



Adipose-Derived Mesenchymal Stem Cells and Conditioned Medium Attenuate the Memory Retrieval Impairment During Sepsis in Rats

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Abstract

In this study, we hypothesized that sepsis induction impairs memory retrieval in rats while transplanted mesenchymal stem cells (MSCs) and MSC-conditioned medium (MSC-CM) application are capable of attenuating those complications. MSCs were obtained from adipose tissue of rats and at the second culture passage; MSCs and MSC-CM were collected. Rats were randomly divided into four experimental groups: sham, CLP, MSC, and MSC-CM. Sepsis was induced by cecal ligation and puncture (CLP) model in the CLP, MSC, and MSC-CM groups. The MSC group received 1×10^6 MSCs/rat (i.p., 2 h after CLP surgery); the MSC-CM rats received the conditioned medium (CM) from 1×10^6 MSCs intraperitoneally 2 h after sepsis induction. Novel object recognition test, sepsis score, and blood pressure measurement were performed 24 h after the treatments. The right hippocampus was taken for western blot analysis. CLP rats showed a significantly higher sepsis score and systolic blood pressure. They also had a significant increase in the phosphorylated form of CAMKII- α , cleaved caspase 3 and Bax/Bcl2 ratio, and a reduction in c-fos protein in the hippocampus tissue samples compared with the sham group. MSC transplantation and MSC-CM administration significantly decreased the mean sepsis score and prevented sepsis-induced attenuation of blood pressure compared with the CLP rats. Animals in the MSC and MSC-CM groups showed a better memory retrieval, attenuation in phosphorylated form of CAMKII- α , cleaved caspase 3 and Bax/Bcl₂ ratio, and an increase in c-fos protein expression compared with the CLP group. It seems that CAMKII and c-fos are inversely involved in regulating memory processes in hippocampus. Phosphorylated form of CaMKII- α overexpression may impair the ability of object recognition. Our findings confirmed that MSC-CM application has more advantages compared with transplanted MSCs and may be offered as a promising therapy for inflammatory diseases such as severe sepsis.

Keywords Sepsis · Mesenchymal stem cells · Conditioned medium · CAMKII · C-fos proteins · Memory · Hippocampus

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Introduction

Until recently, sepsis was characterized as excessive inflammatory response complicated by refractory hypotension to an infection whereas nowadays, it is defined as a dysfunction of the host reaction, leading to multiple organ failure and death [1, 2]. In clinical settings, septic shock is characterized by failure in maintaining the blood pressure above 65 mmHg by adequate resuscitation, requiring the use of vasoconstrictors [3]. Currently, there is no cure for sepsis and septic shock conditions and the management is mostly symptomatic containing antibiotics and catecholamine infusion [4].

Sepsis associated encephalopathy (SAE), as one of the main complications of sepsis, seems to be an ignored cause of impaired mental state and delirium in seriously ill septic patients. This is due to the lack of well-established screening

tools and biologic markers to estimate brain dysfunction occurring during sepsis [5]. In many patients, SAE symptoms usually emerge in the early phase of sepsis in a wide range from mild disorientation or agitation to impaired consciousness and coma even before diagnosis of the other organs dysfunction [6]. Although SAE is often defined as an acute and reversible syndrome, a growing body of evidence supports the view that it may cause considerable long lasting cognitive dysfunctions, including disorders in mental processing-speed and memory retrieval which may remain several years after recovery from sepsis [7]. The extent and details of pathophysiology of sepsis-induced memory impairment are unknown. It seems that the exacerbated host immune response, vascular damage, and inflammatory cytokines finally increase the blood-brain barrier permeability, facilitating the infiltration of immune cells from the bloodstream into the brain. These changes together with the brain immune response, severe microglial activation, and oxidative stress inducers will cause the brain damage particularly within the limbic system [8].

Over the last decade, stem cell therapy was introduced as a potentially novel approach for the treatment of various disorders including sepsis and septic shock [9, 10]. In this regard, mesenchymal stem cells (MSCs) are considered to be the most attractive candidates for experimental and clinical applications [11, 12]. MSCs are introduced as one of the most available primary cells that can be easily isolated from various tissues, such as bone marrow, adipose tissues, umbilical cord blood, and amniotic fluid, as well as placenta and some other sources. The easily culture method and specific biological functions of MSCs have made them a well-known candidate for the cell therapy in preclinical experiments and clinical trials [4]. Regarding the mechanisms underlying these biological functions, it was originally hypothesized that MSCs infiltrate into the damaged tissues, become resident and start to differentiate, and finally replace the injured cells. However, subsequent reports showed very little and transient MSC transplantation and differentiation at damaged tissues and organs. Currently, it seems that MSCs exert their protective effects mainly by trophic factors secretion (MSC-conditioned medium, MSC-CM) [13]. MSC-CM has been administered in different disease models and the results have shown that their functions are similar to those of MSCs, including neuroprotection, immunosuppression, tissue-repair, and anti-inflammatory effects [14, 15].

Novel object recognition (NOR) test is known to be as a standard task for evaluating a rodent's ability to identify a previously presented stimulus and is currently an accepted method to evaluate non-spatial object memory in rodents [16]. During the training session, the rodent faces two identical novel objects presented in a familiar arena. Memory storage (object memory encoding) occurs during the training session. Animal is removed from the box and during this step, the object memory consolidation is occurred. In the following test session, animal is returned to the same arena containing both

the familiar object and a novel one to test the object memory retrieval [17].

Synaptic plasticity is defined as an activity-dependent change in synaptic transmission and is associated with short-term and long-term changes in the cellular and subcellular architecture [18, 19]. Our understanding of the synaptic plasticity and memory formation has been markedly increased by discoveries concerning the role and regulation of CaMKII in the nervous system. Over the past 2 decades, CaMKII has been considered one of the most attractive factors in the nervous system specially hippocampus, and is introduced as the main protein involved in learning, memory [20, 21], and synaptic plasticity [22]. Studies suggest that this enzyme may play a major role in memory consolidation and remote memory formation [23]. In addition, it is reported that c-fos is majorly involved in neuronal plasticity, mandatory for memory processes. This protein's expression is induced following learning which is indicative of a change in neuronal activity [24, 25] and potentially contributed to the recognition of novel objects. Therefore, c-fos expression is used as a marker of the neuronal activation level during memory processes [25, 26].

