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Erythropoietin Improves Effects of Mesenchymal Stem Cells in Experimental Endotoxemia

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Authors' contributions

This work was carried out in collaboration between all authors. Author AVA designed the study and wrote the first draft of the manuscript. Authors AGK and FGZ performed the immunohistochemical and morphological analyses. Author AVS performed the animal handling. Author MAK performed cell culture and statistics. Author VPB performed data analyses and contributed to the manuscript writing and discussion. Author RIK managed the experimental process. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Sepsis is a leading cause of mortality in intensive care units. Development of new strategies such as the therapy with mesenchymal stem cells (MSC) appears beneficial for the treatment of sepsis. In this study we evaluated anti-septic effects of rat MSCs and recombinant erythropoietin (EPO) in a rat experimental model of endotoxemia.
Study Design: Controlled *in vitro* and *in vivo* studies.
Place and Duration of Study: Federal Research Clinical Center of FMBA of Russia, Moscow, Russia, between June 2014 and May 2015.
Methodology: Endotoxemia was induced by intraperitoneal administration of bacterial

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lipopolysacharide (LPS). The animals were then treated either with allogeneic MSCs alone, with recombinant EPO alone, or with a combination of EPO and MCS. After 3 days, the animals were euthanized, and a pathology study of their liver, spleen, thymus, lung, and kidney was performed. The serum levels of IL-1β, IL-6, and TNF-α were quantified. A histochemical analysis of splenic and thymic expression of CD3, CD57, p53, and Bcl-2 was performed.

Results: Essential positive effects of the combined MSC-EPO treatment were observed: 1) The animals treated with MSCs and EPO had the lowest serum concentrations of IL-1β in respect to that of the rats treated with LPS alone (58 ± 22 pg/mL vs. 155±90 pg/mL, P = .01); 2) The treatment of endotoxemic rats with a combination of MSCs and EPO also caused production of the anti-apoptotic factor Bcl-2, while its expression was markedly down-regulated in the other groups of animals; 3) In the MSC + EPO group, the degree of interstitial pulmonary edema was the lowest as compared to the other groups, and a minimal renal injury was detected.

Conclusion: These findings suggest that EPO generally improves anti-inflammatory and anti-apoptotic effects of MSCs injected in the acute phase of experimental endotoxemia.

Keywords: Sepsis; erythropoietin; mesenchymal stem cells; stem cell therapy; rat experimental endotoxemia; systemic inflammation.

1. INTRODUCTION

Sepsis is the leading cause of mortality (not associated with cardiovascular disease) in intensive care units worldwide [1]. Coinciding with the implementation of the International Guidelines for Management of Severe Sepsis and Septic Shock by the Surviving Sepsis Campaign, the 28-day mortality from severe sepsis dropped from 46.9% (1991-1995) to 29% (2006-2009) [2]. However, experts assume that the limit of survival has almost been achieved at this point, and the hopes for the future are associated with the development of new drugs and technologies for severe sepsis and septic shock treatment [3]. The therapy with mesenchymal (stromal) stem cells (MSC) is among the most promising approaches [4].

The pathological septic mechanisms such as enhanced apoptosis, endothelial damage, microcirculatory disorders, immune suppression, and heart injury could in theory be compensated by the paracrine and plastic effects of MSCs. To date, several studies have shown beneficial effects of different types of MSC on the course of sepsis, as well as on its outcome. Xu et al. [5] reported that co-injection of MSCs and bacterial lipopolysaccharide (LPS) in mice prevented both acute lung injury and systemic inflammatory response and significantly reduced the levels of pro-inflammatory cytokines, such as interferon (IFN)-γ, interleukin (IL)-1β, IL-6, macrophage inflammatory protein (MIP)-1α, and IL-8. The MSC efficiency in the clearance of the sepsis-causing pathogens was shown in a sepsis mouse model based on cecal ligation and puncture (CLP) [6]. In this latter paper [6], treatment with MSCs led to a decrease in the levels of circulating systemic inflammatory biomarkers, increased effectiveness of the spleen-assisted bacterial clearance and reduction of the signs of multiple organ dysfunction. In addition, MSCs were found to stimulate the phagocytic activity of macrophages. In a rat endotoxemic sepsis model, treatment with MSCs reduced expression of IL-1β and IL-6 in the serum and heart tissue and prevented myocardial injury [7]. In a human ex-vivo lung perfusion model, using rejected lungs unsuitable for transplantation, McAuley et al. [8] found that intravenous human bone marrow-derived MSCs restored the alveolar fluid clearance in acute injured lungs (similar to a septic lung) to a normal level, thus indicating an improved alveolar epithelial function. In addition to the anti-inflammatory properties, MSCs demonstrated pro-angiogenic effects via producing angiogenic growth factors such as vascular endothelial growth factor and basic fibroblast growth factor and inhibiting cell apoptosis in the damaged tissues [9]. MSCs have also immunomodulatory properties, decreasing neutrophils' migration to the target organs and suppressing the natural killer (NK) cells activation and cytotoxicity [10,11].

