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ORIGINAL RESEARCH ARTICLE

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Antimicrobial effects of mesenchymal stem cells primed by modified LPS on bacterial clearance in sepsis

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Abstract

Background and Objectives: Mesenchymal stem cells (MSCs)-based regenerative therapy is now considered as an alternative approach to revive infectious diseases, including sepsis. Nevertheless, the efficiency of MSC application is limited by the poor survival rate of engrafted MSCs. Hence, preconditioning was established as a strategy to increase the cells' efficiency.

Methods: MSCs were preconditioned with 1 μg/ml of three different lipopolysaccharides (LPSs) of *Pseudomonas* (Pse-LPS), *Acinetobacter* (Ac-LPS), and *Acinetobacter* inactivated lipid A by PagL (Ac-LPS-PagL). Then, preconditioned MSCs were exposed to oxidative stress and serum deprivation followed by evaluation of the antibacterial activity, survival, and apoptosis of MSCs. Then, the murine sepsis model treated with 100 μl phosphate-buffered saline (control group, sepsis group), 100 μl of 1×10^6 wild MSCs (MSC group), and three remained groups received 100 μl of 1×10^6 LPS-preconditioned MSCs (Pse-LPS-MSCs group: LPS purified from *Pseudomonas*, or Ac-LPS-MSCs group: LPS purified from *Acinetobacter*, and Ac-PagL-LPS-MSCs group: detoxified LPS Pagl).

Results: After 4 days, LPS-preconditioned MSC transplantation modulated the immune response and reduced inflammation in septic mice. Apoptosis of Pse-LPS/Ac-LPS-preconditioned-MSCs was obviously reduced in vitro, and the survival rate of engrafted mice was evidently elevated in Pse-LPS-MSCs and Ac-LPS-MSCs groups compared with other three groups.

Conclusion: LPS preconditioning provides an innovative strategy for evolving functional and biological properties of MSCs and ameliorates the survival rate of the mouse model of sepsis after MSC transplantation, protects cells from apoptosis and organ damages, and evaluates therapeutic properties, including immunemodulatory.

KEYWORDS
antibacterial activity, lipopolysaccharides (LPSs), mesenchymal stem cells (MSCs), preconditioning, sepsis, survival

1 | INTRODUCTION

Sepsis is the most common cause of mortality and critical illness in hospitalized cases. Sepsis, being a clinical syndrome that is a consequence of deregulated host inflammatory and immune responses to an infection, could involve in tissue damage, multiple organ dysfunctions, and death (Pedrazza et al., 2017). Sepsis treatment relies mostly on traditional antibiotics and supportive

therapies, which are not sufficient to regress systemic inflammation and organ dysfunction in sepsis (Zhu et al., 2017). Moreover, even after several decades of research, the incidence, mortality rate, antibiotic resistance, and cost of sepsis treatment continue to be high. So, there is a need to consider significant trials to improve and design more effective and operative therapeutic alternatives (Cassini et al., 2016; Johnson, Soeder, & Dahlke, 2017). Over the past years, cytotherapy has become a promising platform for sepsis treatment.

Bone marrow mesenchymal stem cells (BM-MSCs or BMSC) are deliberated as an innovative therapeutic tool to treat sepsis, consistent with their unique properties. They are capable of interfering with different levels of the pathophysiology of sepsis. MSCs have emerged as potent modulators of immune responses, which could return both the innate and the adaptive immune responses to balance. They are able to inhibit bacterial proliferation and then cytokine storm in sepsis through their antimicrobial and anti-inflammatory features. In addition, the cytoprotective and antiapoptotic capacities of MSCs diminish tissue damage and cell death (Gnecchi, Danieli, Malpasso, & Ciuffreda, 2016; Zhang et al., 2015).

Despite medical advantages, MSCs are subjected to the rapid apoptosis and low survival rate after implantation in harsh disease conditions. These conditions can be hypoxia, serum deprivation, inflammatory stimulus, heat shock, and oxygen-free radicals, which considerably reduce their medicinal efficacy. This makes a requirement to ponder an approach to refine the efficacy MSCs and improve the survival rate (Spees, Lee, & Gregory, 2016; van Velthoven, Kavelaars, & Heijnen, 2012). In this regard, preconditioning or pretreatment of MSCs with sublethal environmental stress adjusts them to destructive conditions then protects cells from apoptosis and results in a better survival rate (Liu, Wang, Ji, Yu, & Wei, 2014; Saparov, Ogay, Nurgozhin, Jumabay, & Chen, 2016; Taban, Khatibi, Halabian, & Roushandeh, 2016). Latest data show that endotoxin preconditioning could protect MSCs against apoptosis induced by hydrogen peroxide (H_2O_2) or/and serum deprivation, at the same time as increasing the in vitro viability of MSCs (Amiri, Jahanian-Najafabadi, & Roudkenar, 2015; Zhou et al., 2015).

Endotoxin or lipopolysaccharide (LPS), a surface component of gram-negative bacteria, mainly mediates endotoxemia, sepsis, consequently multiple organ dysfunction syndrome, and endotoxic shock. Other than stimulating innate immunity through switching the exertion of inflammatory agents, LPS might also lead to apoptosis induction and cell death in MSCs.

In the present study, we hypothesized that LPS-MSCs would be more viable than MSCs in experimental septic animals. MSCs were exposed to the LPS, and then their effectiveness in terms of anti-infection, anti-inflammation, and organ-healing capacity was estimated in both in vitro and in vivo sepsis models. The proper dose of LPS treatment could keep MSCs safe against oxidative stress-induced apoptosis (Z. Wang et al., 2009). Because LPS is a toll-like receptor 4 (TLR4) ligand, it helps the proliferation of MSCs through the PI3K/Akt signaling pathways (J. Wang et al., 2013).

Within this context, the therapeutic effects of MSCs were inspected on a well-established live bacterial murine model of sepsis (Johnson et al., 2017; Kanashiro et al., 2017). We preconditioned MSCs with three LPS (*Acinetobacter baumannii* LPS, *A. baumannii* modified LPS, and *Pseudomonas aeruginosa* LPS), and then administered treatment of septic mice. Finally, the effectiveness of MSCs was evaluated for bacterial clearance, and the survival rates were observed.

Furthermore, analysis of transcriptional responses in trial sepsis models has reviewed concerning familiar therapeutic effects of preconditioned MSCs in main objective tissues in sepsis. These effects cause reduction of sepsis-induced mitochondrial dysfunction and downregulation of innate immune proinflammatory transcriptional responses and sepsis-related inflammation. They also can effectively decline vascular leakage and tissue injury, while improving host defense against pathogens and possibly reducing the risk for secondary infections that ameliorate sepsis (Liles, Matthay, Dos santos, Weiss, & Stewart, 2014).

2 | MATERIALS AND METHODS

2.1 | In vitro experiments

2.1.1 | Bacterial strains and growth conditions

Pseudomonas aeruginosa (ATCC® 27853™) and *A. baumannii* (ATCC® BAA747™) (Pasteur Institute, Tehran, Iran) were cultured in Luria-Bertani (LB) liquid medium (Merck, Darmstadt, Germany) at 37°C, overnight. *A. baumannii* ATCC® 19606™ carrying the plasmid pMMB66EH containing the PagL coding sequence was donated by Dr. Badmasti et al. (Pasteur Institute of Iran, Tehran, Iran), which inactivates the lipid A of LPS. The recombinant bacteria were cultured overnight into LB broth. Expression of PagL was induced by the addition of 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG), and cells were grown at 37°C for 16 hr. After harvesting the sedimented bacteria, they were ready for LPS extraction and purification.

2.1.2 | LPS extraction

The LPS extraction process was performed utilizing two methods, the LPS Extraction Kit (#17141; iNtRON Biotechnology, New Territories, Hong Kong), according to the instructions of the manufacturer. LPS was extracted by a hot-aqueous phenol method as described previously by Westpal & Jann (1965) with some modifications. Before extraction, treatment with proteinase K, DNase, and RNase was implemented to eliminate protein and nucleic acids contamination. Then, bacterial suspensions (6×10^8 colony-forming unit [CFU]/ml) were centrifuged, pellets were washed twice in phosphate-buffered saline (PBS), suspended in 10 ml PBS, and sonicated (for 10 cycles each for 30 s on ice). Afterward, an equal volume of hot-phenol 90% (65–70°C) was added to the mixtures and then was shaken strongly (for 15 min). Suspensions were then cooled on ice and centrifuged, and after adding distilled water, phenol phases were extracted. Sodium acetate (0.5 M) and 95% ethanol (10 volumes) were added to the extracts and stored at −20°C overnight to precipitate. They were then centrifuged, and the pellets were

suspended in 1 ml distilled water, completely dialyzed against distilled water to eliminate the residual phenol, and lyophilized. Final purified LPS product was detected by electrophoresis instrument (Bio-Rad Laboratories) in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a standard silver staining protocol.

2.1.3 | Isolation and culture of BMSCs

Five-week-old male Sprague–Dawley rats (n = 15; 150–200 g) were purchased from the Animal Laboratory Medical Faculty, University of Baqiyatallah. The experimental procedure of management and care of the animal studies were approved and performed under the supervision of the local Ethical Committee. The rats were killed, and then femurs and tibias were dissected out. Both femoral and tibias epiphyses were clipped, and the bone marrow cavity was carefully flushed with 3 ml low glucose Dulbecco's modified Eagle's medium (L-DMEM; #10-DM1-500; INOCLON, Tehran, Iran) containing 1% penicillin/streptomycin (Gibco Life Technologies) solution (10,000 IU/10,000 IU) and 10% heat-inactivated fetal bovine serum (FBS; Gibco Life Technologies, Gaithersburg, MD) using a syringe with an 18-gauge. MSCs were incubated at 37°C/5% CO_2/20% O_2/95% humidity. The bone marrow suspensions were cultured in polystyrene six-well plates. The nonadherent cells remained round and suspended in the medium after 2 days and were mostly eliminated from the culture by serial washes in PBS and subsequent fresh media changes. The cell expansion continued, and the medium was changed during proliferation every 2 days and passaging. Once adherent cells were expanded as monolayer cultures and reached almost 90% confluence, they were washed with PBS twice and detached with trypsin (0.25%)/EDTA (0.02%) (Gibco Life Technologies, Gaithersburg, MD), and subcultured in a T25 cm^2 cell culture flask (SPL Life Sciences, Gaithersburg, MD) at a plating density of 3×10^5 cells/flask, then replated in T75 culture flasks in a 1:3 ratio. Third to eight passages of MSCs were used for all the following tests (Alcayaga-Miranda et al., 2015).

