Treatment With Human Wharton’s Jelly-Derived Mesenchymal Stem Cells Attenuates Sepsis-Induced Kidney Injury, Liver Injury, and Endothelial Dysfunction

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ABSTRACT

The pathophysiology of sepsis involves complex cytokine and inflammatory mediator networks. Downregulation of endothelial nitric oxide synthase contributes to sepsis-induced endothelial dysfunction. Human Wharton’s jelly-derived mesenchymal stem cells (WJ-MSCs) are known to reduce expression of proinflammatory cytokines and markers of apoptosis. We hypothesized that treatment with WJ-MSCs would protect renal, hepatic, and endothelial function in a cecal ligation and puncture (CLP) model of sepsis in rats. Rats were randomly divided into three groups: sham-operated rats; rats submitted to CLP and left untreated; and rats submitted to CLP and intraperitoneally injected, 6 hours later, with $1 \times 10^6$ WJ-MSCs. The glomerular filtration rate (GFR) was measured at 6 and 24 hours after CLP or sham surgery. All other studies were conducted at 24 hours after CLP or sham surgery. By 6 hours, GFR had decreased in the CLP rats. At 24 hours, Klotho renal expression significantly decreased. Treatment with WJ-MSCs improved the GFR; improved tubular function; decreased the CD68-positive cell count; decreased the fractional interstitial area; decreased expression of nuclear factor $\kappa B$ and of cytokines; increased expression of eNOS, vascular endothelial growth factor, and Klotho; attenuated renal apoptosis; ameliorated hepatic function; increased glycogen deposition in the liver; and improved survival. Sepsis-induced acute kidney injury is a state of Klotho deficiency, which WJ-MSCs can attenuate. Klotho protein expression was higher in WJ-MSCs than in human adipose-derived MSCs. Because WJ-MSCs preserve renal and hepatic function, they might play a protective role in sepsis.

SIGNIFICANCE

Sepsis is the leading cause of death in intensive care units. Although many different treatments for sepsis have been tested, sepsis-related mortality rates remain high. It was hypothesized in this study that treatment with human Wharton’s jelly-derived mesenchymal stem cells (WJ-MSCs) would protect renal, hepatic, and endothelial function in a model of sepsis in rats. Treatment with WJ-MSCs improved the glomerular filtration rate, improved tubular function, decreased expression of nuclear factor $\kappa B$ and of cytokines, increased expression of eNOS and of Klotho, attenuated renal apoptosis, and improved survival. Sepsis-induced acute kidney injury is a state of Klotho deficiency, which WJ-MSCs can attenuate.

INTRODUCTION

Sepsis is the leading cause of death in intensive care units. Worldwide, the incidence of and mortality associated with sepsis, severe sepsis and septic shock have increased, resulting in higher costs to health care systems [1]. Sepsis syndrome is defined as documented infection with a systemic inflammatory response [2]. In the hyperinflammatory phase of sepsis, the innate and adaptive immune systems are activated to eliminate the pathogens causing the disease [3]. Nearly 50% of all patients with sepsis develop acute kidney injury (AKI). In sepsis-induced AKI, impaired renal function [4], as well as renal inflammation, microvascular dysfunction, endothelial cell injury, and other conditions, is attributable to nitric oxide pathway activation, leukocyte adhesion, reactive oxygen species (ROS), and inflammation [5]. The activation of nuclear factor $\kappa B$ (NF-$\kappa B$) plays an important role in the pathophysiology of sepsis.
We isolated ADMSCs from subcutaneous lipoaspirates collected from one adult donor undergoing elective liposuction surgery. Lipoaspirates were washed with phosphate-buffered saline and digested for 1 hour in 0.1% collagenase type 1, supplemented with 1% bovine serum albumin and 2 mM CaCl₂. The stromal fraction cells were separated by centrifugation at 300g at 21°C. The cells were cultured and expanded, in the same culture medium as the WJ-MSCs, until they reached 80% confluence. The adherent cells were harvested by trypsin digestion, and the cells were characterized after P3 or P4.

The sample collection protocol for obtaining MSCs was approved by the Medical Ethics Committee of the University of São Paulo University Hospital (protocol no. 1278/13) and by the Medical and Research Ethics Committee of the University of São Paulo School of Medicine Hospital das Clínicas (protocol no. 0003/11). All participating mothers gave written informed consent.