Recently, MSCs have been found to produce an antimicrobial peptide cathelicidin (LL-37) suppressing the growth of gram-negative bacteria, a feature which explains MSCs' efficiency in the treatment of infectious diseases. When cathelicidin expression was down-regulated, the anti-infective activity of MSCs

showed an almost twofold reduction [12]. Based on these findings, Krasnodembskaya et al. [12] concluded that the direct antibacterial effect of MSCs on a pulmonary infection was no less important than their traditionally mentioned plasticity and paracrine activity. Unfortunately, the high apoptotic rate of MSC grafts limits their application in the treatment of sepsis and acute lung injury. The apoptosis is caused by pro-apoptotic agents such as tumor necrosis factor (TNF)-α and by oxidative stress, which also promotes death of MSCs and progenitor cells [13,14].

Based on the transcriptomic analysis, dos Santos et al. [15] found that the positive effects of the MSC sepsis treatment were not limited to paracrine, immunomodulatory and antimicrobial pathways, but they also involved a coordinated expression of transcriptional programs serving as a blueprint for MSC-conferred protection against sepsis at the cellular level in multiple organs. Networks of MSC-modulated gene sets coordinate the complementary activities, affecting the host cell metabolic and inflammatory responses.

Recombinant erythropoietin (EPO), a hematopoietic growth factor, is a bioactive agent which has been repeatedly used in the sepsis treatment. In various sepsis models, a number of EPO beneficial effects including vasopressor, anti-apoptotic, cytoprotective, and immunomodulating activities were observed [16,17]. However, the results of clinical trials involving treatment with EPO are contradictory. Potential adverse effects of EPO are associated with increased thrombosis and thromboembolic risk [18]. Decreasing the EPO dosage reduces the risk of thrombosis but also lowers the treatment efficiency [19]. Interestingly, subcutaneous injection of EPO in patients receiving hemodialysis showed increased efficacy as compared to intravenous injection [20]. The mechanisms of non-hematopoietic effects of EPO in the systemic inflammatory response should also be considered [21].

Bone marrow MSCs were found to carry EPO receptors controlling their mobilization and down-regulation of the aging-related apoptosis [22]. Since one of the approaches to improve the MSCs' survival and efficiency is a search for new signaling pathways [23], we hypothesize that a co-treatment with MSCs and EPO should prevent apoptosis of the cell grafts and enhance the beneficial effects of cell transplantation.

2. MATERIALS AND METHODS

2.1 Ethics and Regulation

The study was approved by The Animal Research Ethics Committee of the Medical Radiological Research Center. All procedures involving experimental animals were performed according to the EU Directive 2010/63/EU.

2.2 Mesenchymal Stem Cells

MSCs were derived from bone marrow of female Wistar rats according to the modified protocol [24]. The donor bone marrow cells were obtained from the femoral bones after a deep anesthesia with a Pentobarbital injection (70 mg/kg). A total of 1×10^7 of bone marrow cells were placed in heparin-containing test tubes (100 U/mL of punctate). After storing the red blood cells for 2 hours at room temperature, the supernatant was aspirated with a Pasteur pipette, the isolated cells were washed with Medium 199 (Sigma-Aldrich, St. Louis, MI, USA) and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with penicillin (100 U/mL), amphotericin B (100 ng/mL), 2 mM L-glutamine, and 20% fetal calf serum. A total of 5×10^6 of bone marrow cells were cultured in 8 mL of growth medium added to 25 cm^2 Carrel plastic vials (Sigma-Aldrich). The cells were cultured at 37℃ and 5% of carbon dioxide and placed into a larger (175 cm^2) culture flask to reach a population of (1-2) $\times$ 10^8 cells by week 6, sufficient for transplantation to the recipient rats. The flow cytometry analysis showed that the cultured MSCs had a $CD34^-CD45^-CD90^+CD105^+CD73^+$ phenotype, characteristic of adult multipotent MSCs. In addition, MSCs were able to differentiate into osteocytes, chondrocytes and adipocytes, depending upon the culturing conditions and differentiation inducers.

2.3 Study Design

A total of 50 Wistar female rats, of three months of age, with a body mass of 180-200 g, were randomized into 5 groups, each group containing 10 animals (Fig. 1). Group 1 included control rats, the other animals (Groups 2-5) were intraperitoneally injected with bacterial LPS (20 mg/kg) from *Escherichia coli* strain 0111:B4 (Sigma-Aldrich). Group 2 was used as an endotoxemia control group. Two hours post-

injection, the animals from Groups 3-5 received the following intravenous regiments: Group 3 received 4 × 10^5 allogeneic MSCs, Group 4 received 8.5 μg (1000 IU) per rat of recombinant EPO-β (Hoffmann-La Roche), Group 5 was treated with MSCs and EPO in the same doses as those used for Groups 3 and 4. The survived animals were euthanized with an intraperitoneal injection of Pentobarbital (Sigma-Aldrich) 72 hours after the LPS injection. At the 72 hours time point, we performed the blood sampling from the beating heart for the hematology analysis and immunoenzymometric assay and the tissue sampling (liver, spleen, thymus, lung, kidney tissues) for the pathology study. For the lung tissue fixation, 10% formalin was instilled intratracheally *via* a catheter under the pressure of 20 cm H_2O until the lungs were fully expanded. Then the trachea was ligated and a complex of the heart and lungs was extracted from the thoracic cavity. The kidneys, liver, spleen, lungs and thymus were treated with 10% neutral formalin for 3 days. Then 0.4 cm-thick slides from each organ, except the thymus, were prepared. The thymus was examined separately. The isolated tissue specimens were dehydrated with ascending grades of alcohol, embedded in paraffin blocks, and 4-5-μm-thick sections were prepared according to a standard protocol. The specimens were stained with hematoxylin and eosin and with Van Gieson's picrofuchsin. The qualitative, quantitative and semi-quantitative evaluation of the obtained slides was performed using a Nikon Eclipse 50i microscope (Nikon, Tokyo, Japan), a Nikon DS-Fi1 camera and the NIS-Elements BR analysis software (Nikon). The degree of interstitial and intra-alveolar pulmonary edema and thymic cortico-medullary distinctions were evaluated qualitatively. The following signs of injury were chosen for analysis: dystrophy of hepatocytes, tubular dystrophy and kidney interstitial edema. The volume of the splenic white pulp, the count of T lymphocytes of the thymus cortex, and the thickness of alveolar septa were also quantitatively assessed. Blood leukocytes were counted with an automatic Hematology Analyzer MEK-8222J/K (Nikon Konden, Tokyo, Japan).

