity and specificity.

Initially, full-scan mass spectra of the supernatant were acquired (Figure 4A). Among the ions observed, the major production at m/z [Dex+H]+ (m/z 393.2) was selected for the selected reaction monitoring (SRM) transition. Subsequently, quantification analyses were performed by monitoring optimized SRM transitions for both standards and samples. Representative SRM chromatograms of the supernatant are depicted in Figure 4B.

The findings revealed that the optimal loading efficiency was attained at an EVs to Dex concentration ratio of 2:1, yielding an efficiency of 25.67%. This optimized ratio demonstrates a balance between the effective loading of Dex onto EVs and maintaining the stability and functionality of the resulting Dex-loaded EVs.

3.4 Clinical scoring of septic mice based on the MSS scoring system

As part of the treatment response to sepsis, mice were followed for 24 hours, and their clinical symptoms were assessed using the MSS scoring system (Table 1)(Mai et al., 2018). Based on Figure 5, it is evident that the CLP group has the highest sepsis scoreamong all groups ($P < 0.001$), and L-Dex-EV is the most effective treatment for reducing septic symptoms in septic models. Dex+EV also decreased the MSS compared to the EV group ($P<0.05$). Although EV and Dex treatments individually were able to reduce the MSS compared to the CLP group ($P<0.001$), no significant difference was observed found between them.

3.5. Lung tissue histopathology in treatment groups

Histological evaluations of the lung tissue, according to the scoring in Table 2, are shown in Figure 6. Tissue damage indicators such as infiltration of immune cells into the intercellular spaces, congestion, and edema were observed. Examination of slides from various groups showed a relative improvement in lung tissue in the investigated damage factors in all the treated groups compared to the sepsis group ($P<0.001$). The results showed that Dex+EV treatment, and especially L-Dex-EV, caused a significant reduction in the damage indicators (Figure 6 D), including reduced cell infiltration (Figure 6 C) compared to the EV and Dex groups ($P< 0.01$). However, there was no significant reduction in pulmonary congestion in the Dex+EV group compared to the EV group (Figure 6 B).

3.6. Liver tissue histopathology in treatment groups

The histopathology evaluations in Figure 7 showed that the liver tissue in the sepsis group experienced tissue damage, including hepatocyte necrosis, vacuolar degeneration, and congestion in the liver tissue. As a consequence, these changes may result in impaired liver function. Based on the definitions provided in Table 2, examination of slides from the treated groups (Figure 7 E) showed a significant reduction in damage indicators and a relative improvement in the liver tissue compared to the sepsis group ($P<0.01$). Liver tissue necrosis and congestion in mice treated with L-Dex-EV were significantly reduced compared to the groups treated with Dex and EV individually ($P<0.001$). However, the reduction of degeneration in the L-Dex-EV treated group was not significant compared to the EV group (Figure 7 B-D). In the Dex+EV treated group, a significant decrease in liver tissue necrosis was more pronounced than in Dex group, along with a reduction in degeneration compared to both Dex and EV groups ($P<0.01$).

3.7. Kidney tissue histopathology in treatment groups

The histological analysis of kidney tissue using H&E staining is presented in Figure 8. The kidney tissue in the sepsis group suffered from tissue damage such as tubular cell necrosis, vacuolar degeneration, congestion, and edema resulting in impaired kidney function (Figure 8A). The treated groups (Figure 8 E) show a significant reduction in damage indicators and relative recovery of kidney tissue compared to the sepsis group ($P<0.01$). As illustrated in panels B, C, and D of Figure 8, the L-Dex-EV treated group exhibited significantly reduced congestion, tubular cell necrosis, and kidney tissue degeneration compared to the EV, Dex, and Dex+EV groups ($P<0.001$). Also, renal congestion and degeneration in the Dex+EV-treated group were significantly reduced compared to the Dex-treated group ($P<0.05$).