Flow Cytometry

Flow cytometry was performed with allophycocyanin-conjugated or phycoerythrin-conjugated antibodies against CD3, CD45, CD34, HLA-DR, CD105, CD73, and CD90 (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com), analyzed in a FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences).

Immunofluorescence

Using a commercial kit (Human Mesenchymal Stem Cell Characterization Kit; EMD Millipore, Billerica, MA, http://www.emdmillipore.com/), we performed immunofluorescence with antibodies against CD14, clone 2D-15C (1:500); CD19, clone FMC63 (1:500); CD44, clone F10-44-2 (1:500); CD90, clone F15-42-1 (1:500); and CD105, clone P1H12 (1:500). The labeling was visualized with a fluorescein isothiocyanate-conjugated secondary antibody (anti-mouse IgG, 1:250; Sigma Chemical, St. Louis, MO).

Lineage Differentiation

Lineage differentiation was performed with P2 cells seeded in six-well plates (1 x 10⁵ cells/well). Adipogenesis, osteogenesis, and chondrogenesis were tested over periods of 14, 21, and 14 days, respectively, with commercial differentiation kits (StemPro; Thermo Fisher Scientific Life Sciences). Differentiation media were changed every 3–4 days.

Induction of Sepsis

Rats were anesthetized with isoflurane, after which a midline incision was made and the cecum was exposed. A 4.0 silk ligature was placed 1.5 cm from the cecal tip. The cecum was punctured twice with a 16-G needle and gently squeezed to confirm leakage of cecal contents. The abdominal incision was closed in two layers with 3-0 silk sutures. Control animals were submitted to the same procedure, minus ligation and puncture. To ensure adequate fluid resuscitation, each animal received 0.15 M NaCl (25 ml/kg body weight [b.wt.] i.p.) immediately after the procedure. Additional fluid therapy (0.15 M NaCl, 25 ml/kg b.wt. i.p.) was started at 6 hours after CLP and then repeated every 12 hours, as was antibiotic therapy with imipenem/cilastatin (14 mg/kg b.wt. i.p.; Merck, West Point, PA, http://www.merck.com).

**MATERIALS AND METHODS**

**Animals and Experimental Protocol**

All procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. The study was approved by the Research Ethics Committee of the University of São Paulo School of Medicine, São Paulo, Brazil (protocol no. 378 603).

Male Wistar rats, weighing 200–280 g, were obtained from the animal facility of the University of São Paulo School of Medicine. Animals were fed standard rat chow and given ad libitum access to tap water. Before the experiments, the rats were randomly divided into the control, CLP, and CLP+WJ-MSC groups.

**Isolation and Culture of MSCs**

We collected WJ-MSCs from healthy mother–infant pairs at the University of São Paulo University Hospital, São Paulo. Wharton’s jelly was surgically extracted less than 24 hours after delivery. Small (1–5 mm) explants were plated in culture dishes and cultured with α-modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), supplemented with sodium bicarbonate at pH 7.3 (Sigma-Aldrich), penicillin (300 U/ml; Life Technologies, Carlsbad, CA), streptomycin (300 μg/ml; Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com) and 20% fetal bovine serum (Sigma-Aldrich). We incubated explants at 37°C in 5% CO₂, without changing the medium, for 10–15 days. When cells began to migrate from the explants, the medium was changed every 3–4 days; when they reached 80% confluence, the explants were removed and cells were treated with 0.25% trypsin-ethylenediaminetetraacetic acid (Thermo Fisher Scientific Life Sciences), to be seeded as passage (P) 1 cells. Animals were injected with P3–P5 cells.

Shock [6]. Inhibition of NF-κB has been shown to improve survival in an animal model of polymicrobial sepsis [7]. In addition, studies indicate that ENOS expression is decreased after the onset of sepsis, an effect that might be related to endothelial dysfunction [8], which, together with the production of ROS, induces activation of the mechanism of apoptosis [9, 10].