2.4 Immunochemical Assay

The serum levels of IL-1β, IL-6, and TNF-α were quantified by Quantikinine ELISA Kits (R&D Systems, Minneapolis, MN, USA). Immunofluorescence assay was performed using an automatic ELISA Analyzer BEP 2000 (Siemens, Berlin, Germany). 3 μm-thick paraffin specimens were stained with immunohistochemical markers and placed onto highly adhesive slides (Polysine Slides, Gerhard Menzel GmbH, Braunschweig, Germany). To detect CD3, CD57, p53 and B-cell lymphoma 2 (Bcl-2) markers, primary monoclonal murine antibodies (Spring Bio, Pleasanton, CA, USA) and secondary HRP-labelled antibodies (A3682, Sigma-Aldrich) were used. Immunohistochemical staining was conducted using an automated BenchMark ® ULTRA system (Ventana, Tucson, AZ, USA). The immunohistochemical reaction was assessed with a semi-quantitative method, which estimates the number of marker-expressing cells per 100 cells in total, within three randomly selected fields of view (Table 1).

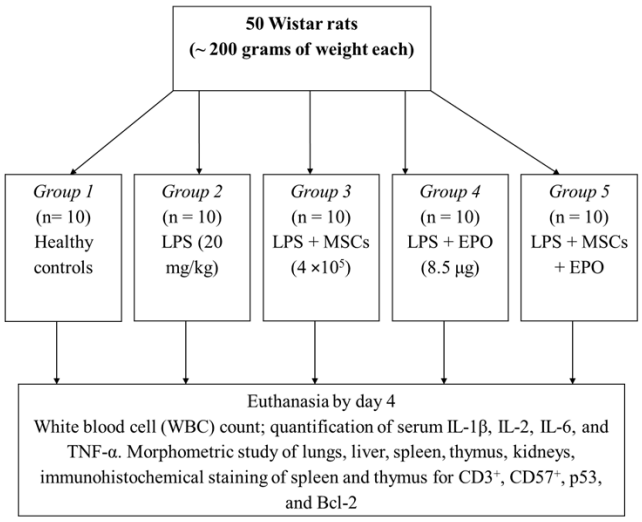

Fig. 1. Study design

Table 1. Immunohistochemical assessment test

Assessment grade	Intracellular immunohistochemical marker expression (cytoplasm and nuclei)	Level of marker expression
–	<5%	No expression
+	5 – 40%	Slight
++	40 – 70%	Moderate
+++	>70%	Intense

2.5 Statistical Analysis

The Statistica for Windows, version 7.0 software package (StatSoft Inc, Tulsa, OK, USA) was used. The data were presented as Mean ± Standard Deviation (S.D.). The statistical confidence interval of the measured indices between the studied groups was calculated by a one-way ANOVA test. The differences between the mean values were considered statistically significant at P = .05.

3. RESULTS AND DISCUSSION

Our main objective was an assessment of the biochemical and morphological differences between the experimental groups, thus, we did not focus on the survival rate statistics. We did observe death of animals in the experimental groups (2 rats in Group 2, 2 rats in Group 3, 3 rats in Group 4 and 2 rats in Group 5) within the first days of the experiment. Among the causes of death, there were acute respiratory distress syndrome (7 animals) and its combination with acute renal failure (2 animals). We excluded those rats from the morphological study because of potential artifacts of fixation caused by autolytic changes.

The highest statistically significant white blood cell (WBC) counts - $(8.15\pm1.4)\times10^9$ cells/L (P = .05) - were found in the group treated with the combination of EPO and MSCs (Group 5) (Fig. 2). In Group 3, treated with MSCs only, the WBC counts were $(4.05\pm2.35)\times10^9$ cells/L, and in Group 4 (treated with EPO alone) the WBC index was $(5.6\pm2.7)\times10^9$ cells/L. In Group 2 (treated with LPS only), the WBC level was $(6.52\pm1.9)\times10^9$ cells/L. All groups treated with LPS had significantly higher WBC numbers compared to those of control animals, which had the WBC index of $(2.15\pm0.41)\times10^9$ cells/L (P = .01).