3.8. Liver and kidney function in treatment groups

As illustrated in Figures 9-A and B, the induction of sepsis without treatment in the CLP group led to a significant increase in liver enzymesALT and AST. In the treated groups, the serum levels of these enzymes were reduced. The reduction in ALT level in the L-Dex-EV group was significantly greater than inthe EV, Dex, and Dex+EV groups ($P<0.05$). Also, the serum bilirubin level significantly decreased in all treated groups compared to the CLP group (Figure 9 D). However, this reduction in bilirubin level in the L-Dex-EV group was statistically significant only when compared to the EV group ($P<0.05$). Figure 9C shows that the serum creatinine levels significantly decreased in all treated groups compared to the CLP group.

3.9. Local immunological changes of lung tissue in treatment groups

After 24 hours of sepsis induction and treatment administration, the mice were euthanized, and various tissues were harvested for subsequent analysis. The expression levels of genes associated with inflammatory pathways, including caspase 1, caspase 3, NLRP3, and HMGB-1, were analyzed using real-time PCR. According to Figure 10, the expression levels of all four genes were significantly elevated in untreated septic mice compared to the healthy group, and their expression in the treated septic mice was significantly reduced compared to the CLP group ($P<0.001$). HMGB-1 expression was significantly lower in the L-Dex-EV group than in the EV and Dex+EV groups, but not significantly different from the Dex group (Figure 10 A). According to Figure 10 B, C, and D, the expression of NLRP3, caspase 1, and caspase 3 genes in the L-Dex-EV and Dex+EV groups was significantly decreased compared to the EV and Dex groups ($P<0.001$).

3.10. Cytokine production in BAL

24 hours after model induction and group-specific treatment, the mice were euthanized, and bronchoalveolar fluid was collected. The amount of cytokines in BAL was measured using the ELISA method. Based on Figure 11, the expression of two inflammatory cytokines, TNF-α and IL-6, in the BAL sample of untreated septic mice (CLP group) showed a significant increase compared to healthy mice,

which indicates an elevated pulmonary inflammation . At the same time, the expression of these two cytokines in sepsis-treated mice in different groups was significantly reducedcompared to the CLP group ($P< 0.001$). Also, in the L-Dex-EV group, the expression of IL-6 was significantly lower than in the EV group , and the expression of TNF-α was significantly reduced compared to the Dex and Dex+EV groups (Figure 11 A, B) ($P< 0.001$). On the other hand, the expression of anti-inflammatory cytokines IL-10 and TGF-β in treated groups increased significantly compared to the CLP group ($P< 0.001$). The expression of IL-10 in the L-Dex-EV and Dex+EV groups was significantly increased compared to the EV and Dex groups, while TGF-β expression was significantly increased in the Dex+EV and Dex groups compared to the EV group (Figure 11 C, D).

3.11. Systemic immunological changes in treatment groups

To investigate systemic inflammatory changes, the expression of Arg-1 and iNOS genes in spleen were measured by Real-time PCR. iNOS expression was significantly elevated in the CLP group compared to the healthy controls (Figure 12A). However, it'sexpression was significantly decreased in all treated mice ($P<0.0001$). Also, the decrease in the expression of this gene in the L-Dex-EV group compared to the Dex, EV, and Dex+EV groups was significant. In the Dex+EV treated group, the expression of Arg-1 and iNOS genes altered significantly compared to EV. As shown in Figure 12 B, the expression of Arg-1 significantly upregulated in all treated groups compared to the CLP group. The increase in the expression of this gene in the L-Dex-EV and Dex+EV groups was also significant compared to EV and Dex groups ($P<0.001$).

Figure 12 C shows that TNF-α levels were elevated significantly in CLP mice compared to health controls, while all treated groups exhibited a significant reduction . The decrease of TNF-a in the L-Dex-EV group was also significant compared to the Dex+EV and Dex-treated groups. The results of IL-10 cytokine serum changes in the studied groups can be seen in Figure 12 D. According to the results, this cytokine concentration significantly increased in all treated mice compared to untreated septic mice.