In the present study, sepsis was induced by cecal ligation and puncture (CLP) model; this produces a source of necrotic tissues similar to the clinical sepsis, especially after severe tissue trauma. In the present study, sepsis induction was confirmed by evaluating sepsis score and systolic blood pressure indices. Here, we hypothesized that sepsis induction impairs memory retrieval in rats, and transplanted MSC and MSC-CM administration are capable of attenuating those complications. First, we used the novel object recognition test to investigate the cognitive consequences of sepsis and septic shock states. Then, in confirmation of our aforementioned aims, we evaluated phosphorylated form of CaMKII- α (Thr 286)/CaMKII- α ratio and c-fos protein levels as well as apoptotic parameters (cleaved caspase 3 and Bax/Bcl2) in the hippocampus tissues. Finally, we compared the positive effects of adipose-derived MSC and MSC-CM administration on these parameters.

Materials and Methods

Isolation, Culture, and Expansion of Rat Adipose-Derived MSCs

Under sterile conditions, adipose tissue samples were collected from inguinal fat pads of six rats killed by CO₂ asphyxiation. Adipose tissues were minced into the small pieces before digesting in 4 mg Collagenase Type I solution with final concentration of 0.1% (Invitrogen Gibco) under gentle agitation for 15 min at 37 °C [27, 28]. The digested mixture was diluted with 4 ml of culture medium (Dulbecco's modified Eagle's medium [DMEM] including 15% fetal bovine serum [FBS])

and then centrifuged at 1500 rpm for 15 min to separate cell fraction (pellet) from adipocytes. The supernatant was disposed, and the cellular pellet was then filtered through a 200- μm nylon mesh to remove undigested tissues and was cultured in DMEM-HG containing 15% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, then incubated (37 °C and 5% CO₂). The first medium substitution was performed about 2 days after culture initiation in which the non-adherent cells were omitted. Thereafter, this was repeated every 48 or 72 h. After achieving 80–90% confluency, MSCs were incubated with trypsin 0.05% (Sigma, USA) and 0.02% EDTA for new passage and were cultured until passage 2 [27–29] (Fig. 1a).

Characterization of MSCs by Flow Cytometric Analysis

At the second passage, MSCs were trypsinized, washed by phosphate buffer saline (PBS), and resuspended in PBS containing FBS (1%). A 100 μL aliquot of suspended MSCs was incubated for 45 min at 4 °C with one of the following anti-mouse monoclonal antibodies (mAb): phycoerythrin (PE)-conjugated CD34, or fluorescein isothiocyanate (FITC)-conjugated CD45 and CD44, or PerCP conjugated CD90 (BioLegend, USA) along with Rat IgG2b isotypic antibodies (BioLegend, USA) as control. After labeling the cells, they were evaluated using BD FACS Calibur™ flow cytometer (BD, USA) and analyzed using the Flow Jo 7.6 Software (Fig. 2).

Characterization of MSCs by Differentiation Assay

MSC differentiation ability into osteocyte and adipocyte lineages was evaluated at the second passage.

Osteogenic Differentiation

MSCs (1×10^4 cells/well) were seeded in 24-well plates (SPL, Korea) and incubated at 37 °C for 24 h; osteogenic differentiation media (100 mM dexamethasone, 10 mM β -glycerophosphate, and 5 g/mL ascorbic acid) was added to the cells every 72 h during 3 weeks. The cells were fixed with 4% paraformaldehyde and mineralization of them was determined by Alizarin Red S staining (Fig. 1b) [29, 30].

Adipogenic Differentiation

MSCs (15×10^3 cells/well) were cultured in 24-well plates (SPL, Korea) and incubated at 37 °C. After 24 h, adipogenic differentiation media (100 mM indomethacin, 0.5 mM 3-isobutyl-methylxanthine, 250 mM dexamethasone and 5 mM bovine insulin) was added to the cells every 3 days and incubated for 2 weeks. Adipose vacuoles were detected by Oil Red O staining after fixing the cells with 4% paraformaldehyde (Fig. 1c) [29, 30].

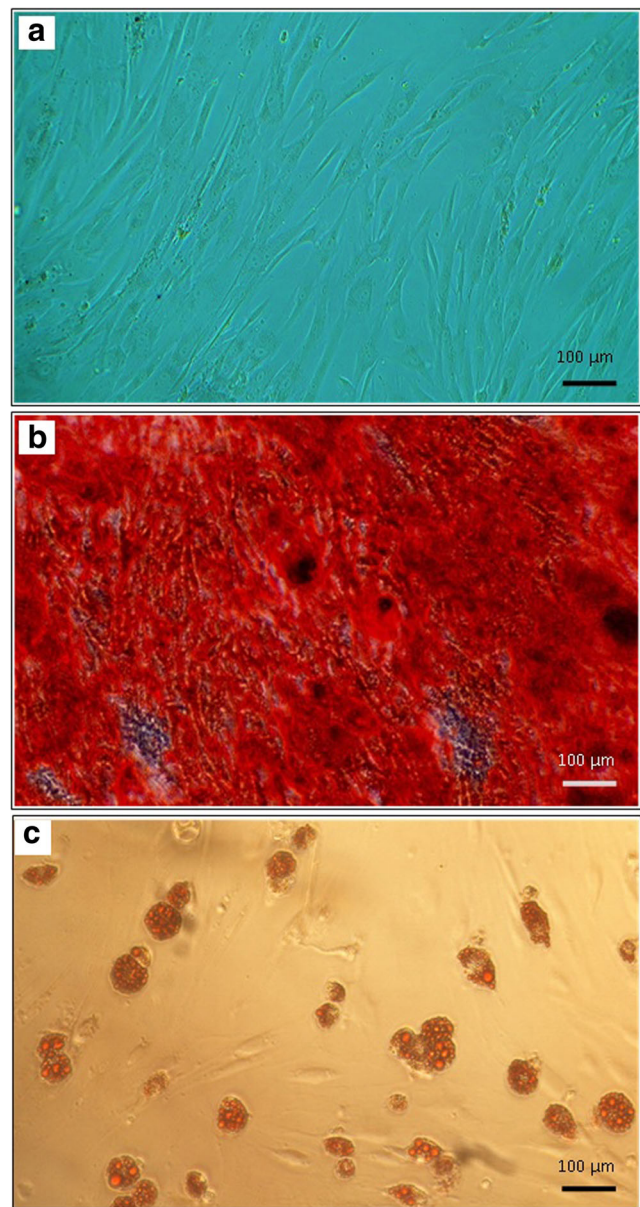


Fig. 1 Photomicrographs of the cultures prepared from adipose tissue-derived MSCs. The adherent cells were observed mainly as spindle-shaped cells at the primary cultures (a). Osteogenic foci appeared as red area following alizarin red staining method (b). Lipid droplets developed following adipogenic differentiation of the cells was stained red with oil red staining method (c). Magnification of all images: $\times 100$

Collection and Concentration of MSC-CM

A conditioned medium collection protocol has been described in several reports [27, 28]. Briefly, in our study, MSC-CM was collected after 48 h incubation of MSCs at the second passage in serum-free culture media. The prepared supernatant was centrifuged, filtered, and immediately injected to animals in the MSC-CM group. The protein concentration of MSC-CM was measured to be 800–1200 $\mu\text{g}/\text{ml}$ using the protein assay kit (Thermo Fisher Scientific, Pierce™ BCA).