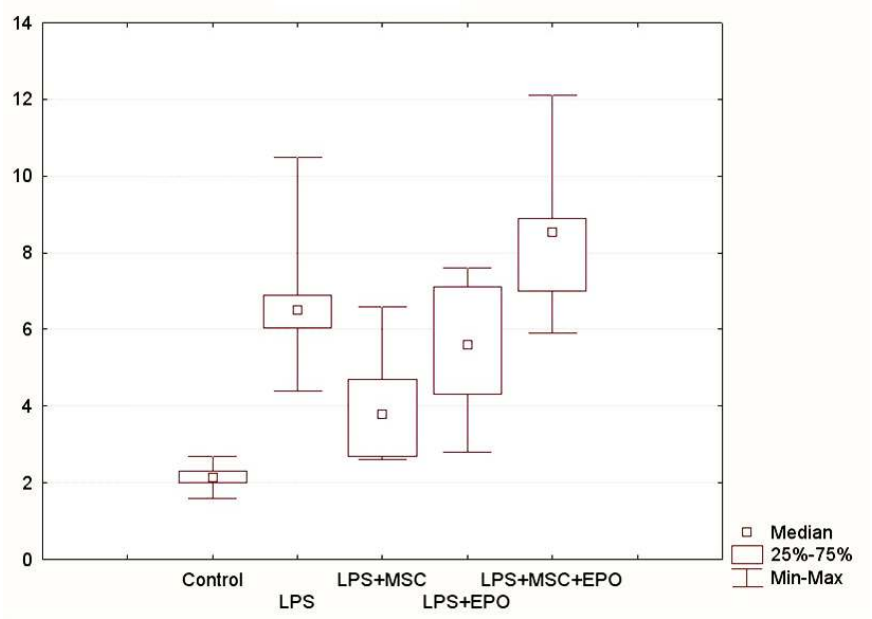

Fig. 2. WBC counts ($\times10^9$ cells/L) in the studied groups of rats

The data are presented as 25% - 75% percentile with the median and extreme values. The WBC count was performed with the MEK-8222 automatic hematology analyzer (Nihon Konden, Japan)

We did not observe any significant difference in the serum cytokines levels between the groups (data not shown) except for IL-1β. The serum IL-1β concentration for the rats treated with EPO +MSCs was significantly lower (58±22 pg/ml) than that for the animals treated with LPS alone (155±90 pg/ml, P = .01) and was close to the control group levels (38±13 pg/ml). In all the other groups, we did not observe significant differences in the serum IL-1β concentration compared with that for the LPS group. Indeed, this finding is suggestive of anti-inflammatory properties of MSCs.

The main results of the pathology analysis of different organs in the studied groups are shown in Table 2 and Fig. 3. The morphometric analysis of the lungs, kidneys, and lymphoid organs revealed a significant difference between the control animals and the Groups 2, 3, and 4 (P = .05). The morphometric changes revealed severe alveolar and interstitial pulmonary edema in the rats treated with LPS, as well as kidney tubular dystrophy and edema, hepatic and myocardial dystrophy (Fig. 3. I, IV, V). On the contrary, we did not observe any significant differences in the morphometric data between the control animals and Group 5 (Table 2). It means that Group 5 (MSCs+EPO) had the lowest degree of interstitial pulmonary edema and reduced tubular dystrophy and renal necrosis, when compared to Groups 2, 3, and 4, (Fig. 3. I, IV). In rats with engrafted MSCs, we found a tendency to reduction of both interstitial pulmonary edema and kidney tubular edema. However, the structural recovery was not as remarkable as that in Group 5 where the lowest rate of hepatocyte dystrophy was observed (Fig. 4).

In addition to edema and dystrophic changes in rats after the LPS administration, we also revealed a reduction in the splenic white pulp volume and a decrease in the number of cells in the thymic cortex (Table 2, Fig. 3. II). The treatment with MSC, EPO and EPO+MSC reversed this reduction of the lymphoid component; in the cases of EPO and EPO+MSC administration, a hyperplasia of both splenic white and red pulp was observed (Table 2, Fig. 3. II).

The immunohistochemical study confirmed a significant decrease in the number of CD3-positive T-cells in the splenic white pulp and in the cortex of thymus in rats treated with LPS (Fig. 4). In the group treated with EPO+MSCs, the cellular CD3 expression in the spleen and thymus was significantly increased both in the central (+3) and peripheral zones (+1-2) of the splenic white pulp, and also in the thymic cortex (+1) and medulla (+3) (Fig. 4).

Along with the reduction of the number of CD3-positive cells, a remarkable increase of CD57 (marker of NK cells) and p53 (apoptotic marker) expression was observed in the spleen and the thymic cortex in the LPS-treated group (Fig. 5). A weak CD57 and p53 expression was detected in the spleen and thymic cortex in the EPO and MSCs groups, and the corresponding staining tended to be negative in the control group and in the EPO+MSCs group. Additionally, we detected a moderate increase in the Bcl-2 expression in the EPO and EPO+MSCs groups (+1 - +2) compared to the other experimental groups of animals, where the Bcl-2 expression was almost absent (Fig. 6).

Our results generally confirm the hypothesis that EPO improves the effects of MSCs in experimental endotoxemia. Nevertheless, the mortality rate, which is one of the key indicators of successful treatment, was not statically different between the studied intervention groups, even though the degree of organ injury in the survived animals differed significantly.