2.1.4 | MSCs viability assay after preconditioning with LPS

Viability assessment of MSCs after preconditioning was performed with 0.4% trypan blue (TB) solution (Sigma-Aldrich, Taufkirchen, Germany) exclusion method and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Taufkirchen, Germany) colorimetric assay. To investigate the cytotoxic effects of LPS on MSCs' survival and proliferation, MSCs were seeded at a density of 1.5×10^3 cells/well in a 96-well plate overnight in the complete medium. After determining the optimum dose of LPS for MSC preconditioning, primarily the medium was exchanged with DMEM without FBS and antibiotics until they grew to confluence. According to our previous study (Kooshki, Ghollasi, & Halabian, 2018), they were treated with different doses of all three LPS types (0, 0.5, 0.8, 1, 1.2, 1.4, 1.8, 2, 3, 4, and 10 μg/ml) in a triplicate pattern to determine the cell viability and lethal dose (LD_{50}) of endotoxins in each concentration group using the MTT assay (Mosmann, 1983). Hence, 20 μl/well of 5 mg/ml MTT was added to cells. After 4-hr incubation, the supernatant was withdrawn from the plate, and formazan crystals were dissolved in 100 μl/well dimethyl sulfoxide. Then, the absorbance was assessed using a microplate reader (Bio-Rad, California, CA) at 570 nm (Hou et al., 2015). The percentage of viable cells was measured by the following equation:

$$\text{Percentage of viable cells} = \frac{\text{OD of exprimental groups}}{\text{OD of control}} \times 100$$

2.1.5 | Collecting the LPS-MSC-conditioned medium

MSCs were seeded in T75 flasks in standard culture medium, as described above, to almost 80% confluence. Confluent cultures were stimulated with optimized doses of LPS (as MSC-conditioned medium [CM] and LPS-MSC-CM) for 24 hr. The supernatant was collected and filtered through a 0.22-μm filter to eliminate cellular debris. Filtered supernatant concentrated 25-fold using ultrafiltration units with a 3–10 kDa molecular weight cutoff (Amicon Ultra-PL 3; Millipore, Merck, New Jersey, NJ) and frozen at −20°C for further in vivo analyses. Hence, LPS-MSC-CM and MSC-CM are CMs have been obtained after stimulation with or without LPS.

2.1.6 | Characterization of LPS-MSCs

The International Society for Cellular Therapy (ISCT) planned a standard set of minimal criteria to define the identity of MSCs. The characteristics of expanded MSCs at the second and third passages were verified before and after preconditioning, by inspection of morphological changes and the plastic adherent, immunephenotyping, and differentiation capacities. The multipotency and immunophenotype of preconditioned MSCs were confirmed by osteogenic, adipogenic differentiation, and flow cytometry, respectively (Dominici et al., 2006).

1. *Shape and plastic adherent*. The characteristics of MSCs were tested by observing morphology using an inverted microscope.

2. *Immunophenotypic characterization*. Typical immunophenotyping of cells determine MSCs by detecting surface antigens panel with flow cytometric analysis. A combination of positive and negative surface markers confirms the integrity of MSCs. The suspension of detached MSCs was incubated with fluorescein-isothiocyanate-labeled monoclonal antibodies against MSC-specific surface antigens (CD105, CD29, and CD44) and the hematopoietic markers (CD14, CD34, and CD45; eBioscience, Inc., Massachusetts, MA) at 37°C in the dark for 30 min. Then, according to standard procedures, flow cytometry (FACS Calibur, BD Bioscience, New Jersey, NJ) and FlowMax software were used for data analyses.

3. *Differentiation potentials*. Along with self-renewal, MSCs are also multipotent and capable of undergoing in vitro differentiation to some cell lines, which is the gold standard for defining MSCs. Consequently, MSCs were evaluated for their capacity to differentiate to adipogenesis and osteogenesis by using the StemPro

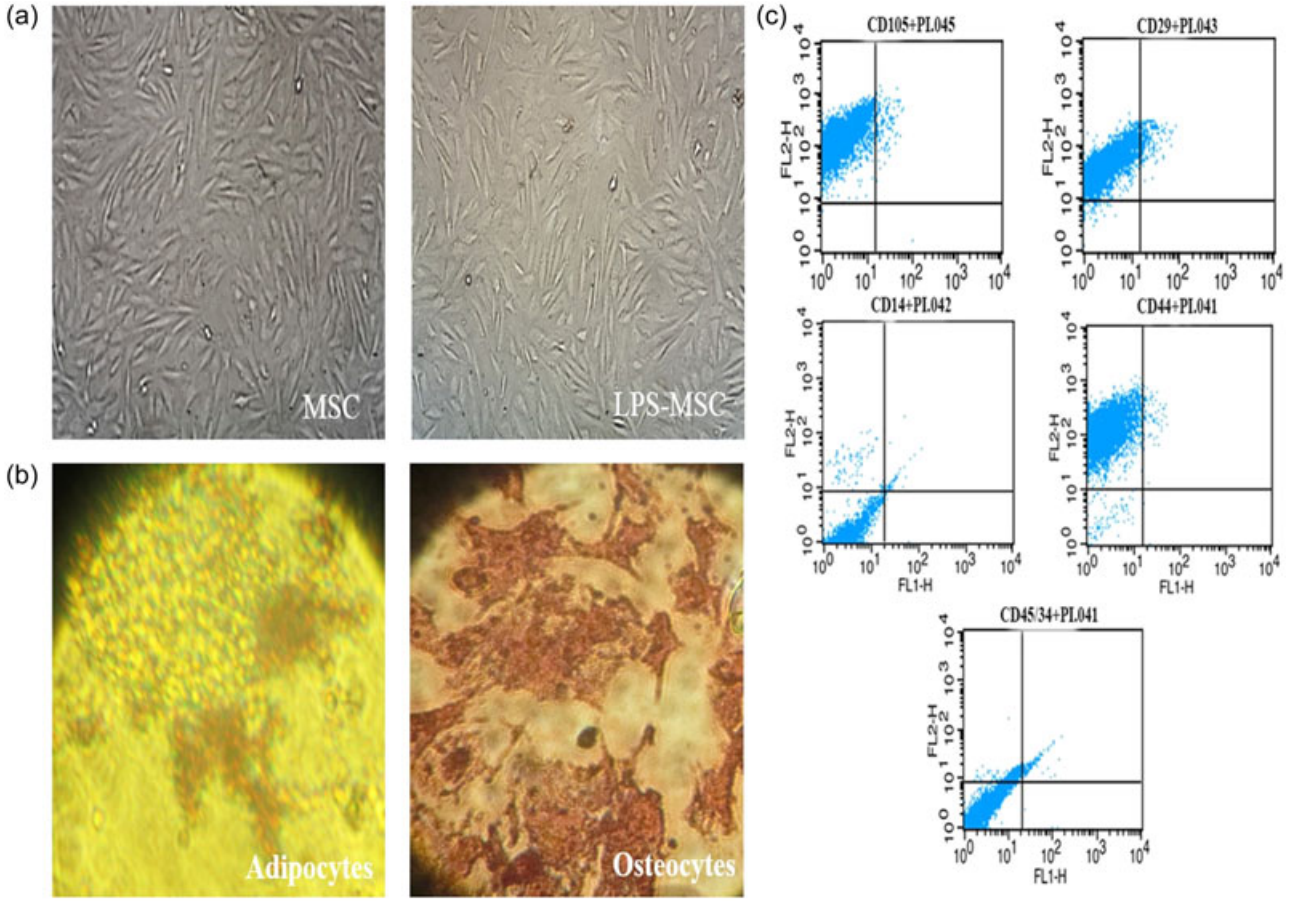

FIGURE 1 Characterization of LPS-MSCs. (a) Fibroblastic-like morphology of MSCs and LPS-MSCs after 3 days from seeding. (b) LPS-MSCs differentiation potential of LPS-MSCs into adipocytes and osteocytes. (c) Flow cytometry analysis indicated the expression of CD105, CD44, CD29, and no expression of CD34, CD45, and CD14. All LPS preconditioned-MSCs used in this study met the standard criteria of ISCT and retained plastic adherence, spindle-shaped morphology, expression of typical MSCs markers, and differentiation as before preconditioning. Ac: *Acinetobacter baumannii*; ISCT: International Society for Cellular Therapy; LPS: lipopolysaccharide; MSCs: mesenchymal stem cells; Pre: preconditioning; Pse: *Pseudomonas aeruginosa* [Color figure can be viewed at wileyonlinelibrary.com]

Differentiation Kits (A1007001 and A1007201; Gibco, Gaithersburg, MD), according to the manufacturer's instruction. In brief, MSCs were seeded in a six-well plate (5×10^3 cells/well for control; 10^4 cells/well for differentiation assay) in complete DMEM medium in a triplicate pattern. Control cells' media were replaced every 3–4 days.

(a) *Adipogenic differentiation of MSCs*. For adipogenic differentiation, after 24 hr, standard media were replaced with complete adipogenesis differentiation medium containing a basal medium and a supplement (in a ratio of 10:1), and the cells were serially fed every 3–4 days. After 14 days, the cells were washed twice with PBS, fixed with 4% formaldehyde for 30 min at room temperature (RT), and lipid droplets, indicating the differentiation of BMSCs into adipocytes, were identified by oil red O (Sigma-Aldrich-O0625, Taufkirchen, Germany) staining (0.7 g oil red O dissolved in 200 ml isopropanol and diluted to 60% with distilled water). The stained plates were washed thoroughly with tap water, dried, and photographed.

(b)*Osteogenic differentiation of MSCs*. To induce osteogenic differentiation, MSCs were incubated and grown up to 21 days in complete osteogenesis differentiation medium containing a basal medium and a supplement (in a ratio of 10:1). The medium was renewed every 3 days. To detect mineralized matrix, the cells were washed twice with PBS, fixed with 4% formaldehyde for 30 min at RT, and rinsed twice again. The matrix was stained with 2% fresh Alizarin Red S (Sigma-Aldrich-A5533, Taufkirchen, Germany) solution (2 g alizarin red dissolved in 100 ml distilled water, pH 4.2) for 2–3 min at RT. The stained plates were washed at length with tap water, dried, and photographed.

2.1.7 | Assessment of cytotoxic effect of LPS on MSCs

MSCs viability was evaluated using TB and MTT assay as mitochondrial activity. The effects of oxidative stress (H_2O_2) and serum deprivation (SD) on MSCs mimic the in vivo harsh and rigorous microenvironment. Cell viability after preconditioning and the subsequent incubation in H_2O_2/SD was examined with the TB solution exclusion assay (data not shown). Then, again, the cytotoxic effects of H_2O_2/SD were determined by the MTT colorimetric assay.