Although many different treatments for sepsis have been tested, sepsis-related mortality rates remain high [11]. An experimental study conducted in 2007 was the first to evaluate the use of mesenchymal stem cells (MSCs) for the treatment of sepsis [12]. The authors demonstrated that MSC treatment decreased proinflammatory cytokine production and minimized sepsis-induced lung injury. It has been suggested that bone marrow-derived MSCs (BMSCs) are more effective than are adipose-derived MSCs (ADMSCs) for the treatment of experimental sepsis in mice [13]. In vitro studies have shown that umbilical cord blood-derived MSCs are better than BMSCs and ADMSCs in reducing proinflammatory cytokine production [14]. Additional in vitro studies have shown that MSCs derived from the umbilical cord matrix, known as Wharton’s jelly-derived MSCs (WJ-MSCs), present characteristics similar to or better than those of other MSCs and show promise as a treatment for various diseases [15, 16]. We hypothesized that treatment with human WJ-MSCs would protect renal, hepatic, and endothelial function in a cecal ligation and puncture (CLP) model of sepsis in rats.
WJ-MSC Treatment
At 6 hours after CLP, animals in the CLP+WJ-MSC group were injected intraperitoneally with 1 × 10^6 freshly recovered P3–P5 WJ-MSCs diluted in 1 ml of saline. At the same time point, animals in the control group were injected intraperitoneally with 1 ml of saline.

Study outline
We conducted our study in three phases. In the first phase (survival study), we used nine animals per group. In the second, we used 6 sham group animals and 5 CLP group animals to perform inulin clearance studies at 6 hours after CLP or sham surgery. In the third phase, we studied animals at 24 hours after CLP or sham surgery, using eight animals per group for metabolic cage studies; inulin clearance studies; analysis of blood and urine; immunohistochemistry; histomorphometry; and Western blotting.

Phase 1
Survival curves were plotted, initially at 6 hours after CLP and then every 6–12 hours, over a 120-hour period.

Phase 2
This phase was aimed at determining whether the glomerular filtration rate (GFR) was already decreased before the WJ-MSC treatment. At 6 hours after CLP or sham surgery, animals were subjected to inulin clearance studies. Before the inulin clearance studies, each animal was anesthetized with an injection of thiopental sodium (50 mg/kg b.wt. i.p.). The trachea was cannulated with a PE-240 catheter, and spontaneous breathing was maintained. To allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. To collect urine samples, a suprapubic incision was made and the urinary bladder was cannulated with a PE-240 catheter. After the surgical procedure, a loading dose of inulin (100 mg/kg b.wt.) was administered through the jugular vein. We started and maintained constant infusion of inulin (10 mg/kg b.wt. at 0.04 ml/min). A total of three urine samples were collected at 30-minute intervals. Blood samples were obtained at the beginning (0.3 ml) and end (4–6 ml) of the experiment. Using the anthrone method, we determined inulin in plasma and urine.

Phase 3
After CLP or sham surgery, animals were moved to individual cages and maintained on a 12-hour/12-hour light/dark cycle (with ad libitum access to water only). After 24-hour urine samples had been collected, water intake and urine volume were measured.

Analysis of Blood and Urine
The volume of each 24-hour urine sample was measured gravimetrically. Urine samples were centrifuged in aliquots to remove suspended material, and the supernatants were analyzed. Plasma and urinary levels of sodium and potassium were measured with ion-selective electrodes (Nova Biomedical, Waltham, MA, http://www.novabio.us/), whereas serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and urea were measured with kinetic techniques. We measured urine and plasma osmolality using a freezing-point osmometer (3D3; Advanced Instruments, Norwood, MA, http://www.aicompanies.com/).

Osmolar clearance was calculated by the following formula:

$$C_{\text{osm}} = \frac{(U_{\text{osmolality}} \times U_{\text{volume}} / 1,440\text{min})}{P_{\text{osmolality}}}$$

where $C_{\text{osm}}$ is osmolar clearance, $U_{\text{osmolality}}$ is urine osmolality, $U_{\text{volume}}/1,440\text{min}$ is urine output in microliters per day, and $P_{\text{osmolality}}$ is plasma osmolality.