Table 2. Morphometric characteristics of the examined tissues in the experimental groups

Index	Control	LPS	LPS + MSCs	LPS + EPO	LPS + EPO +MSCs
Alveolar septal thickening (μm)	10.32±2.05	31.25±2.9	15.3±2.29	18.6±1.1	10.82±1.08
Kidney tubular dystrophy (points)	0	2.54±0.31	2.22±0.29	1.90±0.12	0.74±0.19
Kidney tubular edema (points)	0.21±0.07	0.72±0.09	0.43±0.03	0.32±0.05	0.24±0.04
Hepatocyte dystrophy (points)	0	2.43±0.43	1.22±0.45	1.92±0.37	2.03±0.30
Percentage of T cells in thymus cortex (%)	86.24±2.88	34.59±1.34	49.37±2.16	38.21±1.73	81.48±2.63
Spleen white pulp volume (%)	23.95±2.8	11.15±3.8	19.9±2.92	22.8±0.98	27.15±2.89

P=0.05 (for the LPS group – as compared to the control group; for the other experimental groups – as compared to the LPS group)

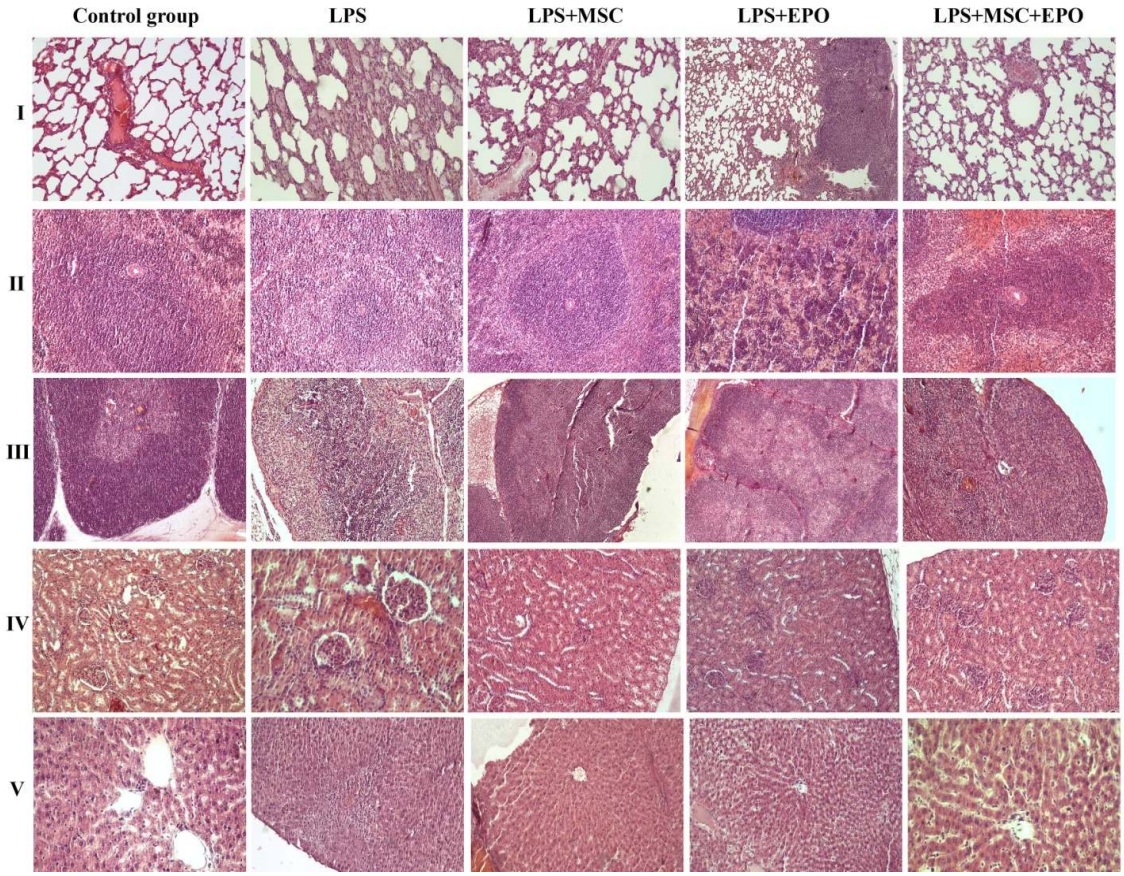

Fig. 3. The results of histological staining

I. Lung specimens. Control group, no histological alterations. Infiltration of leukocytes into interstitial and alveolar spaces, alveolar edema, thickening of alveolar septa (LPS treated group). Edema and leukocyte infiltration are moderately reduced (MSC-treated group). Slight decrease of acute lung injury, lymphoid hyperplasia (EPO-treated group). Significant reduction of inflammatory changes, hyperplasia of lymphoid nodes (EPO+MSCs-treated group). II. Spleen. Control group, normal structure of the red and white pulp. LPS - reduction of the white pulp. MSCs – the tendency of the white pulp to normalization. EPO, EPO+MSC – hyperplasia both in the white and red pulp. III. Thymus. Control group – normal ratio of the cortex and medullary area. LPS – Loss of the structural cortex and medulla distinctions. MSC, EPO –recovery of the T-cell number in the cortex. EPO+MSC – hyperplasia both in the cortex and medullary layers. IV. Renal specimens. The signs of edema and tubular dystrophy in LPS-treated animals. V. Liver. Lobular tissue dystrophy and necrosis in LPS-treated animals. Tissue samples 72 h post-LPS injection (stained with hematoxylin and eosin, ×100)