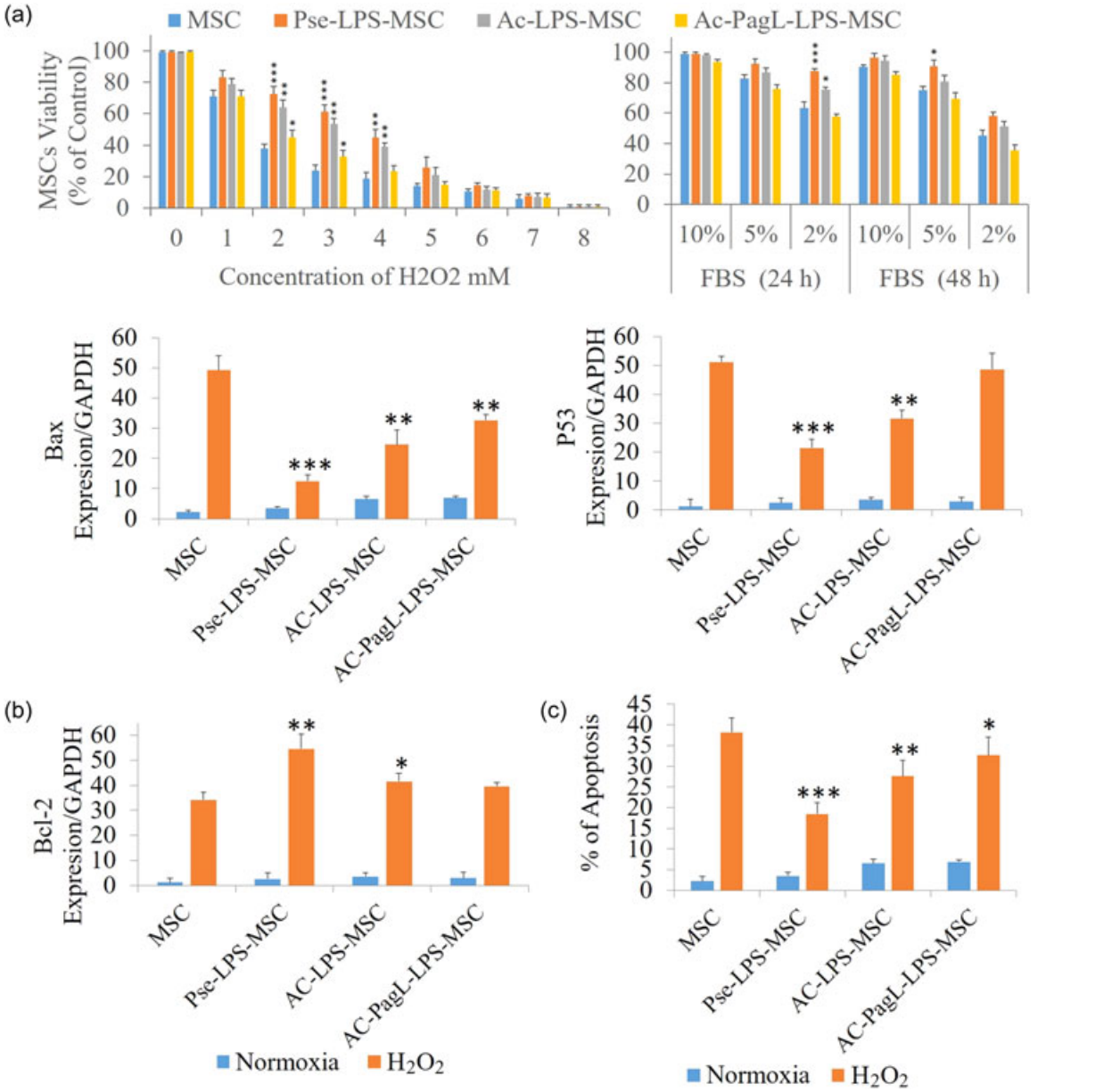

FIGURE 2 Cytotoxic and apoptotic assays of preconditioned MSCs. LPS preconditioning protects MSCs from H_2O_2/SD-induced apoptosis. (a) MSCs after appropriate LPS stimulation showed the obvious alterations by MTT assay. Data are presented as the mean±*SEM* of three separate experiments. Percentage of cell survival is defined as relative absorbance of treated versus nontreated cells. The *Y*-axis shows cells' viability and the *X*-axis the different H_2O_2 concentrations (mM) and the different time points after serum deprivation (hours). (b) Expression of p53, bax, and bcl-2 mRNAs was estimated with real-time PCR. All methods were used for assessment of apoptosis in H_2O_2-treated MSCs and LPS-MSCs after preconditioning. The *Y*-axis shows apoptosis gene expression relative to control and the *X*-axis the treated and untreated cells. (c) Effect of H_2O_2 on apoptosis of MSCs in normoxia and oxidative stress. After treatment of MSCs and LPS-MSCs with H_2O_2 for 24 hr. Apoptosis levels of the cells were measured with annexin V/PI staining and determined using flow cytometry. The proportion of apoptotic LPS-MSCs was lower than the MSCs. Data are presented as the mean ± standard deviation. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control (untreated cells). All samples was triplicate. Ac: *Acinetobacter baumannii*; LPS: lipopolysaccharide; mRNAs: messenger RNAs; MSCs: mesenchymal stem cells; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR: polymerase chain reaction; PI: propidium iodide; Pre: preconditioning; Pse: *Pseudomonas aeruginosa*; SEM: standard error of the mean [Color figure can be viewed at wileyonlinelibrary.com]

To perform the MTT assay, 1×10^4 preconditioned MSCs/well were seeded in a 96-well plate. After 24 hr, they were exposed to different concentrations of H_2O_2 (0, 0.5, 0.8, 1, 1.2, and 1.5 μM) and SD (10%, 5%, and 2% FBS percentages) for determining their sublethal doses in a triplicate pattern.

2.1.8 | Apoptotic assay

Analysis of genes expression by quantitative reverse transcription polymerase chain reaction analysis. Total RNA was extracted from nontreated MSCs and LPS preconditioned MSCs using CinnaPure-RNA

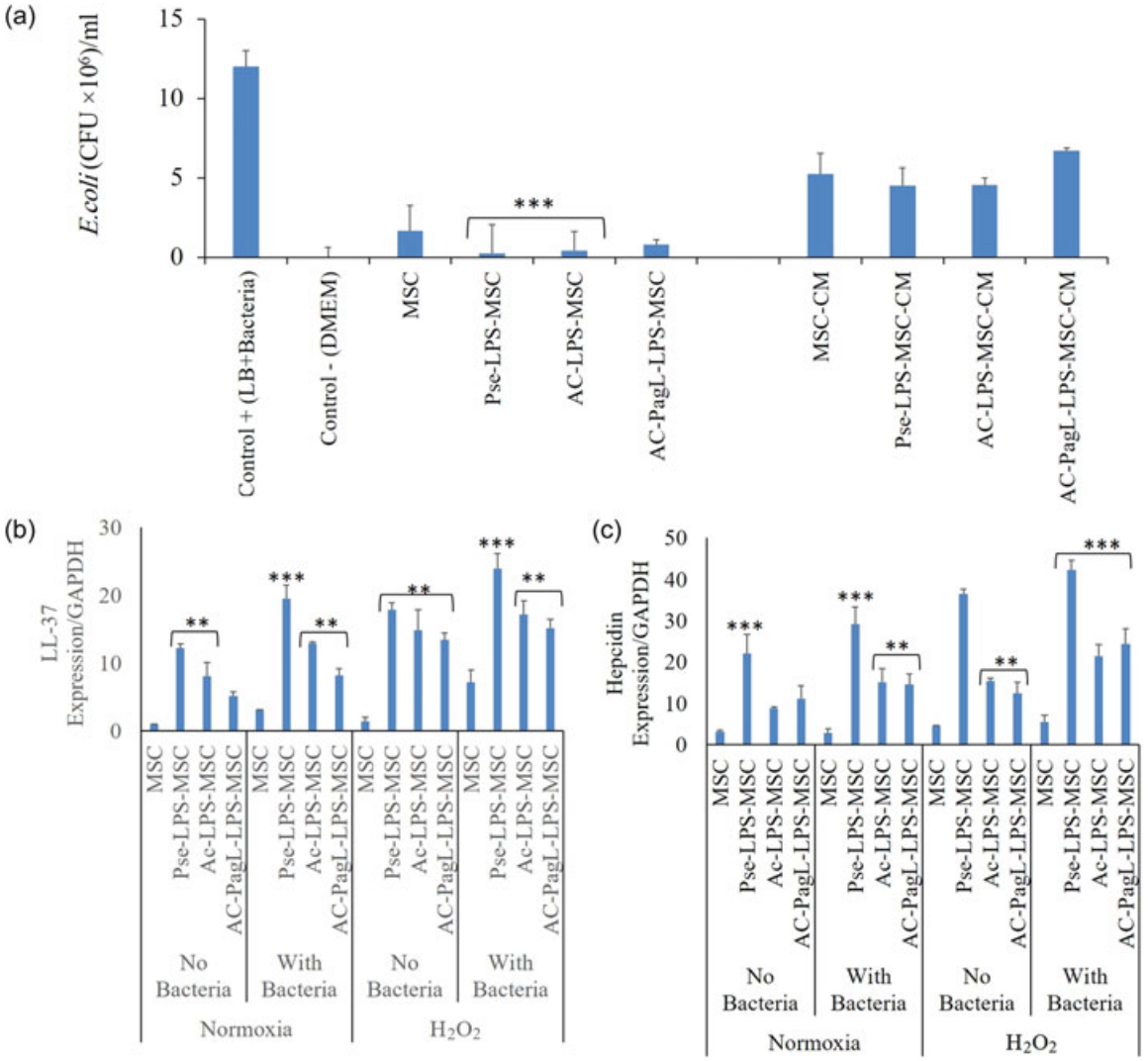

FIGURE 3 Antibacterial effects of MSCs and CMs was assessed by CFU counting and AMPs expression assessment. (a). Direct antibacterial activity. CM obtained from *E. coli*-infected-MSCs plated on blood agar and tested by CFU counting. Indirect antibacterial activity. Filtered CMs of MSCs was incubated with *E. coli* again, and their antimicrobial activity was estimated by total bacterial counts. (b). Relative expression of AMPs. The expression levels of hepcidin and LL-37 under normoxia and oxidative stress conditions in both stimulated and nonstimulated MSCs were determined by real-time PCR. Oxidative stress inhibits the relative expression of hepcidin and LL-37. The results were statistically significant compared with the control group (Bacteria or MSCs with bac.) ($p < 0.001$). CFU: colony-forming unit; CM: conditioned medium; MSCs: mesenchymal stem cells; PCR: polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

extraction kit (SinaClon, Tehran, Iran) according to the manufacturer's protocol. The concentration and purity of RNA were quantified with an ND-2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, MA); 260/280 nm absorbance ratios of 1.8–2.0 indicated a pure RNA sample. Complementary DNA (cDNA) synthesis was performed using 1 μg RNA template by using a reverse transcriptase kit AccuPowercycleScript RT PreMix (BIONEER, Alameda, CA) following manufacturing technical information. For evaluation of apoptosis induction after MSC preconditioning, real-time polymerase chain reaction analysis (PCR) was carried out using the different primers sequences of apoptosis genes (p53, bax, and bcl2; Supporting Information Table 1). It was also performed by utilizing SYBR Green PCR Super Mix (BioRad, California, CA) and conducted after the one-step reverse transcription (RT)-PCR procedure defined by the constructor.

Analysis of H_2O_2-induced apoptosis using annexin V/propidium iodide staining and flow cytometry. Cell viability was also evaluated after preconditioning using an Apoptosis Kit, following the manufacturer's instructions (BD Biosciences, New Jersey, NJ). Briefly, MSCs were cultured in six-well plates (10^6 cells/well), preconditioned with 1 μg/ml of all three LPSs (Pse-LPS, AC-LPS, and AC-pagL-LPS) for overnight, and treated with H_2O_2 (100, 150, 250, 500, 750, and 1,000 μM) for another night. After treatment, cells undergoing apoptosis were harvested using trypsin–EDTA and washed, resuspended in cold sterile PBS, and labeled with Annexin V and propidium iodide (PI) for 20 min. Afterward, the cells were analyzed by a flow cytometre (FC500; Beckman Coulter, Brea, CA) using FC500 MPL CXP2.1 software. All assays were run in at least three parallel repeated times.