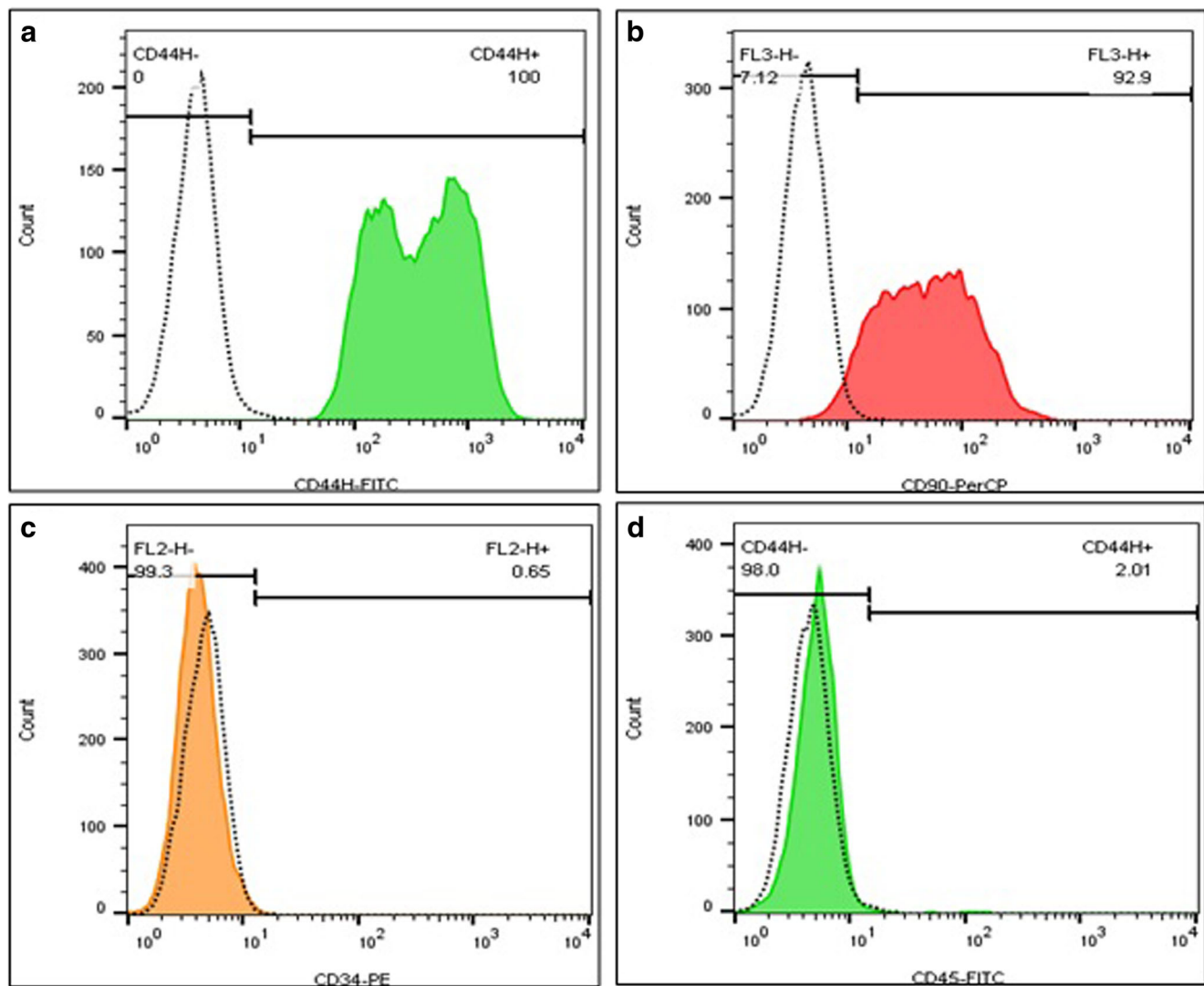


Fig. 2 Characterization of rat adipose tissue-derived MSCs by flow cytometry analysis. **a** and **b** MSCs with high expression of mesenchymal markers [CD44 and CD90]. **c** and **d** Low levels of hematopoietic markers [CD34 and CD45]

Animal Study

Male Wistar rats were obtained from Tehran University of Medical Sciences. Forty-eight rats weighing 220–250 g were maintained in an animal house under standard conditions (12 h light-dark cycle; 20–22 °C) and had free access to food and water. All procedures and animal care methods in the experiments were approved by the Animal Ethics Committee of Tehran University of Medical Sciences (Project number: 41504, Approval ID: 230).

Sepsis Induction by Cecal Ligation and Puncture Model

Under isoflurane anesthesia, a 2-cm midline incision was made and cecum was precisely separated to avoid injury to the blood vessels. Next, the cecum was tightly ligated with a

4.0-silk suture at its base, below the ileocecal valve, punctured twice with an 18-gauge needle and gently squeezed to extrude a small amount of feces from the perforation site into the peritoneal cavity. Then, the abdomen was sutured in two layers with 4.0-silk sutures, followed by fluid resuscitation [saline (subcutaneously, 3 mL/100 g body wt)] and returning animal to its cage [31]. If needed, analgesia was provided by an intra-muscular injection of 0.86 mg/kg ketorolac.

The rats were randomly assigned into the four experimental groups (12 in each): sham, CLP (cecal ligation and puncture), MSC, and MSC-CM. Rats in the CLP, MSC, and MSC-CM groups underwent CLP for the induction of sepsis. In the sham group, the abdominal cavity was exposed without CLP surgery. Two hours after the CLP procedure, animals in the MSC group received MSCs (1×10^6 cells/rat, at passage 2, intraperitoneally) suspended in 50 μ L sterile PBS. Animals in the MSC-CM group received the conditioned medium (CM) from

1×10^6 MSCs intraperitoneally (CM volume was 7–10 mL) while the sham and CLP groups received only PBS [27–29]. All of the rats were kept in their cages. Twenty-four hours after the treatment, novel object recognition (NOR) test was performed for the assessment of learning and memory, then sepsis score was evaluated, and finally, the blood pressure was measured. Rats were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally and then decapitated. The right hippocampus was snap-frozen in liquid nitrogen and stored at 80 °C for western blot analysis.