Free water clearance ($C_{\text{H}_2\text{O}}$) was also calculated:

$$C_{\text{H}_2\text{O}} = U_{\text{volume}}/1,440\text{min} - C_{\text{osm}}$$

Inulin Clearance and Organ Preparation
After the inulin clearance studies, organs were perfused with phosphate-buffered saline, and the kidneys were immediately removed. Some kidneys were frozen in liquid nitrogen and stored at −70°C for subsequent immunoblotting for endothelial nitric oxide (eNOS), vascular endothelial growth factor (VEGF), Klotho, NF-κB, Bcl-X, and Bax. For immunohistochemical analysis, livers and kidneys were immersed in formaldehyde (10%), after which fragments were embedded in paraffin and cut into 4-mm sections.

Histology
For CD68 immunostaining, samples were processed in 4-mm paraffin sections. After deparaffinization, endogenous peroxidase activity was blocked with 0.3% H2O2 in water for 10 minutes at room temperature. Sections were then incubated overnight at 4°C with CD68 antibody (clone ED1, 1:100; AbD Serotec, Oxford, U.K., https://www.abdsorotec.com/). This was followed by incubation with biotinylated mouse anti-rat IgG for 30 minutes at room temperature. The reaction product was detected with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, http://vectorlabs.com/). The color reaction was developed with 3,3-diaminobenzidine (Sigma-Aldrich), and the sections were counterstained with methyl green. To obtain the mean numbers of infiltrating CD68+ cells in the renal cortical tubulointerstitium, all fields (0.087 mm² each) were evaluated and the mean counts per kidney were calculated.

Four-micrometer histological sections of kidney tissue were stained with hematoxylin-eosin or Masson’s trichrome and examined under light microscopy. We quantified fractional interstitial area (FIA) by analyzing tubulointerstitial involvement. For histomorphometry, the images obtained by microscopy were captured on video via an image analyzer (Axiovision; Carl Zeiss, Eching, Germany, http://www.zeiss.com/). We analyzed 30 grid fields (0.087 mm² each) per kidney cortex. The interstitial areas were demarcated manually, and the proportion of the field they occupied, excluding the glomeruli, was determined.

To quantify hepatic glycogen deposition, we submitted liver tissue samples to periodic acid-Schiff immunohistochemical reactions to identify sites rich in glycogen or neutral polysaccharides containing groups β-(1,2)-glucan. The results were evaluated by determining glycogen stores (in percentages) in 10 of 15 fields for each portion of the liver parenchyma.

Staining for Renal Cell Apoptosis
Renal apoptosis was assessed by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) staining with an in situ cell death detection kit (Roche, Penzberg, Germany, http://www.roche.com). The TUNEL+ cells were quantified in 30 randomly chosen fields per slide by an investigator.
Figure 1. Characterization of the WJ-MSCs used. (A): Wharton’s jelly explant (blue arrow), with cells proliferating on the surface, on day 18 of incubation (magnification, ×4). (B): Cells in cultures from Wharton’s jelly (magnification, ×10). (C, D): Densitometry and immunoblotting of Klotho expression in WJ-MSCs and ADMSCs. (E): Fluorescence-activated cytometry of WJ-MSCs for CD34, CD45, HLA-DR, CD73, CD90, and CD105. (F): Immunofluorescence analysis of WJ-MSCs for CD14, CD19, CD44, CD90, and CD146. Gray line: 50 μm. (G): Analysis of the differentiation capacity of the WJ-MSCs; adipogenesis, osteogenesis, and chondrogenesis were identified by staining with Oil Red O, alizarin, and Alcian blue, respectively. Blue line: 50 μm. Abbreviations: ADMSC, adipose-derived mesenchymal stem cell; DAPI, 4′,6-diamidino-2-phenylindole; WJ-MSC, Wharton’s jelly-derived mesenchymal stem cell.
who was blinded to the groups. To obtain the mean numbers of TUNEL+ cells in the tubules, all fields (0.087 mm$^2$ each) were evaluated and the mean counts per kidney were calculated.

**Kidney Fractions**

Kidney samples were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5) containing a protease inhibitor cocktail (Sigma Chemical) in a homogenizer (PT 10/35; Metrohm, Herisau, Switzerland, http://www.metrohm.com). To remove nuclei and cell debris, homogenates were centrifuged at 2,000 g for 15 minutes at 4°C. Supernatants were isolated, and protein was quantified by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, http://www.bio-rad.com).