This observation appears to be related to the specifics of the given endotoxemia experimental model. In animals treated with LPS, the peak of the inflammatory response along with the cytokine release was observed within the first hour after the injection [25]. We believe that the treatment with MSC and EPO within 2 hours after the LPS administration could not be effective in animals with dramatic pulmonary and renal injury. Mei et al. [6] reported no difference in the mortality level between the control group and animals treated with MSCs. However, among the animals receiving an antibiotic therapy from the 1st day, a 7-day survival rate in the MSC group was 50%, while in the placebo group it was less than 20%. Immunosuppressive effects of MSCs on IL-2, IL-6 and TNF in wild-type mice were not associated with an improved survival in experimental staphylococcal toxic shock syndrome [26]. Kim et al. [26] suggest that immunosuppression mediated by MSCs has no effect on the critical pathways and mediators for the septic shock-associated mortality. We also assume that an injection of MSCs in the acute phase of septicemia might induce an additional antigenic response and cytokine release, being a potent stimulator of systemic inflammation. Notably, the dose of MSCs used in our study (4 ×

10^5 cells) was 5-7 times lower than the standard effective dose of (2-3) × 10^6 cells used for animals of this weight [27]. The choice of a low MSC dosage was not random and was made to estimate the EPO effects more precisely. Since standard MSC doses have been shown to produce rather promising results in the previous studies, an additional treatment with EPO in such a situation would not provide a considerable advantage. The low MSC dosage explains why these effects were not detected in Group 3 (MSCs only), in contrast to other experiments.

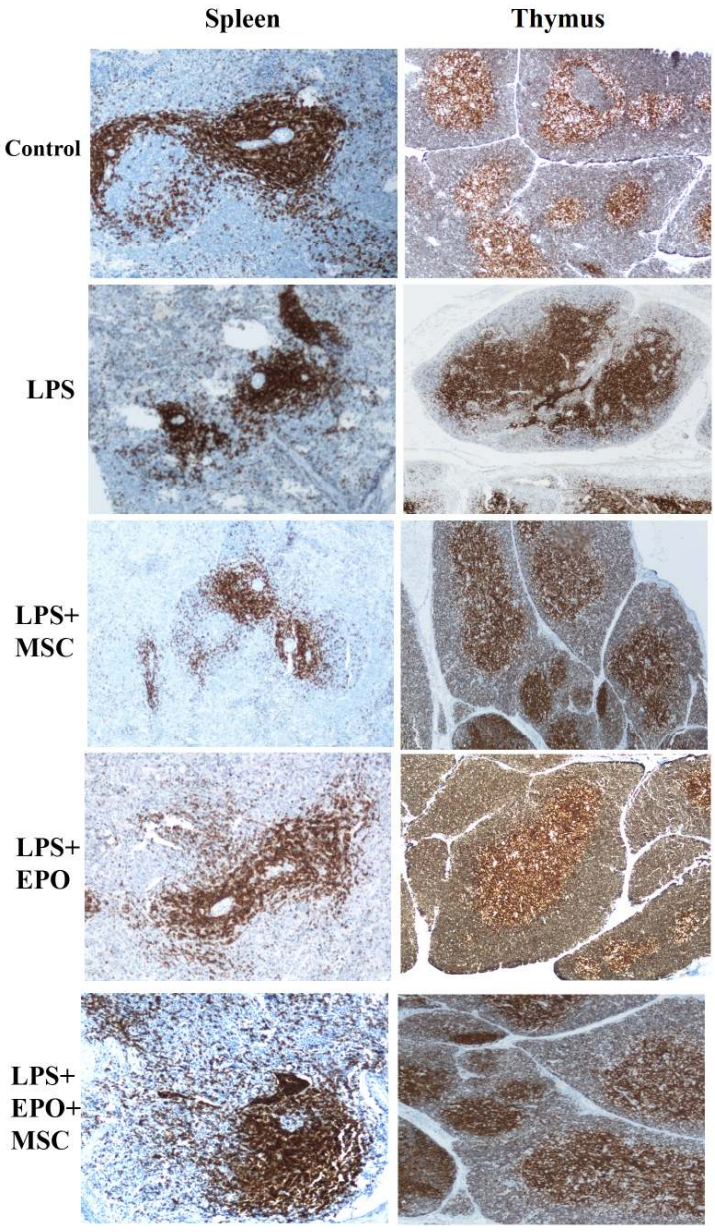

Fig. 4. Immunostaining of CD3+ T-cells in the spleen and thymus slices of experimental animals

The lack of CD+ cells in the white pulp and thymic cortex of LPS-treated animals. Hyperplasia of T-cells in the spleen white pulp and in the thymus cortex after the combined endotoxemia treatment. Secondary antibodies with HRP (A3682, Sigma Aldrich), DAB staining