Sepsis Score

Twenty-four hours after the treatment, one of the co-authors, who was blinded to the treatment procedure, evaluated the rats in their cages (the lids were removed for better monitoring). Appearance (i.e., degree of piloerection), level of consciousness, spontaneous activity, response to touch and auditory stimuli, eyes opening and secretion, and respiratory rate and quality (labored breathing or gasping) were scored according to Shrum's scoring system [32]. The scores of each item mentioned above were between 0 and 4. A higher score means the greater severity of sepsis.

Blood Pressure Measurement

Systolic blood pressure was recorded non-invasively twice, prior to the surgery and at the end of the study prior to the decapitation using PowerLab Tail cuff system. In this regard, one conscious rat was placed into a rat restrainer. After cleaning the tail, the tail-cuff was placed on it. Then, the non-invasive blood pressure sensor was placed distal to the tail-cuff. To measure systolic blood pressure, the tail-cuff was deflated and at this time, the blood pressure was monitored and recorded. Systolic blood pressure was measured for three successive times. A difference less than 5 mmHg was considered the average pressure.

Novel Object Recognition Test

The novel object recognition test is based on the innate tendency of rodents to differentially explore novel objects over familiar ones. The test of novel object recognition (NOR) task took place in a 40 × 50-cm open field apparatus surrounded by 50-cm high walls. The procedure includes three phases: habituation, training, and testing. One day prior to the experiments, animals were submitted to a habituation session where they were allowed to freely explore the open field for 10 min. No objects were placed in the box during the habituation trial. On the experiment day and before the sepsis induction, training was conducted by placing individual rats for 10 min in the field, in which two identical objects (objects A1 and A2; both being cubes) were positioned in two adjacent corners. The number of seconds of exploration of each object was recorded

by two separate timers. Any rats that explored the objects for < 30 s excluded from the experiment. On the following day, testing was performed by placing individual rats in the field in the presence of one familiar (A) and one novel (B, a trapezoidal-shaped) object. Exploration time for each object was recorded for 10 min [25, 33]. All objects had similar textures (smooth), colors (red), and sizes (weight 100–150 g), but distinctive shapes.

The discrimination index [d1] and discrimination ratio [d2] were calculated by following formulas:

$$d1 = [\text{novel (sec)} - \text{familiar (sec)}]$$

$$d2 = d1 / [\text{total novel (sec)} + \text{familiar (sec)}]$$

Western Blot Analysis

The hippocampus tissue samples were homogenized using lysis buffer, and then, total protein extract was obtained by centrifugation in 15,000 rpm for 5 min. The protein amount in the supernatants was quantified using the Bradford's method [34]. Lysates (60 µg of protein each) were loaded on 12% SDS-PAGE and then transferred to a PVDF membrane (Chemicon Millipore Co. Temecula, USA). Membranes were blocked in 2% Electrochemiluminescence (ECL) advanced kit blocking reagent (Amersham Bioscience Co. Piscataway, USA) and incubated individually with primary antibodies overnight. After 3 times washing with TTBS, the blots were incubated with rabbit IgG-horseradish-peroxidase (HRP) conjugated secondary antibody (1/3000, Cell Signaling Technology Co. New York, USA) for 1 h at room temperature. Furthermore, the reactive bands were detected using a chemiluminescence kit reagent (Amersham Bioscience Co. Piscataway, USA). The blots were quantified by the ImageJ software. Rabbit anti β-Actin (1/1000, Cell Signaling Technology Co. New York, USA) was used as internal control.

Statistical Analysis

The data were presented as mean ± standard error of mean. Repeated measures ANOVA was used for sepsis score and systolic blood pressure analysis and the other variables were analyzed using one way ANOVA. Tukey's test was selected as post hoc analysis and $p < 0.05$ was considered statistically significant.

Results

Effects of Adipose-Derived MSC and CM Administration on Sepsis Score During Sepsis Induced by CLP Model

In this study, a scoring system was applied to predict severe sepsis during the experimental timeline. Appearance, level of

consciousness, spontaneous activity, response to touch and auditory stimuli, eyes opening and secretion, and respiratory rate and quality were scored according to Shrum's scoring system [32]. The scores of each item pointed out above were between 0 and 4. A higher score means the greater severity of sepsis. Rats typically recovered quickly after the surgical procedure and started to drink. Approximately 10 h after CLP, they still appeared to be completely normal. Twelve hours following operation, most rats began to show some illness signs (Fig. 3). At this time, the mean sepsis scores in the CLP and MSC groups were 8 ($p < 0.05$) and 5.6 ($p < 0.05$) respectively and no significant difference was seen between these groups. MSC-CM administration prevented the increase in the mean score of this group (mean score: 2.4) compared with the CLP ($p < 0.05$) and MSC groups ($p < 0.05$).

Compared with the sham-treated rats with a mean score of 1 after 24 h, septic rats significantly showed higher sepsis scores (mean score: 26, $p < 0.01$). MSC transplantation and MSC-CM administration significantly decreased the mean sepsis score to 8.2 ($p < 0.01$) and 6 ($p < 0.01$) respectively compared with the CLP rats (Fig. 3).

Effects of Adipose-Derived MSC and CM Administration on Systolic Blood Pressure During Sepsis Induced by CLP Model

In addition to sepsis score, we have also measured systolic blood pressure, since low blood pressure is an important clinical feature of sepsis and is associated with increased mortality rate in these patients. Systolic blood pressure was recorded non-invasively, twice, prior to the surgery and at the end of

the study prior to the decapitation using PowerLab Tail cuff system. At the beginning of the experiments, the systolic blood pressure of all animals was measured to be in normal range. Twenty-four hours after the treatment, blood pressure was significantly declined to lower levels in the CLP group (63.4 mmHg \pm 3.02) compared with the base state (116.25 mmHg \pm 4.24) and with the sham group (116.48 mmHg \pm 5.1) ($p < 0.01$). MSC and MSC-CM therapy significantly prevented the hypotension induced by sepsis (106.4 mmHg \pm 2.24, 116.81 mmHg \pm 7.5 respectively, $p < 0.01$) (Fig. 4).