4. Discussion

The overproduction of cytokines after overstimulation of the innate immune system in response to a wide variety of infectious and non-infectious stimuli can cause inflammation, tissue damage, and other complications, known as cytokine storm (CS). Sepsis is one of the most common causes of cytokine storm occurrence, and in affected patients, sepsis and CS represent two sides of the same coin. Hence generating the septic animal model can provide an appropriate condition to study CS, various techniques exist to induce sepsis in animal models and explore diverse features and novel treatments. In comparison with human sepsis, CLP-induced sepsis has the most similar hemodynamic cycle and cytokine profiles (Buras, Holzmann, & Sitkovsky, 2005). Therefore, the CLP model is considered the gold standard among sepsis animal models (Buras et al., 2005). Another main advantage of the CLP method is its possibility to manage the severity of induced sepsis (i.e., from moderate to severe) (Buras et al., 2005). In other words, based on the length of the ligated cecum (the most important determining factor), the size of the needle used for the puncture, and the number of punctures performed, the severity of sepsis in animals will be controlled (Khosrojerdi, Soudi, Hosseini, Khaligh, et al., 2021; Rittirsch, Huber-Lang, Flierl, & Ward, 2009). The severity and quality of immune responses, as well as susceptibility to infections, are influenced by sex-specific genes and hormones and differ between males and females (Klein & Flanagan, 2016; McClelland & Smith, 2011; Takahashi & Iwasaki, 2021) .So to minimize unintended variables in experimental outcomes, it is important to use animals of one single sex in experimental studies (except those to study the effect of gender on outcomes). Based on previous studies in our group and also others' studies (Ahangari et al., 2024b; T. Zhao et al., 2016), we selected male mice for the induction of the sepsis model and the evaluation of our intervention's effects.

As mentioned earlier, sepsis is associated with damage to the vital organs of the body, and the lung is one of the first vulnerable tissues, so in this studywe focused on evaluating pulmonary damage in septic mice to assess treatment effects.

Various treatments have been proposed for CS, including monoclonal antibodies, corticosteroids, and and other agents(Fajgenbaum & June, 2020; Lutgens, Joffre, van Os, & Ait-Oufella, 2021; Molinaro et al., 2020; P. Morgan, Arnold, Hsiao, & Shu, 2021). However, according to the MSC-EVs' immunomodulatory potential, their use in the treatment of inflammatory diseases has been suggested (Khosrojerdi, Soudi, Hosseini, Eshghi, et al., 2021; Motallebnezhad et al., 2023). However, studies have shown that EVs alone have limited therapeutic efficacy, which can be enhanced through combination strategies (Hazrati, Soudi, & Hashemi, 2022; Malekpour, Hazrati, Soudi, Roshangar, et al., 2023). On the other hand, Dex is known for its potent anti-inflammatory effects. Hence, it was utilized in this study in conjunction with MSC-EVs, either as a physical mixture or as a loaded formulation. Incubation, electroporation, sonication, and extrusion are generally accepted methods for loading small molecules into EVs, although these methods vary in protocol complexity and loading efficiency (N. Wang, Cheng, Li, Wang, & Chen, 2019). Based on our previous study, Dex was loaded into EVs using electroporation (Mirsanei et al., 2024). Notably, previous studies on dexamethasone-loaded EVs have reported loading efficiencies below 20%, largely due to the molecule’s size and hydrophobicity (Kalinec, Cohn, Whitelegge, Faull, & Kalinec, 2019; Yan et al., 2020; J. Zhao et al., 2021). In our study, by optimizing EV and Dex concentrations and fine-tuning the loading process, we achieved a loading efficiency of 25%–30%, representing a significant improvement using minimal Dex quantities (Mirsanei et al., 2024).

After characterization of stem cells and loading Dex into MSC-EVs, as well as validation of the induction of sepsis in mice after CLP surgery, we investigated the potential of Dex and stem cell-

derived vesicles together in reducing the inflammatory condition and tissue injuries in septic mice. The study's results showed that the simultaneous administration of Dex and MSC-EVs, either in a loaded form or mixture led to a significant reduction in the clinical index. In addition, serum factors (ALT, AST, Bil, and Cre) and H&E staining confirmed that combination treatments (L-Dex-EV and Dex+EV) resulted in the greatest alleviation of kidney and liver damage . These results align with the study conducted in our group, which demonstrated that co-administration of MSC-EVs and azithromycin improves clinical and histopathological recovery in septic mice (Ahangari et al., 2024a). Another study conducted in 2021 by Jiawei Zhao *et al*. used dexamethasone loaded in MSC-EVs to treat mice with autoimmune hepatitis (J. Zhao et al., 2021). The results of liver tissue histology, as well as the evaluation of serum factors in these mice, confirmed the significant therapeutic effect of Dex-loaded MSC-EVs. Moreover, they reported that this combination had no toxic effect on other tissues, such as the heart, kidneys, and lungs (J. Zhao et al., 2021).