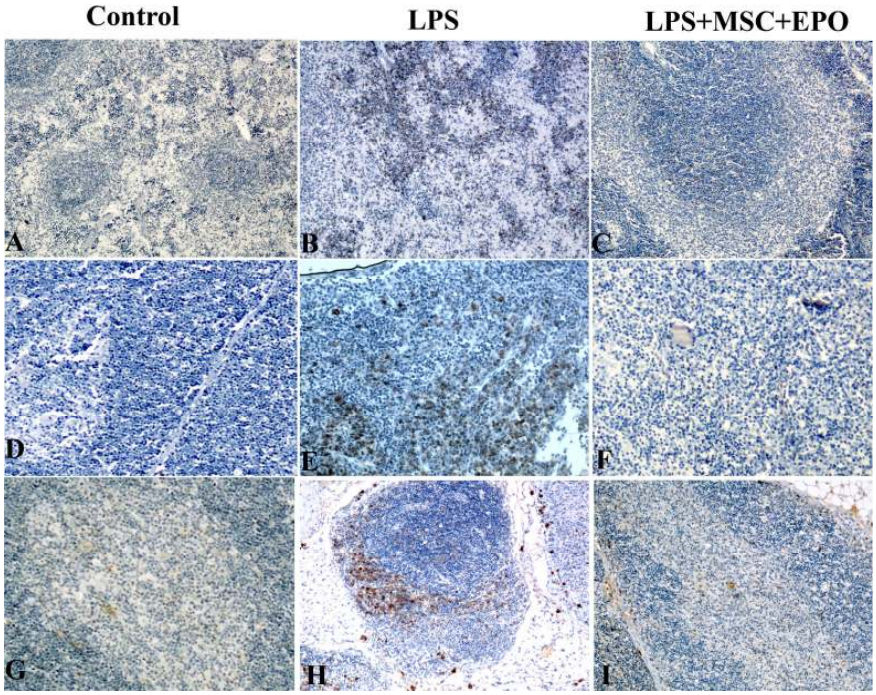

Fig. 5. p53 and CD57 immunostaining of the spleen and thymus slices

A. Spleen, p53, control group. B. Increase of p53+ cells in the spleen of rats from the LPS-treated group. C. Spleen. P53 staining, combined therapy with EPO and MSCs. D. Thymus, p53 control group. E. Thymus, appearance of p53 expression after the LPS treatment. F. Thymus, p53 staining after combined therapy with EPO+MSCs. G. Thymus, CD57 staining, control group. H. An increase of the CD57 expression in the thymus after the LPS administration. I. A reduction of CD57+ NK cells in the thymus of the EPO+MSC-treated rats. Immunostaining with secondary HRP-conjugated antibodies (Sigma Aldrich), DAB, hematoxylin co-staining, ×100

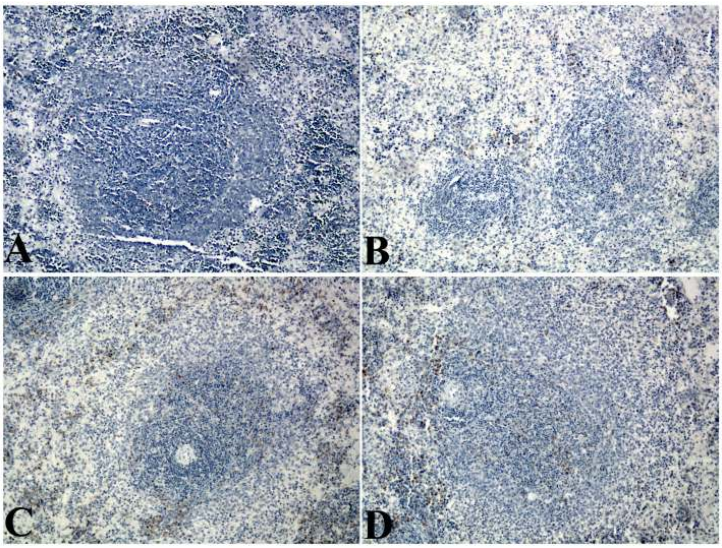

Fig. 6. Bcl-2 Immunostaining of the thymus

A. Control group (negative staining). B. MSC-treated group. C. EPO-treated group. D. EPO+MSC. Moderate Bcl-2 expression revealed after the EPO and EPO+MSC treatment. Immunostaining with secondary HRP-conjugated antibodies (Sigma Aldrich), DAB, hematoxylin co-staining, ×100

The absence of a significant difference in IL-1β, IL-6, and TNF-α expression can be explained by consistency in the cytokine dynamics in the LPS endotoxemia model. The peak bronchoalveolar lavage fluid concentration of these inflammatory mediators (which is more sensitive than the blood serum concentration) was observed 3 hrs after the LPS injection. In 6 hours, the TNF-α and IL-6 levels were lower by 2-3 times than the maximum level and became normal by day 2 [28]. The elevated levels of IL-1β remained high until day 5.

The WBC numbers in the MSC + EPO rats were significantly higher than those in the other groups. Considering the WBC level only as a marker of the inflammation severity, one could possibly come to a conclusion that the treatment was inefficient. However, if to consider this index as a marker for the immune response boost, it should be emphasized that the MSCs + EPO combination is more effective than MSCs or EPO alone. The results of the hematology study correspond to the data of the morphological investigation of the thymus and spleen which showed marked hyperplasia of the cortex and white pulp in these organs. Indeed, the LPS-treated groups showed various degrees of lymphoid tissue atrophy.