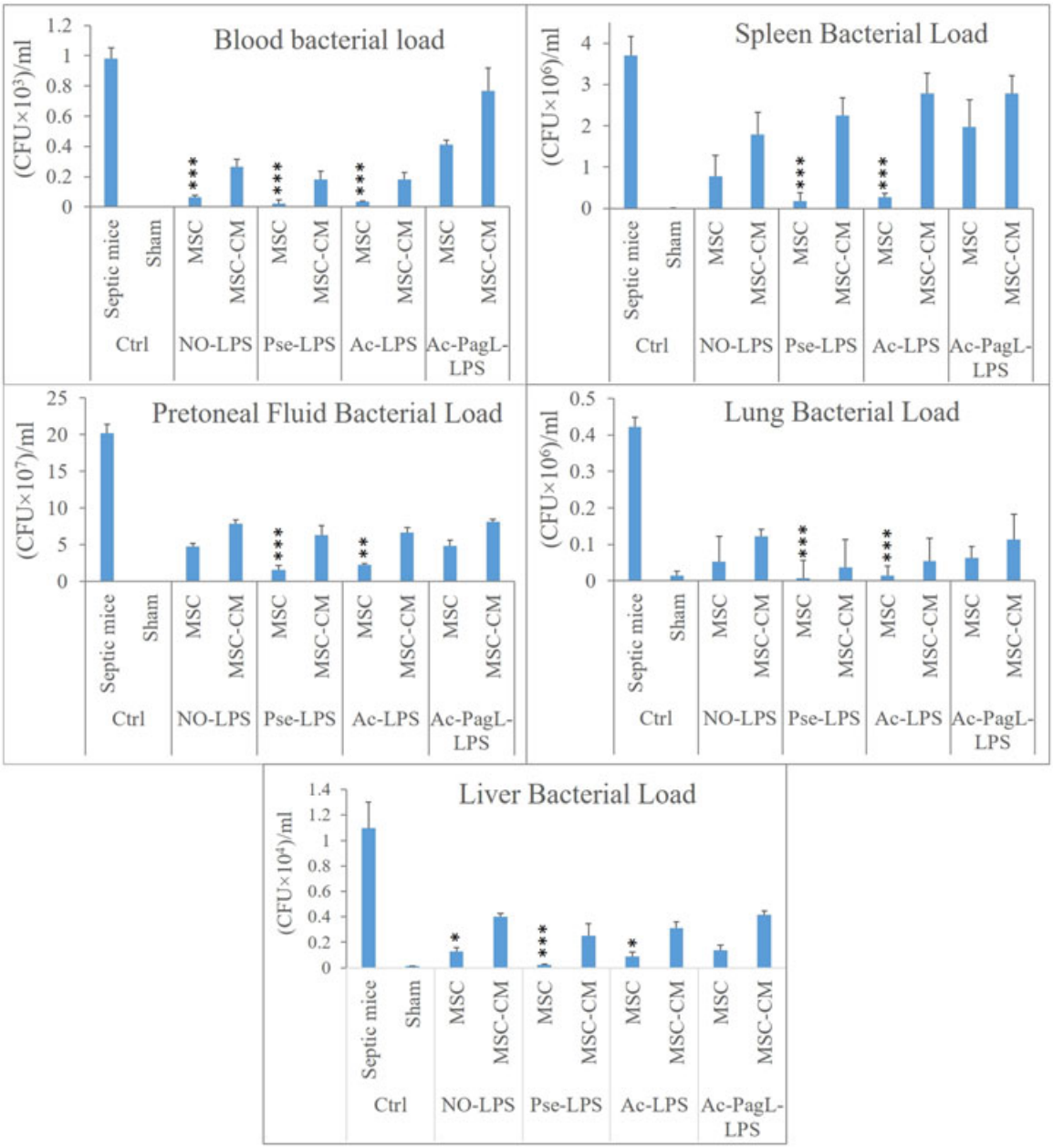

FIGURE 4 Preconditioned MSCs enhances bacterial clearance more than un-preconditioned-MSCs in sepsis. Administration of MSCs or preconditioned MSCs, 16 hr after *E. coli* instillation mainly reduced bacterial load in blood; spleen; peritoneal fluid; lung; and liver homogenates of mice 24 hr after treatment. Bacterial loads in each group were determined after incubating at 37°C overnight and were compared and expressed as CFUs per milliliter. For each groups $n = 10$, control (containing septic group and sham), MSC and CM group (NO-LPS), Pse-LPS-MSC and CM group, Ac-LPS-MSC and CM group and Ac-pagL-LPS-MSC and CM group). Mice treated with MSCs burdened fewer *E. coli* CFU compare with CMs in all 10 groups ($n = 10$ per groups). The *Y*-axis shows the number of CFUs/ml in each organ and the *X*-axis the bacterial load with MSCs and CMs treatment. Data are presented as the mean ± standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. CFU: colony-forming unit; CM: conditioned medium; MSCs: mesenchymal stem cells; PBS: phosphate-buffered saline [Color figure can be viewed at wileyonlinelibrary.com]

2.2 | Antibacterial activity assay

2.2.1 | Bacterial culture for antimicrobial assays

Escherichia coli was isolated from the blood of a patient with septicemia at the Medical Center of Baqiyatallah. First, the purity of inoculum was checked using gram staining, shape, biochemical tests, growth on selective media, and molecular level identification. Then, the pure *E. coli* cultured in LB broth medium overnight at 37°C. Before each experiment, the bacterial cell pellet of midlog-phase was washed once, resuspended in PBS, and adjusted to McFarland turbidity standards (as OD600 = 0.45 corresponds to 6×10^8 CFU *E. coli* /ml).

Assessment of antibacterial activity by culturing and colony-forming unit (CFU) counting. The antibacterial activity of MSCs and their

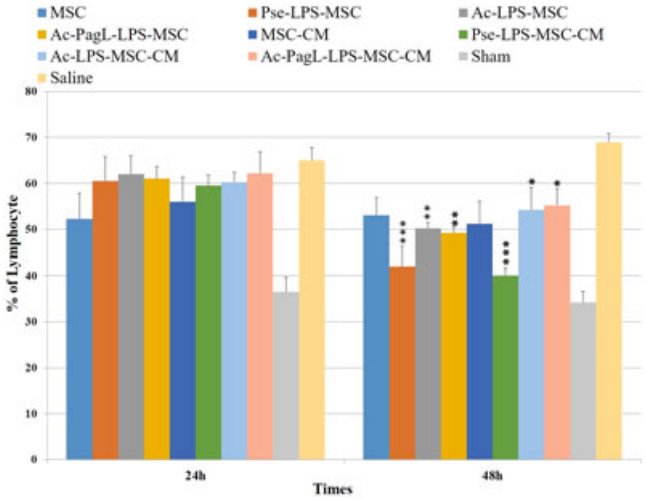

FIGURE 5 percentage of lymphocyte in the CBC test for identification of the hematological values in septic mice before and after treatment with preconditioned and nonpreconditioned MSCs and CMs. After sepsis lymphocyte was raised and when sepsis was treated % of lymphocyte was decreased. CBC: complete blood count; CM: conditioned medium; MSC: mesenchymal stem cell [Color figure can be viewed at wileyonlinelibrary.com]

condition mediums (CMs) was evaluated by two different methods: direct and indirect assay. The direct assessment of the bacterial growth inhibition of MSCs and CMs was determined by CFU counting. Briefly, 2×10^3 MSCs/well were seeded in 96-well plates overnight in DMEM without antibiotics supplemented with 10% FBS for 24 hr. Then, they were preconditioned with 1 μg/ml of three LPSs overnight again. Then, the suspension of 100 CFUs *E. coli*/ well was exposed to serial dilution of the supernatant of MSCs and LPS-MSCs, incubated overnight at 37°C. After 16 hr, the CMs were collected, serially diluted, and plated on blood-agar plates, and colonies were counted. The indirect evaluation of the antibacterial effects of CMs (MSC-CM and LPS-MSC-CM) was performed by filtering CMs through a 0.22-μm filter (F8148, Sigma, Jefferson City, MO) and centrifuging at high speed, and multiple freezing and thawing cycles to remove any contamination. The samples of CMs were assessed in both normoxia (5% CO_2) and oxidative stress (H_2O_2), and with or without bacterial stimulation. CMs were exposed to 100 CFUs *E. coli* per well; after 16 hr incubation at 37°C, CFUs were plated on blood agar and counted after 24 hr (Krasnodembskaya et al., 2010). The results are representative of parallels repeated experiments performed in triplicate.

Assessment of antibacterial activity by minimum inhibitory concentration and minimum bactericidal concentration determination. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of MSCs and CMs were assessed by the broth (Alcayaga-Miranda et al., 2015) and agar dilution methods (Pottumarthy, Fritsche, & Jones, 2006), respectively, with some changes. Broth dilution is a method used for determining the MIC. An equal volume (100 CFU *E. coli*/well) of bacterial suspensions in a 96-well plate was treated with serial dilution of the supernatant of LPS preconditioned MSCs and CMs, incubated overnight at 37°C. Finally, bacterial growth inhibition was evaluated in a microplate reader (Bio-Rad) at 600 nm. The MIC was considered as the concentration of CMs that completely suppressed the formation of bacterial colonies. All experiments were carried out in triplicate. Assessing MBC was specified by values of inhibition zone by MSCs and CMs. A standardized inoculum of *E. coli* was inoculated on the surface of Muller Hinton agar plate. Instantly, wells were made on the plate, the bottom of the wells was covered (with LB agar), and then the MIC of preconditioned MSCs and CMs were poured. After overnight incubation at 37°C, the diameter of minimum inhibition zones was precisely measured using a very precise ruler. MBC was determined by subculturing last clear MIC tube and inspecting for bacterial growth. The MIC and the zone diameter of inhibition have inverse correlations. The inhibition rates, specifying the means of at least three experiments, can be valued using equation as follows:

$$\%\ \text{Inhibition rate} = 100 \times \frac{(\text{OD treated} - \text{OD well})}{(\text{OD untreated} - \text{OD blank})}$$

Assessment of antibacterial peptide genes' expression by real-time PCR. The expression level of antibacterial peptides' genes, Hepcidin, and LL-37 (of Cathelicidin family) was evaluated by the real-time (quantitative) PCR normalizing with the gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping gene.

Total RNA from MSCs and LPS-MSCs were isolated as described before, cleaned up by treatment with RNase-free DNase, and quantified by nanodrop measurement at 260 nm. After cDNA synthesis from a certain quantity of total RNA, the optimum temperature (Tm) was obtained by PCR. Negative controls were included in each real-time PCR run using the Sybr green PCR master mix (Roche, Germany).

2.3 | In vivo experiments

The effect of preconditioned MSCs on the treatment of sepsis model of animals was compared, dividing 100 mice randomly into 10 groups: control +/− group, MSC group, Ac-LPS-MSC group, Ac-pagL-LPS-MSC group, Pse-LPS-MSC group, MSC-CM group, Ac-LPS-MSC-CM group, Ac-pagL-LPS-MSC-CM group, Pse-LPS-MSC-CM group. Each group contained 10 mice.