Effects of Adipose-Derived MSC and CM Administration on Novel Object Recognition Test During Sepsis Induced by CLP Model

Novel object recognition is a well-established task, used to assess learning and memory in rodents. During the initial acquisition episode, animals observe a pair of identical objects, and the recalling of this learned information allows the healthy animal to discriminate between a familiar and a novel object during the recognition phase of the task (memory retrieval). CLP rats showed a decrease in the discrimination index [d1] (7.1 ± 2.03 , $p < 0.001$) and an increase in the discrimination ratio [d2] [0.86 ± 0.1 , ($p < 0.01$)] compared with the sham group [d1: 152.5 ± 24.8 , d2: 0.62 ± 0.06]. MSC transplantation and MSC-CM administration significantly increased d1 index [107.5 ± 27.53 s, ($p < 0.01$); 110.75 ± 21.2 , ($p < 0.01$) respectively] meanwhile decreased d2 [0.42 ± 0.08 , ($p < 0.05$); 0.45 ± 0.1 , ($p < 0.05$) respectively] compared with the CLP group (Fig. 5).

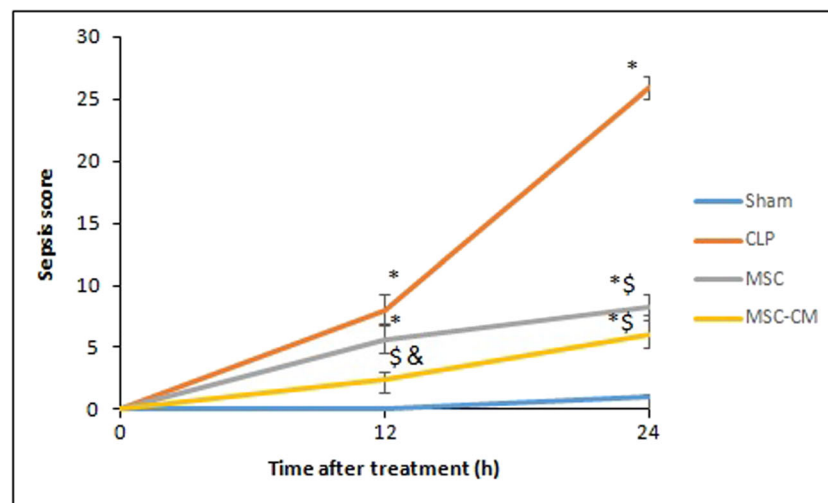


Fig. 3 Effect of MSC and MSC-CM administration on sepsis score during sepsis induced by CLP model. Data are presented as the mean \pm standard error of mean ($n = 10$ in each group). * $P < 0.05$ compared with the sham group. \$ $P < 0.05$ compared with the CLP group. & $P < 0.05$ compared with the MSC group. CLP, sepsis was induced by cecal

ligation and puncture; MSC, 2 h after the sepsis induction, the rats received mesenchymal stem cells (1×10^6 cells/rat, i.p.) suspended in 50 μ L sterile PBS. MSC-CM, 2 h after the sepsis induction, the rats received the conditioned medium (CM) from 1×10^6 MSCs intraperitoneally

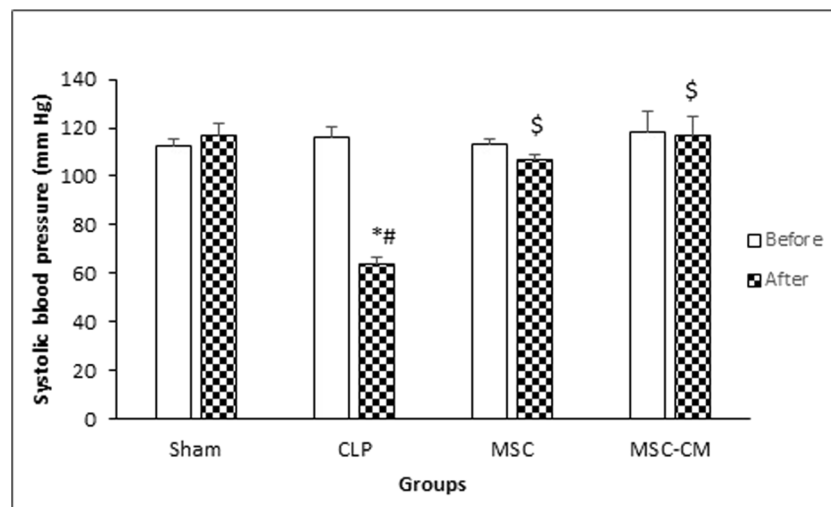


Fig. 4 Effect of MSC and MSC-CM administration on systolic blood pressure during sepsis induced by CLP model. Data are presented as the mean \pm standard error of mean ($n=8$ in each group). # $P<0.05$ compared with the base state. * $P<0.05$ compared with the sham group. \$ $P<0.05$ compared with the CLP group. CLP, sepsis was induced by

cecal ligation and puncture; MSC, 2 h after the sepsis induction, the rats received mesenchymal stem cells (1×10^6 cells/rat, i.p.) suspended in 50 μ L sterile PBS. MSC-CM, 2 h after the sepsis induction, the rats received the conditioned medium (CM) from 1×10^6 MSCs intraperitoneally

Effects of Adipose-Derived MSC and CM Administration on Phosphorylated Form of CAMKII- α and c-Fos Protein Expression in the Hippocampus Tissue During Sepsis Induced by CLP Model

Since during sepsis condition, hippocampal and associated areas are likely to be damaged and memory retrieval is impaired, we evaluated the beneficial effects of MSC and MSC-CM on p-CaMKII- α (Thr 286)/ CaMKII- α ratio and c-fos proteins in the hippocampal tissue samples as two possibly important molecules involved in memory processes. CLP rats had an increase in the phosphorylated form of CAMKII- α ($p<0.01$) and a reduction in c-fos ($p<0.05$) protein in the hippocampus tissue samples compared with the sham group. Treatment with MSC and MSC-CM reduced phosphorylated form of CAMKII- α ($p<0.05$) and increased c-fos protein expression ($p<0.05$) compared with the CLP group (Fig. 6).