**Electrophoresis and Immunoblotting**

Kidney samples were run on polyacrylamide mini-gels. After transfer by electrophoresion to nitrocellulose membranes (GE Healthcare, Little Chalfont, U.K., http://www3.gehealthcare.co.uk), blots were blocked with 5% nonfat dry milk in Tris-buffered saline. Blots were then incubated overnight with antibodies (eNOS, 1:1,000; VEGF, 1:500; Klotho, 1:500; NF-κB, 1:500; Bax, 1:1,000 [Santa Cruz Biotechnology, Dallas, TX, http://www.scbt.com/]; and Bcl-X, 1:500 [Millipore, Oxford, U.K.]). The labeling was visualized with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, 1:2,000, or anti-goat, 1:10,000; Sigma Chemical) and enhanced chemiluminescence (ECL) detection (GE Healthcare Life Sciences).

**Proteins**

We scanned the ECL films with an imaging system (Alliance 4.2; UVitec, Cambridge, U.K., http://www.uvitec.co.uk), and the data were analyzed with Bio-Plex Manager software, normalizing the bands to α-actin expression.

**Cytokines**

To determine kidney tissue levels of interleukin (IL)-1α, IL-6, interferon (INF)-γ, tumor necrosis factor (TNF)-α, IL-4, and IL-10, we submitted samples to multiplex cytokine assay (Bio-Plex Rat 9-Plex kit; Bio-Rad, Hercules, CA, http://www.bio-rad.com/). The assay was read on the Bio-Plex suspension array system, and the data were analyzed with Bio-Plex Manager software, version 4.0.

**Statistical Analysis**

Differences among the means of multiple parameters were analyzed by one-way analysis of variance, followed by the Student-Newman-Keuls test. Quantitative data are expressed as mean ± SEM. A $p$ value < .05 was considered to represent a statistically significant difference. Survival analyses were compared by a log-rank test. The statistical software used was GraphPad Prism, version 5 (GraphPad Inc., La Jolla, CA, http://www.graphpad.com/).

**RESULTS**

**Characterization of WJ-MSCs**

Figure 1A shows a Wharton’s jelly explant of a human umbilical cord, with cells proliferating on the surface, on day 18 of incubation. Figure 1B shows passage 3 (P3) cells in Wharton’s jelly culture. Expression of Klotho was higher in WJ-MSCs than in ADMSCs (Fig. 1C, 1D). Flow cytometry, immunophenotyping, and immunofluorescence analysis of P3–P5 cells revealed positivity for the mesenchymal markers CD44, CD73, CD90, CD146, and CD105, together with negativity for the hematopoietic markers CD14, CD19, CD34, HLA-DR, and CD45 (Fig. 1E, 1F). The WJ-MSCs differentiated into adipogenic, chondrogenic, and osteogenic lineages (Fig. 1G).

**Effects of WJ-MSCs on Survival in Sepsis**

We administrated WJ-MSCs at 6 hours after CLP or sham surgery. All studies were conducted at 24 hours after the procedures. As shown in Figure 2, mortality was significantly lower in the rats submitted to CLP and intraperitoneally injected, 6 hours later, with 1 × 10$^6$ human WJ-MSCs (CLP+WJ-MSC group) than in those submitted to CLP and left untreated (CLP group). Five-day survival was 87.5% in the CLP+WJ-MSCs group and 55.6% in the CLP group.

**Effects of WJ-MSCs on Sepsis-Induced AKI**

**Renal Function**

Figure 3A shows inulin clearance (in ml/minute per 100 g b.wt.) at 6 hours after CLP or sham surgery, at which point the GFR was already decreased. Inulin clearance was lower in CLP rats than in sham-operated rats (0.96 ± 0.05 vs. 0.31 ± 0.02; $p < .0001$). Figure 3B shows inulin clearance at 24 hours after CLP or sham surgery. As expected, inulin clearance was lower in CLP rats than in sham-operated (control group) rats (0.26 ± 0.03 vs. 0.87 ± 0.03; $p < .0001$). Inulin clearance was also lower in CLP rats than in CLP+WJ-MSC rats (0.26 ± 0.03 vs. 0.90 ± 0.08; $p < .0001$), although there was no significant difference between CLP+WJ-MSC and control rats.