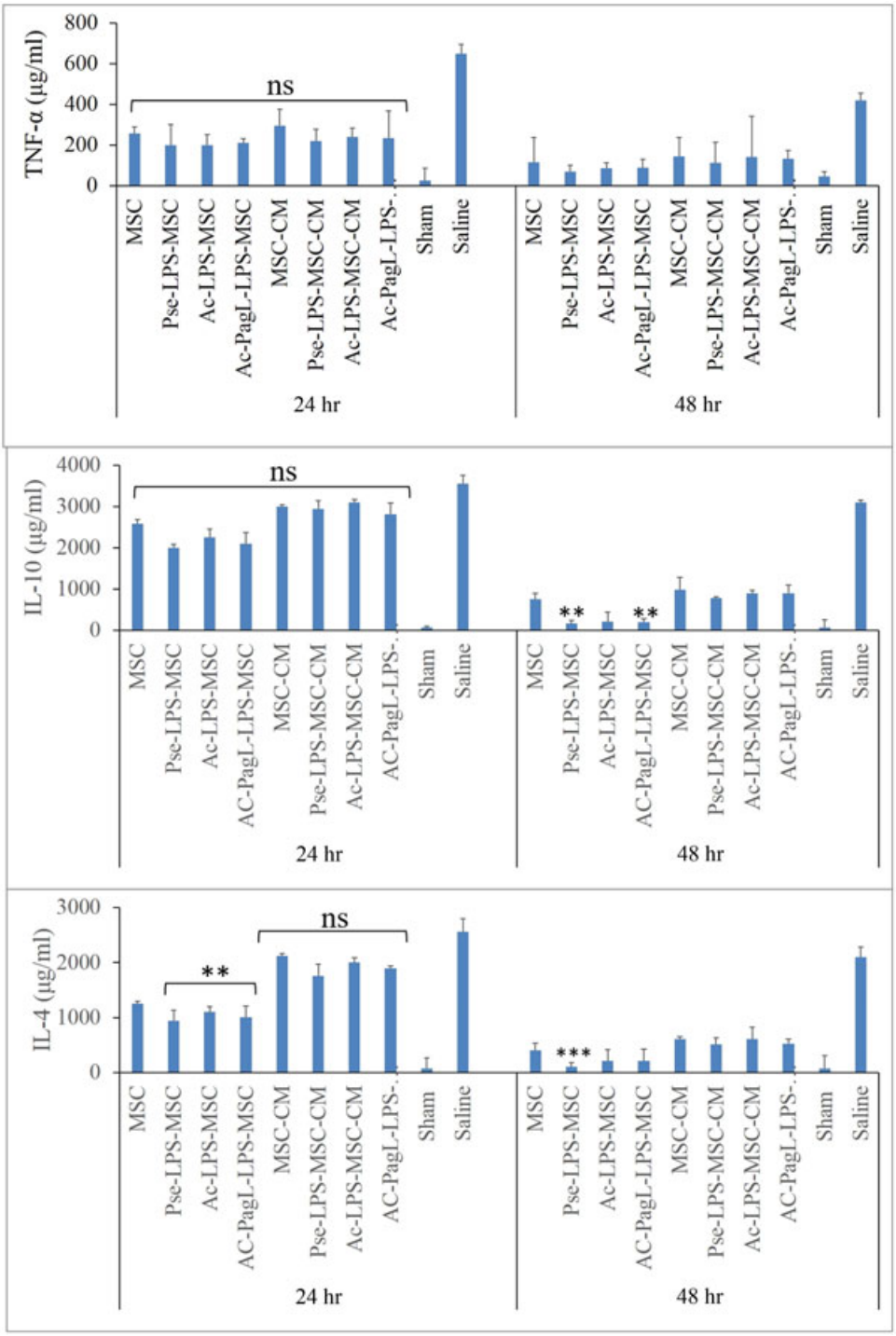

FIGURE 6 LPS-MSCs modulate more effectively the host immune response in sepsis models. (a) At 24 and 48 hr after sepsis induction and treatment, blood samples were collected to evaluate the serum concentration of the inflammatory cytokines IL-10, TNF-α, IL-6, and IL-4. (b) Quantification of mRNA expression of IL-6, IL-4, IL-10, and TNF-α by real-time RT-PCR in MSCs stimulated with LPS normalized relative to GAPDH. CM: conditioned medium; IL-10: interleukin 10; LPS: lipopolysaccharide; mRNA: messenger RNA; MSC: mesenchymal stem cell; RT-PCR: reverse transcription polymerase chain reaction; TNF-α: tumor necrosis factor [Color figure can be viewed at wileyonlinelibrary.com]

2.3.1 | Bacterial culture for sepsis induction

Live-bacteria infusion models of sepsis were induced; 10^7 CFU *E. coli* mice in the volume of 200 μl were instilled into the intraperitoneal (IP) of mice.

2.3.2 | MSC administration

In vitro cultured MSCs of passage 5 were used in this study, preconditioned as described before, then washed twice with PBS, (10^6 cells/mouse) suspended in 200 μl of PBS and injected via the tail

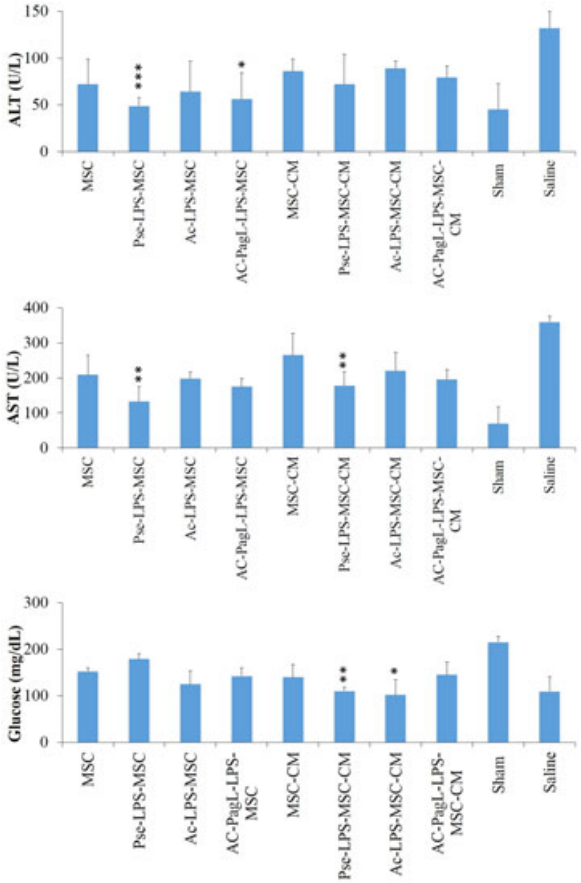

FIGURE 7 Biochemical analysis of serum. Treatment effect of preconditioned and nonpreconditioned MSCs and CMs on sepsis multiple organ dysfunction, using organ failure biomarkers, including ALT, AST, and glucose in plasma. Data are presented as the mean ± standard deviation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. PBS (control group, sepsis group), Sham (untreated group), MSCs (MSC group Pse-LPS-MSCs group: LPS purified from *Pseudomonas*, Ac-LPS-MSCs group: LPS purified from *Acinetobacter*, Ac-PagL-LPS-MSCs group: detoxified LPS Pagl). ALT: alanine aminotransferase; AST: aspartate aminotransferase; CM: conditioned medium; MSC: mesenchymal stem cell; PBS: phosphate-buffered saline [Color figure can be viewed at wileyonlinelibrary.com]

vein 16 hr after sepsis induction. The survival rate in septic mice, after MSC treatment, was evaluated every 24 hr for 28 days (with $n = 10$ in each group).

2.3.3 | Effect of MSC therapy on bacterial clearance in septic mice

Assessments of bacterial clearance were performed on blood and organ samples collected from mice killed 24 and 40 hr after MSC administration. To determine bacterial load in the peritoneum, 2 ml PBS was injected into the peritoneal cavity, and then recollected as IP fluid. Organs (liver, spleen, and lung) were homogenized in 1 ml PBS, the number of viable bacteria was determined by plating total blood, and 10-fold dilution of homogenized organs dissection was done on blood agar plates incubated at 37°C overnight.

A portion of blood was collected for complete blood count (CBC) test in an EDTA-containing tube for analysis of peripheral blood mononuclear cells. Another portion of the blood was centrifuged at 3,000*g* for 5 min to separate the serum and stored at −80°C until being used for determination of circulating cytokine levels.

Blood analyses by CBC test. A CBC is commonly performed as an initial test to emphasize any potential health problems, counting red and white blood cells and platelets. After induction of sepsis in mice, they received LPS-MSCs and MSCs and CMs, and their blood was analyzed after 24 and 48 hr.

2.3.4 | Serum samples preparation

Blood samples were collected from the inner canthus of mice eyes in eppendorf tubes allowed to clot for 2 hr at RT. Samples were spun at 3,000*g* for 5 min, and supernatant (serum) was collected and stored at −80°C until being used for biochemical and cytokine assays.

Serum analysis was performed to evaluate the effect of treatment with LPS-MSCs on the expression of cytokines in plasma. Forty hours after MSC administration, the obtained peripheral blood was allowed to clot for 2 hr at RT. The samples were spun at 3,000*g* for 5 min; the supernatant (serum) was collected and stored at −80°C until determination of circulating cytokine levels. The serum samples were assayed by enzyme-linked immunosorbent assay (ELISA) for interleukin (IL)-4, tumor necrosis factor-α (TNF-α), and IL-10.

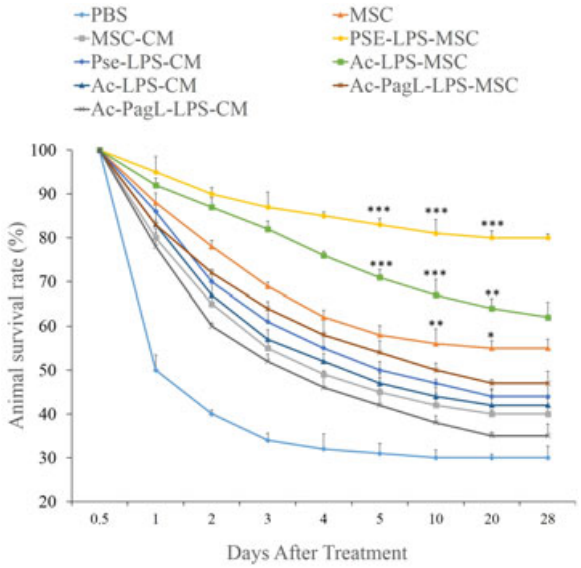

FIGURE 8 LPS preconditioning of MSCs promoted the survival rates of the sepsis model of mice. Survival was evaluated for 28 days, representing in Kaplan–Meier curves in each group. The septic animals treated with LPS-MSCs had lower mortality rates than that of with MSCs or PBS only (n = 10 mice/group). The Y-axis displays the percentage of animal survival rate and the X-axis the days after treatment. Data are presented as the mean ± standard deviation.; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control (PBS). PBS (control group, sepsis group), MSCs (MSC group Pse-LPS-MSCs group: LPS purified from *Pseudomonas*, Ac-LPS-MSCs group: LPS purified from *Acinetobacter*, Ac-PagL-LPS-MSCs group: detoxified LPS Pagl). LPS, lipopolysaccharide; MSCs, mesenchymal stem cells; PBS: phosphate-buffered saline [Color figure can be viewed at wileyonlinelibrary.com]

Serum cytokine analysis by ELISA. Sepsis treatment with MSCs reprograms immune cells, as well as macrophages and dendritic cells to produce more anti-inflammatory cytokines (IL-10, IL-4) and inhibit the production of proinflammatory cytokines (TNF-α, IL-6). The production of mentioned cytokines substantially would change in plasma in response to sepsis. The sandwich ELISA is a proper assay to quantify of cytokine levels in sera samples. The cytokines including IL-10 (mAb 2A5 and 16E3), TNF-α, (mAb MT1C8/23C9 and MT11B10), and IL-4 (mAb 11B11 and BVD6–24G2) were chosen in sera by ELISA kit (Mabtech AB, Nacka, Sweden), according to the manufacturer's instructions. Ninety-six-well plate was coated with coating buffer-containing antibodies and incubated overnight at 4°C. Free-binding sites on plates were blocked by blocking solution and incubated for 1 hr at RT. The plates were washed five times using washing buffer. Diluted samples or standard were added to the wells in triplicate and incubated 1–2 hr at RT. After washing five times, biotinylated detected antibodies against IL-10, TNF-α, and IL-4 diluted in PBS (1 μg/ml) were added to each well and incubated 1–2 hr at RT. Then again, after washing five times, diluted streptavidin–horseradish peroxidase (HRP; 1–2,000 in PBS) was added and incubated 30 min at RT. After the final wash, a colorimetric substrate was added to the wells until the solution color changed. Then, stop solution was added and absorbance was measured at 450 nm in an ELISA reader (Bio-Rad).