A deeper analysis of lung tissue inflammation and damage at three levels of gene expression (HMGB-1, NLRP3, Caspase1, Caspase3), protein expression (IL6, TNF-α, IL10, TGF-β) and tissue staining confirmed the superior efficacy of L-Dex-EV, followed by Dex+EV in ameliorating lung tissue damage. In a recent study by Enhai Cui in 2024, it was reported that in mice suffering from LPS-induced sepsis, the use of MSCs-derived exosomes significantly reduces lung tissue damage (Cui et al., 2024). Similarly, another study showed that intravenous injection of MSC-EVs in LPS-induced septic mice significantly reduced lung, liver, and spleen tissue damage and also reduces biochemical markers in the serum (Eshghi et al., 2022) Our results are similar to those reported by Jiawei Zhao, 2021. His findings demonstrated a notable reduction in TNF-α, IL-6,

IL-12, and IL-1β levels in the group receiving loaded MSC-EV compared to the untreated group (J. Zhao et al., 2021).

Finally, our latest experiments showed that L-Dex-EV and Dex+EV resulted in systemic inflammation as indicated by TNF-α and IL-10 serum levels and iNOS and Arg-1 gene expression in the spleen. These results were consistent with the results of Enhai Cui's study on acute lung injury caused by LPS using umbilical cord MSCs derived exosomes. By evaluating the level of TNF-α, IL-6, and IL-10, they reported a reduction of in inflammatory cytokines and an increase in the amount of IL-10 in the serum of mice treated with these exosomes (Cui et al., 2024).Sepsis causes inflammation and CS by involving multiple signaling pathways in which the innate immune system causes an uncontrolled and excessive release of circulating cytokines (Y.-y. Zhang & Ning, 2021). Free DNA released from apoptotic cells and dead bacteria activates the STING signaling pathway and results in IRF3 and NF-κB transport to the nucleus (Deng et al., 2022). Complement activation results in C3a and C5a production and multiple signaling pathways, such as ERK1/2 and then MAPK/P38, followed by the production of IL-6 (Chiou, Tsai, Yang, & Tsai, 2004). Cell stress-related signals such as ATP or PAMPs also activate the NLRP3 pathway, producing more reactive oxygen species (ROS). NLRP3 also promotes inflammatory cytokines IL-1β and IL-18 production by activating Caspase 1 (Y.-y. Zhang & Ning, 2021). The recognition of HMGB-1 (an example of DAMPs) by immune cells, which is released from damaged cells and tissues and apoptotic cells, leads to the intensification of inflammation and activates the TLR2/4 pathway and the RAGE pathway. Finally, these pathways lead to the activation of the NF-κB transcription factor and the inflammatory cytokines release (Yuan, Liu, Xu, Liu, & Zhang, 2020).

In a study by Li JW, mir-21-p encapsulated exosomes were used to treat septic mice. The results of Li JW’s study revealed a reduction in lung tissue edema, alleviation of macrophage-mediated

inflammation, and a decline in the expression of inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-17, and TNF-a in the BAL (wei Li, Wei, Han, & Chen, 2019). In another study on lung damage caused by cigarette smoke, the injection of stem cells-derived exosomes reduced the expression of HMGB-1 and NF-κB, thereby decreasing inflammation in these mice (Xu et al., 2020). The use of Dex can control the inflammatory pathway by suppressing the ERK/NF-κB pathway and improving patients' conditions (J. Zhang et al., 2016). Several studies also confirm the effect of Dex in reducing the activity of inflammasomes by inhibiting NLRP3 and caspases 1 and 3(Jaiswal, Dash, & Singh, 2022) (Yang et al., 2020). In a study by Jaiswal *et al.* on LPS-induced asthma, it was suggested that the simultaneous inhalation of Dex and curcumin has reduced the activity of inflammasomes by inhibiting NLRP3, thereby reducing inflammation in affected mouse models (Jaiswal et al., 2022). Jia-Wei Yang *et al*. showed that glucocorticoids can improve LPS-induced lung damage by inhibiting the activity of the inflammasome and NLRP3 (Yang et al., 2020). Caspase 1 and 3, and NLRP-3 play a role in the activation of inflammasomes and production of inflammatory cytokines, including IL-6 and TNF-α (Man & Kanneganti, 2015; Sutterwala, Haasken, & Cassel, 2014; Vince et al., 2018).