Fractional excretion of sodium, urea, and water were significantly higher in CLP rats than in control rats and CLP+WJ-MSC rats (Table 1). In addition, urine volume and free water clearance were significantly higher in CLP rats than in control rats, whereas urine osmolality was significantly lower in CLP rats than in control rats.

**Macrophage Infiltration**

Infiltration of the renal tubulointerstitium by macrophages and monocytes, as quantified by CD68+ cell counts (Fig. 4A, 4B), was significantly greater in CLP rats than in control rats (6.72% ± 0.59%; $p < .05$). However, the FIA did not differ significantly between the CLP+WJ-MSC and control rats. The FIA was significantly greater in CLP rats than in control rats (9.76% ± 0.41% vs. 6.54% ± 0.56%; $p < .05$), whereas it was markedly lower in CLP+WJ-MSC rats than in CLP rats (6.72% ± 0.48% vs. 9.76% ± 0.41%; $p < .05$). However, the FIA did not differ significantly between the CLP+WJ-MSC and control rats (supplemental online Fig. 1).

**Apoptosis**

Figure 4C and 4D shows TUNEL staining as an indicator of apoptosis. The proportion TUNEL+ nuclei was significantly higher in CLP+WJ-MSC rats than in control rats (52.4% ± 4.32% and 42.4% ± 3.92%; $p < .03$ vs. 0.41% vs. 0.41%; $p < .0001$). In addition, kidney tissue levels of interleukin-1α, IL-6, interferon-γ, tumor necrosis factor (TNF)−α, IL-4, and IL-10 were significantly lower in CLP+WJ-MSC rats than in CLP rats (6.72% ± 0.59%; $p < .05$). However, the FIA did not differ significantly between the CLP+WJ-MSC and control rats (supplemental online Fig. 1).
and INF-γ and TNF-α levels remained higher. Notably, WJ-MSC treatment had no apparent effect on sepsis-induced increases in the levels of the anti-inflammatory cytokines IL-4 and IL-10.

**Endothelial Markers**

Expression of VEGF was higher in CLP+WJ-MSC rats than in CLP and control rats (208.7 ± 24.4% vs. 106.2 ± 3.22% and 98.4% ± 3.54%; \( p < .05 \); Fig. 5). Expression of eNOS was lower in CLP rats than in control rats (49.0% ± 2.08% vs. 102.5% ± 2.5%; \( p < .0001 \)) but was completely restored in CLP+WJ-MSC rats (97.4% ± 2.79%; Fig. 5).

**Klotho Protein**

Figure 5 shows Klotho protein expression, which was lower in CLP rats than in control rats (43.3% ± 5.52% vs. 100.0% ± 3.16%; \( p < .001 \)) but was partially restored in CLP+WJ-MSC rats (65.25% ± 3.45%; \( p < .05 \) vs. CLP). The difference between CLP+WJ-MSC and control rats was also significant (\( p < .05 \)).

**Effects of WJ-MSCs on Sepsis-Induced Liver Dysfunction**

Table 1 shows plasma levels of the liver enzymes ALT and AST. The ALT level was significantly higher in CLP rats than control rats (48.8 ± 4.76 U/l vs. 29.8 ± 4.19 U/l; \( p < .05 \)), whereas the AST level was significantly higher in CLP rats than in control and CLP+WJ-MSC rats (205.9 ± 25.2 U/l vs. 78.25 ± 5.54 U/l and 142.5 ± 11.4 U/l; \( p < .05 \)), although AST remained significantly higher in CLP+WJ-MSC rats than in control rats (\( p < .05 \)). Glycogen deposition (Fig. 6) was reduced in CLP rats compared with control and CLP+WJ-MSC rats (2.24% ± 1.19% vs. 29.8% ± 2.91% and 11.1% ± 1.54%; \( p < .0001 \) and \( p < .05 \), respectively), although the difference between CLP+WJ-MSC and control rats was also significant (\( p < .0001 \)).