Serum biochemical analysis. After sepsis, multiorgan failure could be assessed by evaluation of biochemical indicators of hepatic, pancreatic, and renal function in serum samples taken 40 hr after treatment. In septic mice, increased plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glucose indicated pancreatic dysfunctions. After administration of MSCs, the plasma levels of these indicators were evaluated using the Piccolo Xpress™ Chemistry Analyzer (Abaxis, Union City, CA), according to the instructions of the manufacturer.

1. *Biochemical tests*. Because sepsis lethality is associated with organ dysfunction, biochemical indicators of organ (hepatic, pancreatic and renal) function were measured in serum samples, taken 40 hr after MSC treatment. In septic mice: increased plasma levels of indicators of kidney failure (blood urea nitrogen and serum creatinine); indicators of hepatic dysfunction (ALT, AST, and bilirubin); and pancreatic dysfunctions' indicator (amylase, albumin, and glucose) was determined. The plasma levels of these indicators were evaluated after MSCs administration, using the Piccolo Xpress™ Chemistry Analyzer (Abaxis; Alcayaga-Miranda et al., 2015).

2. *Cytokine assay*. A proper assay to quantify cytokine levels in sera samples is sandwich ELISA. Ninety-six-well plate was coated with coating buffer-containing antibodies and incubated overnight at 4°C. Free-binding sites on plates were blocked by blocking solution and incubated for 1 hr at RT. Plates were washed five times using washing buffer. Diluted samples or standard were added to the wells in triplicate and incubated 1–2 hr at RT. After washing five times, biotinylated detection antibodies (against IL-10, TNF-α, and IL-4) diluted in PBS (1 μg/ml) were added to each well and incubated 1–2 hr at RT. Then again, after washing five times, diluted streptavidin-HRP (1–2,000 in PBS) was added and incubated 30 min at RT. After the final wash, a colorimetric substrate was added to the wells until the solution color changed. Then, stop solution was added and absorbance was measured at 450 nm in an ELISA reader.

2.4 | Statistical analysis

Data were analyzed by standard statistical methods and analysis of variance followed by the Student–Newman–Keuls post hoc test for multiple comparisons or the nonparametric Kruskal–Wallis test. Group data were expressed as mean±standard error. $p \leq 0.05$ was considered as statistically significant. All tests were repeated in triplicate.

3 | RESULTS

3.1 | In vitro

3.1.1 | LPS extraction

LPS from *P. aeruginosa*, *A. baumannii*, and *A. baumannii* with inactivated lipid A were extracted and purified by two methods: extraction kit and hot-aqueous phenol extraction method according to the standard protocol with some modifications. Purified LPS was fractionated by the SDS-PAGE monitored by silver staining (Supporting Information Figure 1).

3.1.2 | Characterization of MSCs

Morphology surveillance, immunephenotype, and differentiation potentials indicated the characteristics of MSCs. Attached MSCs exhibited a fibroblast appearance and spindle-shaped morphology and were preserved through frequent passages, when assessed by an inverted microscope. In addition, cells were evaluated for expression of cluster of differentiation (CD) markers by flow cytometry, which verified that the cells were positive for CD105, CD44, and CD29 as standard MSCs surface antigens (with 95% purity), but negative for CD14, CD34, and CD45 as standard hematopoietic surface markers. Adipogenesis and osteogenesis of MSCs were carried out as termed before. Oil red O staining was used to dye lipid droplets adipocytes, and alizarin red S staining for osteocytes detected the mineralized matrix in cell culture (data not shown). Altogether, the results showed that the cells used in this study fulfilled the standard criteria for MSCs as determined by the ISCT.

3.1.3 | Identification of cytotoxic concentrations of endotoxins by the MTT assay

A major cause of MSC death is apoptosis. Considering the cytotoxic effect of endotoxins, MSCs were treated with different doses of all three LPS types (0, 0.5, 0.8, 1, 1.2, 1.4, 1.8, 2, 3, 4, and 10 μg/ml) for 24 hr in a triplicate pattern. TB and MTT assays were used to estimating the LPS cytotoxicity and MSCs viability. Exposure to low doses of LPS increased the cell viability, while treatment of MSCs with 2 μg/ml LPS led to a great reduction in cell viability. LPS pretreatment with 2 μg/ml induced apoptosis (lethal dose), while preconditioning with 1 μg/ml LPS not only improved MSCs viability, but also inhibited apoptosis ($p < 0.01$). The most effective cytoprotective dose of LPS for preconditioning of MSCs was selected 1 μg/ml (Supporting Information Figure 2). The MTT results were representative of three independent experiments and similar to that of the TB assay.

3.1.4 | Characterization of LPS-MSCs

Isolated MSCs from bone marrow after three passages were pretreated with 1 μg/ml LPS for 24 hr, and then the characteristics of LPS-MSCs were evaluated. LPS-MSCs, while keeping their plastic adherence property, exhibited a spindle shape and retained the fibroblastic morphology over several passages as before preconditioning (Figure 1a). For further evaluation of the LPS-MSCs, multipotency of preconditioned MSCs was confirmed by successful differentiation of MSCs into adipocytes and osteoblasts (Figure 1b). Also, for assessment of LPS-MSCs immunophenotype, by flow cytometry analysis, the expression of cell-surface markers was established as positive for CD29, CD105, and CD44, while negative for CD45, CD34, and CD14 (Figure 1c). These results confirm that LPS-MSCs are capable of retaining their properties similarly as MSCs.

3.1.5 | Cytotoxic assay of MSCs

MSCs viability (by TB and MTT assay). Appropriate doses of LPS promote the viability of MSCs by increasing metabolic activity in harsh conditions that can be imitated by H_2O_2/SD-induced toxicities/apoptosis. To inspect the effect of endotoxins on MSCs' apoptosis, the MSCs and LPS-MSCs were exposed to various concentrations of H_2O_2 or SD and then measured by the TB and MTT assay in triplicate. After treatment of MSCs with 1–8 mM H_2O_2 for 24 hr, the proportion of apoptotic LPS-MSCs was considerably lower than the MSCs at 1 mM H_2O_2. Just in term of exposure of the cells to SD conditions for 48 hr, the number of apoptotic LPS-MCSs was again lower than the number of apoptotic MCSs. Hence, LPS preconditioning led to much more metabolic activity (or much less apoptotic activity; Figure 2a).

Analysis of apoptotic gene expression by real-time PCR. Total RNA was isolated from MSCs and LPS-MSCs using an RNA extraction kit, and the concentration of RNA was determined by a nanodrop. The RNA concentrations of MSC and LPS-MSC have been provided in Supporting Information Table 1. For cDNA synthesis, 1 μg of RNA reversely was transcribed with random-hexamer primer using an RT PreMix kit. The apoptosis genes (p53, bax, and bcl-2) expression was measured with RT-PCR and real-time PCR and primers sequences can be seen in Supporting Information Table 2. This step was applied for evaluation of apoptosis after MSCs preconditioning (Figure 2b).

Flow cytometric analysis of apoptosis. The prominent cause of MSCs death is apoptosis. To study the MSCs' apoptosis, the MSCs and LPS-MSCs were treated with H_2O_2. MSCs, and LPS-MSCs were exposed to several concentrations of H_2O_2 (μM) as described earlier, and cell viability was analyzed by annexin V and PI staining and flow cytometry. LPS-primed-MSCs exhibit a significant viability and lower apoptosis than MSCs (Figure 2c).

3.1.6 | Antibacterial activity assay

LPS-MSCs inhibit in vitro bacterial growth. Direct and indirect methods were used to evaluate the in vitro antibacterial activity of the MSCs and CMs as described previously (Dominici et al., 2006). The normoxia/oxidative stress groups of MSCs and LPS-MSCs in 96-well plates were infected with *E. coli* and incubated for 16 hr. In the direct assay, serial dilutions of CMs were plated on blood agar, and CFUs were counted (Figure 3a). After evaluating the indirect antimicrobial activity of CMs, they were filtered and then centrifuged at high speeds and freezed and thawed several time. Aliquots of the CM samples were dispensed to a 96-well plate, inoculated with 100 CFU *E. coli*/well, and incubated for 16 hr. CFUs were then plated and counted. LPS stimulation significantly improved the antibacterial activity of MSCs compared with unstimulated MSCs and control medium (DMEM).

MIC and MBC were assessed through broth and agar dilution methods as described earlier (Figure 3a). As a result, we can claim that MSCs further inhibit bacterial growth than CMs; however, the LPS preconditioned ones exhibit more antibacterial activity (Supporting Information Table 3).

Evaluation of hepcidin and LL-37 gene expression in preconditioned MSCs by real-time PCR. Real-time PCR was performed on cDNA of LPS-MSCs and MSCs to quantify the expression of AMPs under basal conditions and toxin stimulation. Hepcidin and LL-37 genes' expression were evaluated relative to the gene GAPDH as an internal control. LPS stimulation of MSCs enhances the levels of hepcidin and LL-37 mRNA expression. It is known that oxidative stress condition affects the expression of AMPs. Therefore, upregulation of AMPs by the oxidative stress condition causes LPS-stimulated-MSCs to express more AMPs than nonstimulated (Figure 3b). These consequences propose that MSCs, after stimulation by LPS, produce some paracrine molecules such as hepcidin and LL-37, which intervene the antibacterial effects of MSCs.

3.2 | In vivo

3.2.1 | Treatment with LPS-preconditioned MSCs enhances bacterial clearance in septic mice

MSC treatment limits bacterial load in septic mice. The contribution of MSC treatment to clear bacterial infection was assessed by examining bacterial load in organs and blood, after MSC administration (Figure 4). Organ and blood samples were taken 24 hr after sepsis induction and cultured on blood agar plates. The bacterial colonies were counted after 24 hr of incubation. The number of CFUs was high in organs of septic mice before treatment. MSCs treated with LPS exhibited much lower organ and blood bacterial load. Taken together, the results of the data set are statistically significant.

3.2.2 | Serum and blood analyses

CBC test. CBC test was conducted to count blood cells and components. Preconditioned and nonpreconditioned MSCs and CMs were administered on septic mice, and after 24 and 48 hr, mice blood was examined (Figure 5).

The effect of MSCs' treatment on the expression of cytokines in plasma (cytokine assay by ELISA). Cytokine assays were performed using sandwich ELISA for determining the levels of IL-10 (mAb 2A5 and 16E3), TNF-α (mAb MT1C8/23C9 and MT11B10) and IL-4 (mAb 11B11 and BVD6–24G2) in sera using an ELISA kit (Mabtech AB) according to the manufacturer's instructions. Treatment with LPS-MSCs at 24 and 48 hr considerably reduced the levels of proinflammatory cytokines, IL-4, and TNF-α in plasma compared with the sepsis group. However, the level of anti-inflammatory cytokine IL-10 was significantly increased compared with that in the sepsis group. MSC treatment downregulates the inflammatory responses in vivo (Figure 6).

Biochemical tests. Biochemical indicators of hepatic, pancreatic, and renal function accompanying multiple organ failure due to sepsis were determined by evaluating serum samples. As the results indicated, MSCs treatment reduced plasma levels of AST and ALT in septic mice compared with positive and negative controls, while a slight increase in blood glucose was sensed by MSCs administration. Overall, the alterations in the biochemical indicators of organ function indicated reduction in organ damages (Figure 7).