Effects of Adipose-Derived MSC and CM Administration on Apoptotic Parameters in the Hippocampus Tissue During Sepsis Induced by CLP Model

Since sepsis-associated encephalopathy may lead to Ca^{2+} overload and excitotoxicity, we decided to evaluate the effects of adipose-derived MSC and CM administration on some apoptotic parameters in the hippocampus tissue samples during sepsis. Compared with the sham group, CLP rats showed an increase in cleaved caspase 3 protein ($p<0.01$) and Bax/Bcl2 ratio ($p<0.05$) in the hippocampus tissues. MSC and MSC-CM administration resulted in

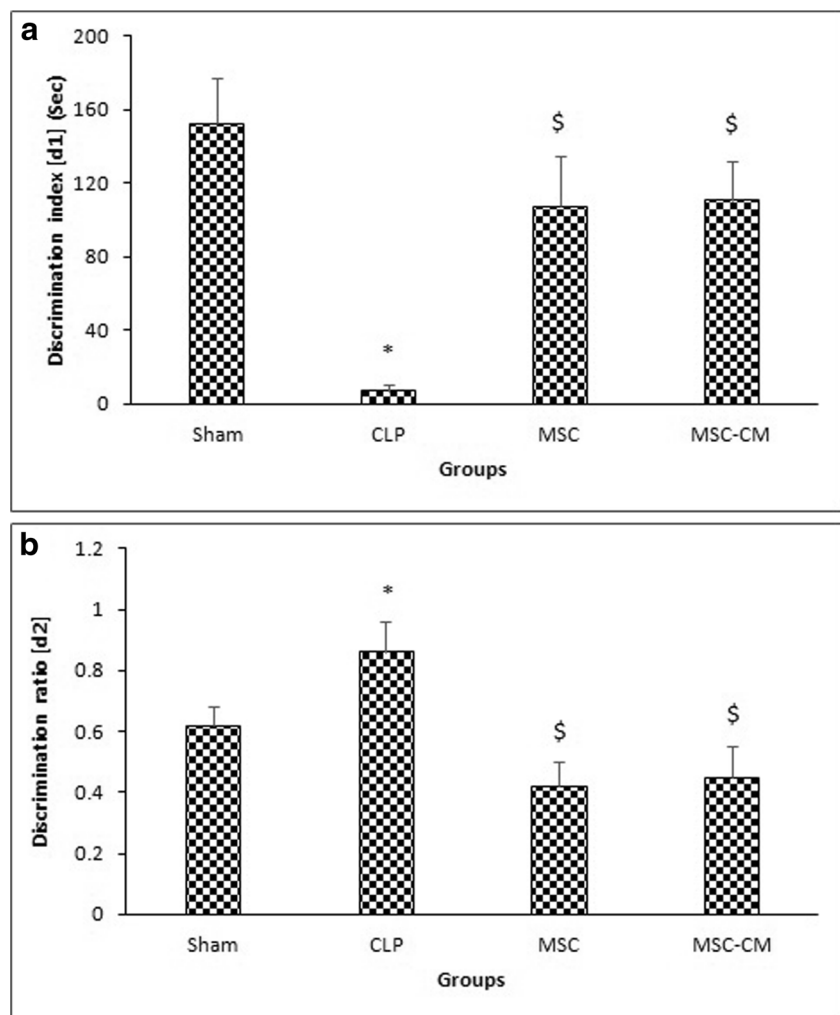
attenuation in cleaved caspase 3 ($p<0.05$) and Bax/Bcl2 ratio ($p<0.05$) compared with the CLP group (Fig. 7).

Discussion

In the present study, a scoring system was applied to predict severe sepsis during the experimental timeline. Shrum et al. have introduced this scoring system for application in mice [32], but it seems to be reliable in rats as well. In our study, 12 h after sepsis induction, no significant difference was seen in the mean score between the CLP and MSC groups whereas a significant decrease was reported in this index in the MSC-CM compared with the CLP and MSC groups. Twenty-four hours after the treatment, the mean score of septic rats was significantly greater than the MSC and MSC-CM groups, and no significant difference was seen between the MSC and MSC-CM groups. This finding confirms that MSC-CM administration is more effective than MSC transplantation, since the beneficial effects of the media appears earlier than the positive effects of the cell therapy.

Systolic blood pressure was recorded before the surgery and also at the end of the study prior to animal decapitation. At the beginning of the experiments, the systolic blood pressure of all animals was measured to make sure that this value is in normal range. Twenty-four hours after the treatment, the blood pressure was significantly declined to the lower levels in the CLP group compared with its base state and to the sham group. MSC transplantation and MSC-CM administration returned blood pressure back to the normal values. Thus, sepsis treatment with MSC and MSC-CM was associated with positive results on systolic blood pressure.

Fig. 5 Effect of MSC and MSC-CM administration on novel object recognition test during sepsis induced by CLP model. **a** Discrimination index [d1] and **b** discrimination ratio [d2]. Data are presented as the mean \pm standard error of mean ($n = 10$ in each group). * $P < 0.05$ compared with the sham group. \$ $P < 0.05$ compared with the CLP group. CLP, sepsis was induced by cecal ligation and puncture; MSC, 2 h after the sepsis induction, the rats received mesenchymal stem cells (1×10^6 cells/rat, i.p.) suspended in 50 μ L sterile PBS. MSC-CM, 2 h after the sepsis induction, the rats received the conditioned medium (CM) from 1×10^6 MSCs intraperitoneally