**DISCUSSION**

This study demonstrates that in rats sepsis causes renal, hepatic, and endothelial dysfunction; increases levels of proinflammatory markers; and induces a Klotho-deficient state. Hu et al. showed that, in rodents, AKI reduces Klotho in the kidneys, urine, and blood [17]. The authors also showed that AKI-induced changes in function and histology were more extensive in Klotho-haploinsufficient mice than in wild-type mice, whereas those changes were milder in transgenic Klotho-overexpressing mice, suggesting that Klotho is renoprotective. In our study, renal Klotho expression was lower in the CLP group. However, it is unclear whether the reduction in renal Klotho was due to sepsis-induced AKI or to the sepsis itself. In a recent study, we showed that sepsis-related multiple organ dysfunction is more severe in Klotho-haploinsufficient mice [18]. The Klotho protein is a potential biomarker of kidney injury. In ischemia/reperfusion injury-induced AKI, epigenetic changes resemble aging, one such change being Klotho suppression [17]. The Klotho protein can induce antioxidant enzyme activity [19], and Klotho deficiency is associated with increased oxidative stress, even in experimental models of kidney disease or in dialysis patients [20]. In the kidneys of aged mice treated with BMSCs, markers of senescence are more numerous among those treated with cells obtained from aged mice than among those treated with cells obtained from other young mice [21]. In our study, we have demonstrated that WJ-MSCs cells presented higher Klotho protein expression than the ADMSCs. We
found an increase in Klotho expression and a decrease in NF-κB protein expression, as shown by Maekawa et al. [22].

The administration of ADMSCs, BMSCs, and other types of MSCs minimizes organ injury in animal models of sepsis [12, 23, 24]. In our study, we attempted to mimic the setting of human sepsis by starting antibiotic therapy at 6 hours after CLP or sham surgery, an interval roughly equivalent to the time from diagnosis to the initiation of treatment in human cases of sepsis, and by using fluid resuscitation to maintain tissue perfusion. We have demonstrated that 6 hours after CLP GFR had already decreased. We found that administration of WJ-MSCs at 6 hours after sepsis induction improved survival, attenuated kidney and liver injury, reduced apoptosis, restored endothelium-related proteins, increased Klotho protein expression, and reduced inflammation.

Sepsis is a major cause of AKI, and sepsis-induced AKI is associated with extremely high mortality [4]. Here, we demonstrated

| Variable | Control | CLP | CLP+WJ-MSC |
|----------|---------|-----|------------|
| FENa (%) | 0.10 ± 0.01 | 1.69 ± 0.56<sup>a</sup> | 0.67 ± 0.12<sup>b</sup> |
| FEK (%)  | 8.51 ± 0.26  | 16.1 ± 4.02 | 10.0 ± 1.60 |
| FEU (%)  | 26.6 ± 1.37  | 74.0 ± 11.4<sup>a</sup> | 49.7 ± 3.43<sup>b</sup> |
| FEH₂O (%)| 0.65 ± 0.09  | 4.77 ± 1.34<sup>a</sup> | 1.53 ± 0.26<sup>b</sup> |
| Urine osmolality (mOsm/kg) | 912.3 ± 60.9 | 368.0 ± 42.6<sup>c</sup> | 620.4 ± 92.7<sup>a,b</sup> |
| Urine volume (ml/minute) | 0.011 ± 0.001 | 0.028 ± 0.002<sup>c</sup> | 0.024 ± 0.005<sup>c</sup> |
| Free water clearance (ml/minute) | −0.02 ± 0.001 | −0.006 ± 0.003<sup>a</sup> | −0.02 ± 0.004<sup>b</sup> |
| ALT (U/l) | 29.8 ± 4.19 | 48.8 ± 4.76<sup>a</sup> | 39.6 ± 4.19 |
| AST (U/l) | 78.25 ± 5.54 | 205.9 ± 25.2<sup>a</sup> | 142.5 ± 11.4<sup>a,b</sup> |

Values are expressed as mean ± SEM.

<sup>a</sup>p < .05 versus control.

<sup>b</sup>p < .05 versus CLP.