LPS-preconditioned-MSCs improve survival in the E. coli septic mouse models. The therapeutic effects of MSCs alone or in combination with antibiotics on septic animal survival have been known for decades. Hence, we inspected the possibility of LPS-preconditioned-MSCs being more effective in sepsis treatment. Over 28 days, just 20% of the septic group treated with only PBS and 55% of those treated with MSCs survived. While the survival rate of animals treated with LPS-MSC was 60%–80%. Animals treated with LPS-MSCs showed a very lower mortality rate (90%–95% survival) after 48 hr compared with the MSC-treated (78% survival) and PBS-treated (40% survival) groups. Overall, the survival rates of septic mice receiving LPS-MSCs were improved with statistical significance compared with those receiving MSCs or PBS only (Figure 8).

4 | DISCUSSION

Sepsis corresponds to a sudden flow of systemic signaling factors, as well as cytokines and growth factors that might be uncontrollable (Bosmann & Ward, 2013; Sagy, Al-Qaqaa, & Kim, 2013). MSCs, perfectly depicted as multipotent stromal cells, have regenerative properties and can fight systemic infection in bacterial sepsis by modulating the immune response to the disease. MSCs are major histocompatibility complex II (MHCII)-negative and capable of lowering mortality by modifying several traits of sepsis, for instance, modulation of the inflammatory response without severe immunosuppression, and promotion of bacterial clearance, the organ dysfunction, almost comparable with that of the conventional (antibiotic) therapy of sepsis. Because in acute life-threatening symptoms, such as sepsis, isolation, and proliferation of autologous MSCs are not accessible, allogeneic MSCs therapy is a preferred

source for treatments (Gu et al., 2014). It allows a clinical-scale allogeneic MSCs transplantation that is easy-to-use and off-the-shelf products, in less time (Lalu et al., 2016). Regardless of its advantages, MSCs therapy has been restrained by low survival ability and death due to the hostile environment at the site of damage; so, designing an operative strategy for effective MSCs transplantation is crucial.

Nowadays, LPS preconditioning of MSCs has become an interesting therapeutic approach for the treatment of inflammatory diseases. MSCs release vast supplies of cytokines and transcription factors to switch bio information within nearby cells, preserving a dynamic and homeostatic microenvironment for tissue repair, which is thoroughly related to endotoxin cross-tolerance. LPS preconditioning slightly reprograms MSCs and drastically improves the paracrine protective effects and regenerative and repair properties of MSCs (Hou et al., 2015; Saparov et al., 2016). Considering paracrine effects as the main therapeutic effect of MSCs, we experienced (pre) MSCs' CM (cell free) as an alternative to direct cell delivery for disease treatment. In this context, LPS preMSCs present some advantages over MSCs CM and MSCs alone, such as more resistance in harsh environment that allow a tolerance when re-exposed to LPS in septic conditions.

Estimating the cytotoxic effect of endotoxins on MSCs revealed that MSCs pretreatment with 2 μg/ml LPS had a great cytotoxic effect while exposure of low doses (1 μg/ml) of LPS not only increased MSCs viability, but also inhibited apoptosis. Our results are in agreement with the results of He et al. (2016), in that LPS significantly improves MSC viability at the concentration of 1 μg/ml.

In terms of characterization of MSCs after LPS preconditioning, all LPS preMSCs used in this study met the standard criteria of ISCT and retained plastic adherence, spindle-shaped morphology, expression of typical MSCs markers, and differentiation as before preconditioning. These results confirm that MSCs do not lose their stemness properties after preconditioning with LPS.

As hypothesized, our experiments demonstrated that LPS preconditioning protects MSCs from H_2O_2/SD-induced apoptosis. After appropriate LPS stimulation, MSCs apoptosis showed obvious alterations. The proportion of apoptotic LPS-MSCs (except PagL-LPS-MSCs) was considerably lower than the MSCs in H_2O_2/SD conditions. The rate of preconditioned-MSCs' viability relative to control revealed that Pse-LPS is the most helpful in refining the MSCs. Then, preconditioning with Ac-LPS can help somewhat, and at last Ac-PagL-LPS does not seem to play a significant role in making MSCs more viable. Therefore, this test revealed that LPS preconditioning leads to significant cell viability (much less apoptotic activity) and much more proliferation (metabolic activity), which results in MSCs resistance. This outcome confirms previous findings (He et al., 2016; J. Wang et al., 2013) and also is in complete agreement with the results of Hou et al. (2015).

Apoptosis assay showed that in oxidative stress conditions, the death percentage of MSCs preconditioned with Pse-LPS is almost half the control, Ac-LPS about 20% and Ac-PagL-LPS 10% of control. However, the percentage of apoptosis of LPS-stimulated MSCs (LPS-MSCs) is very similar to control in normoxia.

In an apoptosis test by assessment of apoptotic genes' expression, it was determined that the expression of proapoptotic genes (Bax and P53) decreased and antiapoptotic gene (Bcl2) increased in successfully preconditioned cells. In detail, Bax and P53 expressions were reduced in MSCs treated by Pse-LPS (five- and twofold), Ac-LPS (2- and 1.5-fold), and Ac-PagL-LPS (1- and 0-fold), respectively. The expression of Bcl2 was increased in cells preconditioned with Pse-LPS (1.5-fold), Ac-LPS (onefold), and Ac-PagL-LPS (0-fold).

The antibacterial activities of nonstimulated MSCs, LPS-MSCs, and CMs were compared in vitro. However, nonpreconditioned MSCs show the antibacterial activity for AMPs expression as proposed by Krasnodembskaya et al. (2010). The evidence we found points to higher antibacterial activity of preconditioned MSCs. Obviously, Pse-LPS-MSCs and Ac-LPS-MSCs showed more significant inhibition of bacterial growth than MSCs alone, PagL-LPS-MSCs, and even CMs. In general, samples including cells (MSCs) were more effective antibacterial than CMs.

The results of the antibacterial assay designated the effect of LPS preconditioning on antibacterial activity of MSCs. The most effective LPS, to improve the antibacterial traits of MSCs, is related to Pse, then Ac, and at last Ac-PagL. However, Ac-PagL-MSCs showed antibacterial activity rather than MSCs. Hence, we cannot decisively claim that Ac-PagL contributes less in MSCs refining their viability. These results were confirmed by assessment of AMPs (hepcidin and LL-37) gene expression. In general, the expression of AMPs in oxidative stress conditions and the presence of bacteria increased significantly than that of normoxia and lack of bacteria. Again, the order of efficiency in stimulating AMPs' gene expression is Pse-LPS, Ac-LPS, and Ac-PagL-LPS.

In the evaluation of the presence of bacteria in organs of septic mice after MSCs' transplantation, most bacteria were found in peritoneal fluid and the least in blood. After treatment, in all organs, MSCs could decrease the bacterial load in order in blood, liver, lung, spleen, and, at last, in peritoneal fluid. Generally, sepsis treatment with MSCs can reduce the bacterial load; the most effective are Pse-MSC and Ac-MSC (equally), and the least effective is Ac-PagL-MSC. Preconditioning by the CMs is also helpful, but not as much as MSCs. Unlike in previous studies, where CMs were more effective than MSCs in sepsis treatment, we consider MSCs more helpful than CMs.

In line with other research works, MSCs are capable of clearing bacterial load in animal organs (Zhu et al., 2017), but interestingly, preconditioned MSC treatment further enhances bacterial clearance in septic mice. Typically, before treatment, the bacterial load (number of CFUs) was high in the blood and organs of septic mice. Treatment with LPS-MSCs (except PagL-LPS-MSCs) exhibited much lower organ and blood bacterial load. Taken together, these results show that mice treated with LPS-MSCs burdened fewer *E. coli* CFU compared with CMs in all groups.

The most remarkable result to emerge from the data is that LPS-preconditioned-MSCs improve survival in the *E. coli* septic mouse models. The survival rates of septic mice receiving LPS-MSCs for 28 days were increased with statistical significance compared with that of receiving MSCs or PBS only. The septic animals treated with

LPS-MSCs (except PagL-LPS-MSCs) had lower mortality rates than that of with MSCs (Zhu et al., 2017), CMs, or PBS only. Contrary to expectations, the results of PagL-modified LPS were not satisfactory.

Foregoing studies revealed that, in addition to immune cells, MSCs express TLR4, which is pivotal in regulating the function and the fate decision of MSCs by inducing proinflammatory or immunosuppressive effects (Najar, Krayem, Meuleman, Bron, & Lagneaux, 2017). LPS targeting TLR4 on the surface of MSCs triggers signaling pathways, and stimulates the production of some mediators that switch inflammatory cytokines' profile of MSCs in vitro and in vivo, involved in cell differentiation, migration, apoptosis, proliferation, and survival (Roger et al., 2009). The suppressive shifts of these inflammatory components potentially induce MSCs protection in vitro against next re-exposure to LPS in vivo, which might be promising for LPS-primed-MSCs therapeutic effects. In detailed, in vitro LPS priming of MSCs reprograms TLR4 results in ligand-specific shift of the inflammatory balance, activating both MyD88-dependent and independent signaling pathways, which downregulates proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling and upregulates anti-inflammatory interferon regulatory factor 3 (IRF3) signaling, respectively. It is striking that the balance between the NF-κB signaling and the IRF3 activity is largely ligand dependent. Moreover, LPS may protect MSCs from stresses-induced apoptosis through the TLR4 and PI3K/Akt signaling pathway In this experiment, the preconditioning of MSCs with LPS has been performed in serum-free medium. Because, FBS contains soluble factors that can induce MyD88 independent (anti-inflammatory) signaling pathway (Chiossone et al., 2016; Zeuner, Patel, Denecke, Giebel, & Widera, 2016).

As might have been expected, our findings were often contradictory for PagL-modified LPS. The diversity of LPS structures can alter TLR4-mediated responses, means different LPS ligands induce different downstream signaling cascades and could trigger diverse autocrine effects in MSCs. The LPS of *A. baumannii* is an actual potent stimulator of the immune response. In this experiment, the PagL gene (PhoP-activated gene) from *Psedumonas aerogenosa*, which encodes a lipid A 3-deacylase (an LPS-modifying enzyme), was expressed in *A. baumannii*. It is most likely valuable tools for detoxification of LPS by deacylating of lipid A by PagL (Badmasti et al., 2015; Chen et al., 2014).

Such modifications of lipid A reduces the immunogenicity of LPS and makes it poor stimulator of the TLR4-mediated signaling pathway, altering the nature of downstream cytokine production differentially. MSCs may gain anti-inflammatory trait, if they stimulate by inflammatory agents and contrariwise. The assumption that PagL-modified LPS molecules might most likely provide the prospect to develop MSCs viability did not proof by the results. Actually, comparing the effects of LPS with activated or inactivated lipid A (PagL) on the survival of MSCs shows that lipid A is an important part of LPS in this process.

The current study does not support previous research in the issue of the antibacterial effects of MSCs and CMs. Former research indicated that CMs superior to MSCs in antibacterial effects (Chen et al., 2014; Saparov et al., 2016). After 24 hr, LPS preconditioned MSCs washed away and incubated with fresh medium for another 24 hr. and CM was collected. However, it seems most of LPSs removed; CM is not completely free of LPS. The little LPS remaining in the CM may effect independent of paracrine factors of MSCs. Therefore, LPS-CMs did not show significant antibacterial effects. Hence, it has made CM inferior instead of superior to MSC.

We compared the survival of MSCs preconditioned with PagL-modified LPS and wild-type LPS of *A. baumannii*. As expected, in our in vitro and in vivo studies survival of Ac-LPS-MSCs efficiently were superior than PagL-LPS-MSCs. Furthermore, we put forward the notion of determining the different LPSs endotoxic activity by evaluating the production of the anti-/proinflammatory cytokine in MSCs preconditioned with Ac-LPS and PagL-LPS by real-time PCR. The results indicated that PagL-LPS did not show endotoxic activity because it could not stimulate MSCs to produce more anti and less proinflammatory cytokines as it can recognize by TLR-4 on the surface of MSCs.