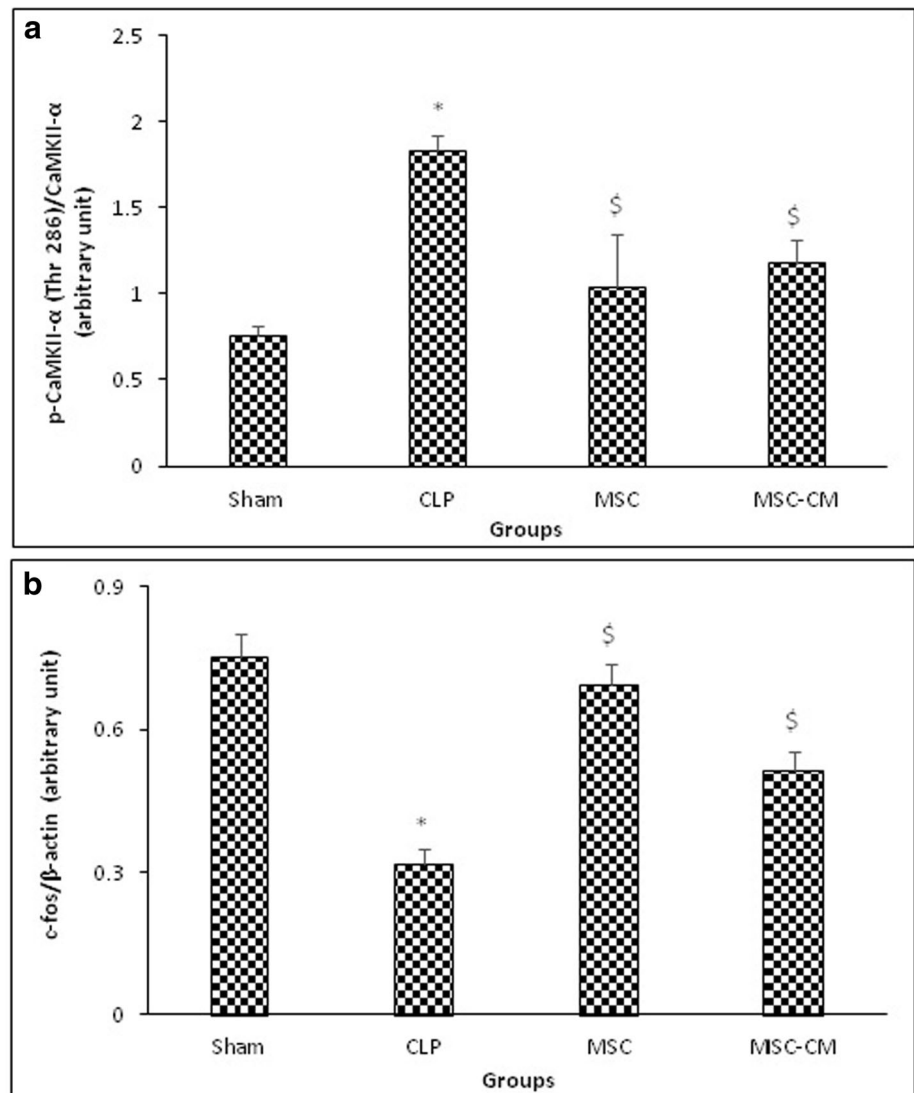
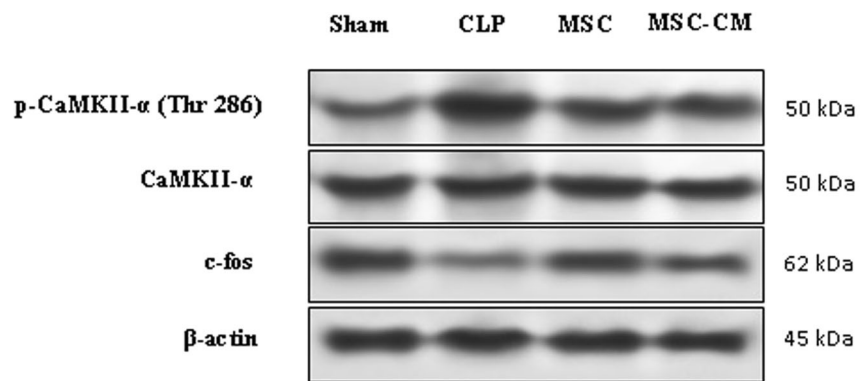
Hippocampus is one of the main brain areas involved in cognitive functions especially in learning and memory. It also plays a major role in new memory formation, contextual memory, and declarative and spatial memory processes [35]. During sepsis, as a result of blood brain barrier (BBB) breakdown, pathogens, inflammatory cytokines, neurotoxic plasma-derived proteins, and oxidative stress inducers enter into the hippocampus which may result in cognitive impairment [7]. Novel object recognition is a well-established task used to evaluate learning and memory in rodents. During the initial acquisition episode, animals observe a pair of identical objects and recalling of this learned information allows the healthy animal to discriminate between a familiar and novel object during the recognition phase of the task (memory retrieval) [17]. In our study, during test session, the septic animals were not able to recognize the novel object while this problem was not seen in animals in the MSC and MSC-CM groups. Some studies reported that retrieval of object memory involves the hippocampus and NOR test session performance increases firing rates of hippocampal neurons [17, 36, 37]. Thus, it seems that during sepsis condition, hippocampal and

associated areas are likely to be damaged and memory retrieval is impaired.

In confirmation of aforementioned statement, we evaluated the beneficial effects of MSC and MSC-CM on p-CaMKII- α (Thr 286)/ CaMKII- α ratio and c-fos proteins in the hippocampal tissue samples as the important molecules involved in memory processes. Following hypo-perfusion/ischemia (as seen in septic shock), sustained elevation of intracellular Ca^{2+} results in the formation of the Ca^{2+} /CaM complex [38, 39]. This complex in turn binds to the regulatory region of CaMKII- α and a conformational change is occurred, which not only leads to the phosphorylation of its substrates, but also to an autophosphorylation at threonine 286 (Thr 286). Thus, autophosphorylated CaMKII- α on Thr 286 can remain active even after a decrease in intracellular Ca^{2+} concentration and therefore has Ca^{2+} independent activity [40]. In the present study, sepsis induction caused a significant increase in p-CaMKII- α /CaMKII ratio compared with the sham group. MSC and MSC-CM administration significantly reduced this ratio to the basal level and the sham group. In this study, overexpression of p-CaMKII- α was observed in the CLP

Fig. 6 Effect of MSC and MSC-CM administration on **a** p-CaMKII- α (Thr 286)/CaMKII- α and **b** c-fos/ β -actin protein expressions in the hippocampus tissue samples during sepsis induced by CLP model. Data are presented as the mean \pm standard error of mean ($n = 4$ in each group).