<sup>c</sup>p < .0001 versus control.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; control, sham-operated rats; CLP, rats submitted to cecal ligation and puncture and left untreated; CLP+WJ-MSCs, rats submitted to CLP and intraperitoneally injected, 6 hours later, with 1 × 10⁶ human Wharton’s jelly mesenchymal stem cells; FEH₂O, fractional excretion of water; FEK, fractional excretion of potassium; FENa, fractional excretion of sodium; FEU, fractional excretion of urea.
that WJ-MSCs improve survival, GFR, and tubular function. Our findings of decreased urine osmolality, as well as increased fractional excretion of sodium, urea, and water, together with greater free water clearance, suggest tubular dysfunction. Renal injury was also evidenced by the increased FIA, which indicated greater leukocyte infiltration, particularly because there was also greater macrophage infiltration [25–27]. In our analysis of the renal histology, we found no significant tubular epithelial swelling, vacuolar degeneration, necrosis, or desquamation. It is well known that sepsis induces acute tubular necrosis only when septic shock occurs [25]. However, sepsis can induce renal apoptosis by mechanisms related to oxidative stress and inflammation [9, 10]. Here, we demonstrated an increase in Bax expression and in the number of TUNEL+ cells in renal tissue. We also demonstrated that WJ-MSCs have an antiapoptotic effect, decreasing TUNEL+ cell counts, and Bax expression. Yagi et al. showed that MSCs administered during endotoxemia significantly reduce apoptosis in the kidney and lung tissue of rats [28]. That antiapoptotic effect might be related to NF-κB inhibition. Inhibition of NF-κB reduces expression of multiple proinflammatory genes, diminishes intravascular coagulation, reduces tissue neutrophil influx, and prevents microvascular endothelial leakage [29]. Other animal studies using a CLP model of sepsis have shown that inhibition of NF-κB significantly improves survival [30].

In sepsis, there is an initial proinflammatory period, characterized by increased production of various cytokines and a Th1 CD4+ T cell response. However, in later stages, patients develop compensatory anti-inflammatory response syndrome (characterized by a Th2-type response), which is a reaction to the proinflammatory state, thereafter progressing to systemic immunosuppression [31]. In our study, the animals with sepsis developed proinflammatory profiles, characterized by increased expression of IL-1α, IL-6, INF-γ, and TNF-α. With the exception of TNF-α, treatment with WJ-MSCs decreased the expression of those cytokines. We found interesting that WJ-MSCs increased the expression of anti-inflammatory cytokines, such as IL-4 and IL-10. Aggarwal et al. found that MSCs induce a reduction in Th1 activity and a significant increase in IL-4, both characteristic of Th2 profile [32]. Other studies have suggested that such modulation can also occur through upregulation of CD4+CD25+forkhead box P3+ regulatory T cells, as evidenced by increased IL-10 production [33, 34].

Sepsis is a model of endothelial dysfunction. We found that WJ-MSC treatment induced a pronounced increase in VEGF.

**Figure 5.** Expression of VEGF, Klotho protein, NF-κB, eNOS, Bax protein, and Bcl-X protein. Densitometry (A–F) and immunoblotting (G) of markers of renal injury, at 24 hours after CLP or sham surgery, in sham-operated rats (control group; n = 8), rats submitted to CLP and left untreated (CLP group; n = 8), and rats submitted to CLP and intraperitoneally injected, 6 hours later, with 1 × 10^6 human WJ-MSCs (CLP+WJ-MSC group; n = 8). Values are mean ± SEM. **p < .05 versus control; ***p < .0001 versus control; #p < .05 versus CLP; ###p < .0001 versus CLP. Abbreviations: CLP, cecal ligation and puncture; eNOS, endothelial nitric oxide; NF-κB, nuclear factor κB; VEGF, vascular endothelial growth factor; WJ-MSC, Wharton’s jelly-derived mesenchymal stem cell.
Previous studies have suggested that downregulation of the VEGF signaling cascade accounts for lipopolysaccharide-induced apoptosis and impaired physiological angiogenesis in lung tissue [35, 36]. In addition to its anti-inflammatory effects, VEGF contributes to reconstituting damaged endothelium and promotes revascularization. It is known that eNOS and VEGF protect against sepsis-induced endothelial dysfunction [37, 38]. In our study, WJ-MSC treatment also reversed sepsis-induced downregulation of eNOS expression.

Sepsis can induce liver injury. We found WJ-MSC treatment to be effective in normalizing liver enzymes, mainly AST, results similar to those reported by Chang et al. [24]. It has been suggested that MSCs protect against sepsis-induced liver injury by decreasing leukocyte infiltration [39, 40], modulating the immune response and decreasing apoptosis [23].

CONCLUSION

In the present study, we found that WJ-MSCs protected renal and hepatic function. Therefore, WJ-MSC administration could play a role in the treatment of sepsis.

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