Although it is difficult to explain precise mechanisms of this phenomenon, it is possible to outline some of the key points. First, stem cells are known to suppress TNF-α expression that causes apoptosis in multiple cell types including lymphocytes [29,30]. Second, EPO also exhibits an anti-apoptotic effect on the thymus and spleen in the endotoxemia model through the suppression of caspase-3, -8, and -11 and initiating NO production [31]. Finally, leukocytes as well as MSCs have EPO receptors on their surface, a phenomenon that could explain EPO-dependent stimulation of the WBC lineage, increasing the life expectancy of the leukocytes [32]. For better understanding the results of our immunohistochemical study, it is necessary to consider the significance of immune cells' apoptosis in sepsis. Severe sepsis was shown to lead to apoptosis of T lymphocytes, highly correlated with the mortality [33]. For patients who died from sepsis, a significant depletion of T cells in the splenic white pulp and increased expression of apoptotic receptors were shown [34].

On the other hand, Bosman et al. [35] reported similar mortality rates in wild-type mice and a murine model of acute polymicrobial sepsis, with mice lacking the recombination activating gene 1 (Rag-1). Spleens of Rag-1-deficient mice were atrophic and completely free of $CD3^+$ T lymphocytes and $CD19^+$ B cells. Indeed, the authors suggested that T and B cells played a more remarkable role in the later stages of sepsis [35]. However, the temporal boundaries between the early and the late phase of sepsis are not precisely established. Muenzer et al. [36] observed that superinfection with *Pseudomonas aeruginosa* in 4 days after the CLP significantly increased the mortality of mice whereas infection on day 7 had no effect on survival. Splenocytes from the animals extracted in 4 days after CLP had a significant decrease in IFN-γ secretion compared to splenocytes from sham-operated animals or animals 7 days post-CLP [36].

In our study, the histological and immunohistochemical analyses were performed 72 h post-LPS, and the findings in the control LPS-treated Group 2 suggest an immune depletion. The maximum and moderate expression of the CD3 antigen in thymic and splenic lymphocytes, respectively, in the group treated with MSC and EPO may be indicative of T-cell proliferation in these organs due to the combined therapy effect.

The immunohistochemical study of the spleen and thymic tissues also showed increased expression of the CD57 antigen in the LPS-treated group and its normalization in the combined MSCs + EPO therapy group, as well as weak expression in the MSC- and EPO alone-treated groups. CD57 is a glycoprotein found mostly on the surface of NK cells and $CD8^+$ T lymphocytes [37]. An up-regulated expression of CD57 in T cells reflects a proliferative inability and/or replicative senescence. One of the survival pathways in $CD57^+$ T cells may be attributed to the up-regulation of various inhibitory NK cell receptors whose expression is associated with decreased susceptibility to apoptosis and elevated levels of anti-apoptotic Bcl-2 [38].

Many studies showed the anti-apoptotic effect of endogenous and exogenous Bcl-2 on immune cells in various animal models. Transgenic mice that selectively overexpress Bcl-2 in T cells have displayed a marked protection against lymphocyte apoptosis and a significantly improved survival in a CLP-induced sepsis suggesting that increased apoptosis in lymphoid organs plays a critical role in the adverse sepsis

outcome [39]. Iwata et al. [40] reported that administration of recombinant human Bcl-2 reduced apoptosis in cardiac, intestinal, and peritoneal neutrophils and improved survival after a CLP-induced sepsis in mice. We found moderate expression of Bcl-2 in the thymic tissue only in the rats treated with a combination of MSCs and EPO which suggested the suppressive effect of the combined therapy on the anti-apoptotic mechanisms. Based on our data, we suggest that the synergy of EPO- and MSCs-mediated effects enhance the efficiency of cell therapy and allows lowering the EPO dose, which in turn may reduce the risk of drug-induced thrombosis in septic patients. Our results confirm that the EPO treatment shows a positive effect of reduction of renal injury and lymphoid tissue apoptosis, but might not be effective enough compared to the therapy with small doses of MSCs and to the combined treatment with EPO and MSCs.

Our results are consistent with the earlier studies on MSC preconditioning in the EPO-containing medium [41]. As was demonstrated in [41], adipose tissue-derived MSC benefited from in vitro preconditioning in the medium with recombinant human EPO (50 ng/L). In the conditions of H_2O_2-induced oxidative stress, the preconditioned MSC had a lower rate of apoptosis and a better survival, as compared with the control cells (29±3.41% of apoptotic cells vs. 63.03±4.96% $p<0.01$). Thus, we believe that the EPO therapeutic effect in the MSC-EPO treatment of sepsis is associated primarily with its positive influence on MSC viability, rather than with its action onto the recipient organism. The significance of our study is in the fact that we first have shown the beneficial effect of MSCs in combination with EPO in the endotoxemic rat model. We suggest that our findings may be applicable to other inflammatory diseases, in which MSC transplantation can be potentially beneficial.

4. CONCLUSIONS

We have shown that, in the endotoxemic rat model, a co-treatment with EPO and MSCs reduces acute lung injury and renal damage, has an anti-apoptotic effect on the lymphoid tissue, and enhances the immune response to a greater extent than the treatment with these agents used separately does. The results of this study may offer new promises for improving the outcomes of sepsis.

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CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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