The anti-inflammatory effect of MSC-derived exosomes has been demonstrated in vitro and in vivo (Hazrati, Soudi, & Hashemi, 2022; Malekpour, Hazrati, Soudi, Roshangar, et al., 2023). Khosrowjerdi *et al.* demonstrated that the simultaneous therapeutic use of MSC-derived exosomes and the imipenem in the CLP-induced sepsis model in mice led to a reduction in inflammatory conditions and serum cytokine levels, along with the improvement of damaged liver tissue (Khosrojerdi, Soudi, Hosseini, Khaligh, & Hashemi, 2023).

According to the results of various studies, it seems that MSC-derived EVs perform their therapeutic effects through the involvement of different mechanisms. MSC-EVs, through the

transfer of growth factors, mRNAs, microRNAs, long non-coding RNAs, anti-inflammatory cytokines, and modulation of inflammatory responses, including effects on macrophages and the transport of mitochondria or their subunits, can help damaged cells to improve tissue damage (Malekpour, Hazrati, Soudi, & Hashemi, 2023). In the meantime, loading dexamethasone into these EVs significantly increases the therapeutic potential of MSC-derived EVs due to their inherent ability in suppressing pathogenic inflammatory responses (Rawat, Dadhwal, & Mohanty, 2021). However future studies—building upon the current findings—may help provide deeper insights into the potential underlying mechanisms.

In general, the findings of this study indicate that the concurrent administration of Dex and MSC-EVs effectively diminishes inflammatory factors and mitigates tissue damage in septic mice. This outcome is probably attributed to the activation of various immunomodulatory pathways and tissue repair mechanisms, leading to a synergistic effect. While these two combined treatments yielded comparable outcomes in many cases, the superior efficacy observed with L-Dex-EVs effects may be due to several factors. Most notably, the encapsulation of Dex within EVs enhances its stability in the bloodstream compared to its free form. Beyond that, sustained release of Dex from the vesicles prolongs its bioavailability in tissues and enhances cellular uptake. Additionally, the simultaneous uptake of Dex and MSC-EVs by target cells is more likely with the loaded formulation.

Given the all above explanation, it's crucial to note that while our findings showed an amelioration in inflammatory conditions and tissue damage in septic mice, sepsis and cytokine storms are complicated phenomena with multitudiness factors. In this study, we only examined a limited number of these factors. Therefore, it is essential to carry out a multifactorial study that emphasizes the signaling pathways involved, including molecular and epigenetic aspects.

5. Conclusion

Considering the critical condition of patients with acute inflammatory diseases, such as sepsis, the synergistic application of well-established anti-inflammatory drugs along with MSC-derived extracellular, which possess strong immunomodulatory and regenerative properties, could be a promising treatment option for these patients. In summary, based on the study findings, combination treatments of Dex and MSC-EVs exert remarkable tissue repair and anti-inflammatory properties, suggesting these regimens are a potential treatment for inflammation-related diseases.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Authors contribution

ZM: Investigation, Formal analysis, Data curation, review & editing the original draft. **FJ-A & SV**: Data curation, Investigation, review & editing. **PE, FA, SS, SK, FS, P-EA, A-AH**: Data curation, Investigation. **AH**: Writing original draft—review & editing. **SS, MS**: Conceptualization, Methodology. **SA-H & SMH**: Conceptualization, Supervision, Validation, Methodology, review & editing.

Ethics approval

The instructions and animal handling followed the laboratory animal care guidelines approved by the National Ethics Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethical code: IR.SBMU.MSP.REC.1400. 831).

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