Cytokine assay after MSCs' transplantation revealed the more anti-inflammatory and less proinflammatory cytokines' production. The best result is regarding again to Pse-MSC, but the next is Ac-PagL-MSC and the last Ac-MSC.

The results of biochemical tests displayed the significant lower levels of ALT and AST and higher quantity of glucose in mice serum after treatment. The most efficient MSCs were Pse-MSCs, Ac-PagL-MSCs, Ac-MSCs, and then MSCs.

Inflammatory reaction causes the reduction of red blood counts and the increase of white blood counts in sepsis. Hence, after MSC and LPS-MSC application, the CBC test was accomplished to achieve the lymphocytes' percentage to see whether therapy has been occurred or not. As expected, evaluation of lymphocytes' percentage in the treated animals exhibited the success and effectiveness of the preconditioning process.

These findings validate the usefulness of LPS-primed-MSCs as a more effective therapeutic tool for sepsis treatment. Despite high translational potential of the study, some limitations and ambiguities need to be resolved in future. First, the fate of LPS-treated MSCs remains vague. Second, how MSCs maintain their multipotency and can differentiate in harsh conditions. Then, the safety of the MSC therapy not ascertained.

Despite very promising, the indication is still undefined whether MSC therapy is superior to conventional treatments. In addition, there are several concerns, which will require more considerations on further trials such as the MSCs immunogenicity, long-term adverse effects of immune suppression, and prospective risks of tumorigenic transformation, and unsolicited MSCs lineage's differentiation. Overall, we still look forward to scientific evidence for most of the risk factors.

5 | CONCLUSION

This study provides evidence that MSCs survived better after sublethal exposure to LPS, by substantially increasing the resistance

of preMSCs to apoptotic and other different insults. This study, involving an in vivo surveillance, has merely confirmed that pretreatment of MSCs with a low dose of LPS actively moderates cellular immune responsiveness to organ damage, attenuates the sepsis squeal and is associated with reduces cellular infiltration and activation in the blood more than MSCs alone.

In conclusion, we found that being more resistant; LPS preMSCs have improved regulatory properties for the amelioration of inflammation. Moreover, antibacterial activity of LPS preMSCs can concurrently activate feedback inhibitory mechanisms restrict the intensity of inflammatory responses. Therefore, preMSCs could be a very promising tool for sepsis treatment.

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REFERENCES

Alcayaga-Miranda, F., Cuenca, J., Martin, A., Contreras, L., Figueroa, F. E., & Khoury, M. (2015). Combination therapy of menstrual derived mesenchymal stem cells and antibiotics ameliorates survival in sepsis. *Stem Cell Research & Therapy*, *6*(1), 199.

Amiri, F., Jahanian-Najafabadi, A., & Roudkenar, M. H. (2015). In vitro augmentation of mesenchymal stem cells viability in stressful microenvironments: In vitro augmentation of mesenchymal stem cells viability. *Cell Stress & Chaperones*, *20*(2), 237–251.

Badmasti, F., Shahcheraghi, F., Siadat, S. D., Bouzari, S., Ajdary, S., Amin, M., … Imani Fooladi, A. A. (2015). Heterologous expression of 3-O-deacylase in *Acinetobacter baumannii* modulates the endotoxicity of lipopolysaccharide. *Journal of Molecular Microbiology and Biotechnology*, *25*(1), 37–44.

Bosmann, M., & Ward, P. A. (2013). The inflammatory response in sepsis. *Trends in Immunology*, *34*(3), 129–136.

Cassini, A., Plachouras, D., Eckmanns, T., Abu sin, M., Blank, H.-P., Ducomble, T., … Suetens, C. (2016). Burden of six healthcare-associated infections on European population health: Estimating Incidence-based disability-adjusted life years through a population prevalence-based modelling study. *PLoS Medicine*, *13*(10), e1002150.

Chen, L., Xu, Y., Zhao, J., Zhang, Z., Yang, R., Xie, J., … Qi, S. (2014). Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. *PLoS One*, *9*(4), e96161.

Chiossone, L., Conte, R., Spaggiari, G. M., Serra, M., Romei, C., Bellora, F., … Bottino, C. (2016). Mesenchymal stromal cells induce peculiar alternatively activated macrophages capable of dampening both innate and adaptive immune responses. *Stem Cells*, *34*(7), 1909–1921.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., … Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, *8*(4), 315–317.

Gnecchi, M., Danieli, P., Malpasso, G., & Ciuffreda, M. C. (2016). Paracrine mechanisms of mesenchymal stem cells in tissue repair. *Mesenchymal Stem Cells: Methods and Protocols*, *1416*, 123–146.

Gu, L. H., Zhang, T. T., Li, Y., Yan, H. J., Qi, H., & Li, F. R. (2014). Immunogenicity of allogeneic mesenchymal stem cells transplanted via different routes in diabetic rats. *Cellular & Molecular Immunology*, *12*, 444–455.

Kooshki, H., Ghollasi, M., & Halabian, R. (2018). Osteogenic differentiation of pre-conditioned bone marrow mesenchymal stem cells with lipopolysaccharide on modified poly-L-lactic-acid nanofibers. *Journal of Cellular Physiology*, 1–11.

He, X., Wang, H., Jin, T., Xu, Y., Mei, L., & Yang, J. (2016). TLR4 activation promotes bone marrow MSC proliferation and osteogenic differentiation via Wnt3a and Wnt5a signaling. *PLoS One*, *11*(3), e0149876.

Hou, Y. S., Liu, L. Y., Chai, J. K., Yu, Y. H., Duan, H. J., Hu, Q., … MA, L. (2015). Lipopolysaccharide pretreatment inhibits LPS-induced human umbilical cord mesenchymal stem cell apoptosis via upregulating the expression of cellular FLICE-inhibitory protein. *Molecular Medicine Reports*, *12*(2), 2521–2528.

Johnson, C. L., Soeder, Y., & Dahlke, M. H. (2017). Concise review: Mesenchymal stromal cell-based approaches for the treatment of acute respiratory distress and sepsis syndromes. *Stem Cells Translational Medicine*, *6*, 1141–1151.

Kanashiro, A., Sônego, F., Ferreira, R. G., Castanheira, F. V. S., Leite, C. A., Borges, V. F., … Cunha, F. Q. (2017). Therapeutic potential and limitations of cholinergic anti-inflammatory pathway in sepsis. *Pharmacological Research*, *117*, 1–8.

Krasnodembskaya, A., Song, Y., Fang, X., Gupta, N., Serikov, V., Lee, J. W., & Matthay, M. A. (2010). Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells*, *28*(12), 2229–2238.

Lalu, M. M., Sullivan, K. J., Mei, S. H., Moher, D., Straus, A., Fergusson, D. A., … McIntyre, L. (2016). Evaluating mesenchymal stem cell therapy for sepsis with preclinical meta-analyses prior to initiating a first-in-human trial. *eLife*, *5*, e17850.

Liles, W. C., Matthay, M. A., Dos santos, C. C., Weiss, D. J., & Stewart, D. J. (2014). Mesenchymal stromal (stem) cell therapy: An emerging immunomodulatory strategy for the adjunctive treatment of sepsis/reply. *American Journal of Respiratory and Critical Care Medicine*, *189*(3), 363–364.

Liu, X. B., Wang, J. A., Ji, X. Y., Yu, S. P., & Wei, L. (2014). Preconditioning of bone marrow mesenchymal stem cells by prolyl hydroxylase inhibition enhances cell survival and angiogenesis in vitro and after transplantation into the ischemic heart of rats. *Stem Cell Research & Therapy*, *5*, 5.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55–63.

Najar, M., Krayem, M., Meuleman, N., Bron, D., & Lagneaux, L. (2017). Mesenchymal stromal cells and toll-like receptor priming: A critical review. *Immune Network*, *17*(2), 89–102.

Pedrazza, L., Pereira, T. C. B., Abujamra, A. L., Nunes, F. B., Bogo, M. R., & de Oliveira, J. R. (2017). Mesenchymal stem cells cannot affect mRNA expression of toll-like receptors in different tissues during sepsis. *Inflammation Research*, *66*, 1–5.

Pottumarthy, S., Fritsche, T. R., & Jones, R. N. (2006). Activity of gemifloxacin tested against *Neisseria gonorrhoeae* isolates including antimicrobial-resistant phenotypes. *Diagnostic Microbiology and Infectious Disease*, *54*(2), 127–134.

Roger, T., Froidevaux, C., Le Roy, D., Reymond, M. K., Chanson, A.-L., Mauri, D., … Calandra, T. (2009). Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(7), 2348–2352.

Sagy, M., Al-Qaqaa, Y., & Kim, P. (2013). Definitions and pathophysiology of sepsis. *Current Problems in Pediatric and Adolescent Health Care*, *43*(10), 260–263.

Saparov, A., Ogay, V., Nurgozhin, T., Jumabay, M., & Chen, W. C. W. (2016). Preconditioning of human mesenchymal stem cells to enhance their regulation of the immune response. *Stem Cells International*, *2016*, 3924858.

Spees, J. L., Lee, R. H., & Gregory, C. A. (2016). Mechanisms of mesenchymal stem/stromal cell function. *Stem Cell Research & Therapy*, *7*(1), 125.

Taban, Z. F., Khatibi, S., Halabian, R., & Roushandeh, A. M. (2016). The effects of preconditioning on survival of mesenchymal stem cells in vitro. *Gene, Cell and Tissue*, *3*(4).

van Velthoven, C. T. J., Kavelaars, A., & Heijnen, C. J. (2012). Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatric Research*, *71*(4-2), 474–481.

Wang, J., Li, Z., Zhang, Y., Liu, X., Chen, L., & Chen, Y. (2013). CX43 change in LPS preconditioning against apoptosis of mesenchymal stem cells induced by hypoxia and serum deprivation is associated with ERK signaling pathway. *Molecular and Cellular Biochemistry*, *380*(1-2), 267–275.

Wang, Z. J., Zhang, F. M., Wang, L. S., Yao, Y. W., Zhao, Q., & Gao, X. (2009). Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via toll-like receptor (TLR)-4 and PI3K/Akt. *Cell Biology International*, *33*(6), 665–674.

Westpal, O., & Jann, K. (1965). Bacterial lipopolysaccharides-extraction with phenol water and further application of the procedure. *Methods in Carbohydrate Chemistry*, *5*, 83–91.

Zeuner, M.-T., Patel, K., Denecke, B., Giebel, B., & Widera, D. (2016). Paracrine effects of TLR4-polarised mesenchymal stromal cells are mediated by extracellular vesicles. *Journal of Translational Medicine*, *14*, 34.

Zhang, J., Huang, X., Wang, H., Liu, X., Zhang, T., Wang, Y., & Hu, D. (2015). The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. *Stem Cell Research & Therapy*, *6*(1), 234.

Zhou, H., Li, D., Shi, C., Xin, T., Yang, J., Zhou, Y., … Chen, Y. (2015). Effects of exendin-4 on bone marrow mesenchymal stem cell proliferation, migration and apoptosis in vitro. *Scientific Reports*, *5*, 12898.

Zhu, Y., Xu, L., Collins, J. J. P., Vadivel, A., Cyr-Depauw, C., Zhong, S., … Thébaud, B. (2017). Human umbilical cord mesenchymal stromal cells improve survival and bacterial clearance in neonatal sepsis in rats. *Stem Cells and Development*, *26*, 1054–1064.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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