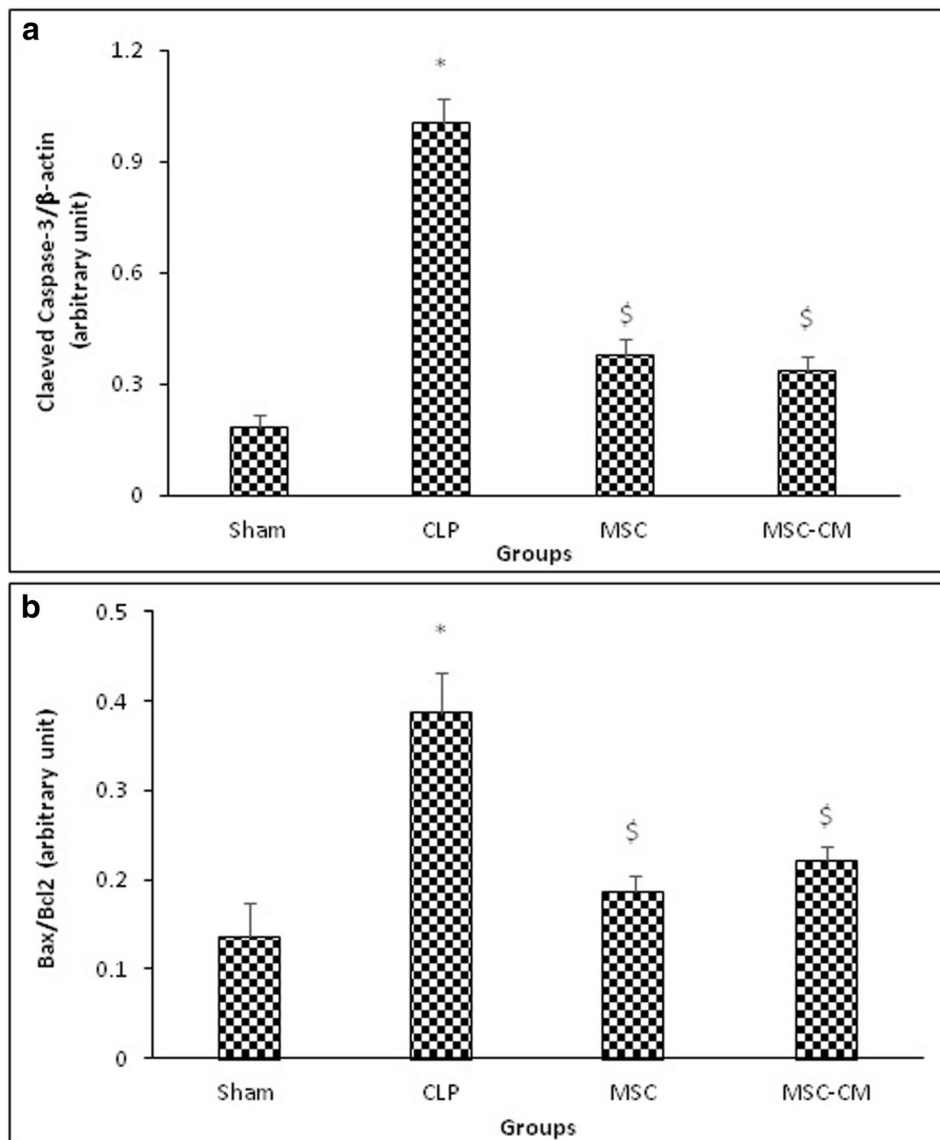
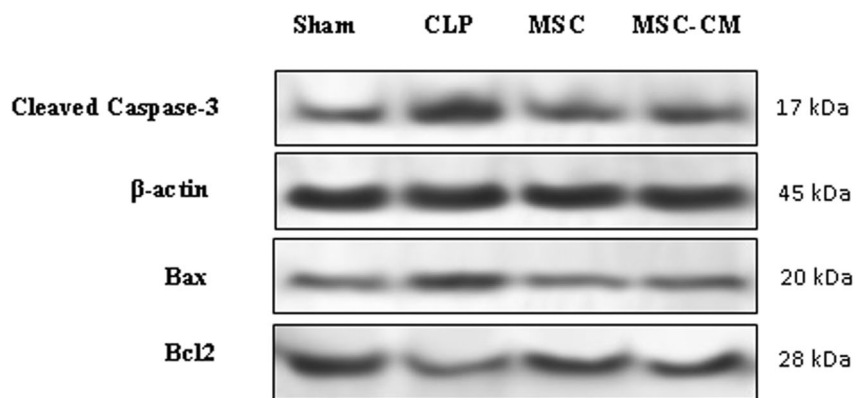
* $P < 0.05$ compared with the sham group. \$ $P < 0.05$ compared with the CLP group. CLP, sepsis was induced by cecal ligation and puncture; MSC, 2 h after the sepsis induction, the rats received mesenchymal stem cells (1×10^6 cells/rat, i.p.) suspended in 50 μ L sterile PBS. MSC-CM, 2 h after the sepsis induction, the rats received the conditioned medium (CM) from 1×10^6 MSCs intraperitoneally



group while in the MSC and MSC-CM groups, this factor showed a significant reduction. Some studies believe that CaMKII plays a key role in memory reconsolidation [41] and at the same time, the others reported the major role of CaMKII in memory destabilization [42]. CaMKII- α role in memory destabilization is of clinical relevance but because of less scientific literature on this topic; more investigations

are needed to introduce this pathway as a clinical tool [43]. Consistent with our study, Cao et al. observed that increased activity of CaMKII- α during retrieval of contextual fear memory causes the memory erasure [42]. Jarome et al. reported that CaMKII inhibitor administration in the amygdala did not impair memory retrieval. They showed that memory retrieval increases proteasome activity and phosphorylation and

Fig. 7 Effect of MSC and MSC-CM administration on **a** Caspase-3/ β -actin and **b** Bax/Bcl2 protein expressions in the hippocampus tissue samples during sepsis induced by CLP model. Data are presented as the mean \pm standard error of mean ($n = 4$ in each group). * $P < 0.05$ compared with the sham group. \$ $P < 0.05$ compared with the CLP group. CLP, sepsis was induced by cecal ligation and puncture; MSC, 2 h after the sepsis induction, the rats received mesenchymal stem cells (1×10^6 cells/rat, i.p.) suspended in 50 μ L sterile PBS. MSC-CM, 2 h after the sepsis induction, the rats received the conditioned medium (CM) from 1×10^6 MSCs intraperitoneally



CaMKII regulates retrieval-induced proteasome activity in vivo and in vitro [44]. It seems that in septic condition, hippocampal damage led to pCaMKII- α (Thr 286) overexpression, does not have a neuroprotective role, and may impair the ability of object recognition.

In the present study, c-fos protein expression showed a significant decrease in the CLP group compared with the sham group, while MSC and MSC-CM administration prevented the attenuation in this variable. Arias et al. suggested that the hippocampus, entorhinal, and temporal

association cortices together form a neural circuit which is involved in contextual memory. They reported that the increased expression of c-Fos protein after learning is indicative of neuronal activity changes [25]. It should be noted that during sepsis, hippocampal dysfunction led to a reduction in c-fos expression and memory impairment as seen in the CLP group. MSC and MSC-CM administration improved the ischemia induced by sepsis and consequent hippocampal damage thereby increased c-fos protein expression and ability to recognize the novel object. In order to determine the definite involvement of CaMKII/c-fos molecules in MSC and MSC-CM signaling pathway, future studies are needed by the administration of inhibitors of these molecules.

Many studies suggest that Ca^{2+} overload following ischemia activates long lasting processes that lead to progressive and delayed cell death [45]. This process is defined as “excitotoxicity,” an underlying mechanism in neuronal cell death elicited by some pathologies, including sepsis-associated encephalopathy. Several protein kinases including CaMKII have been shown to transduce Ca^{2+} signaling to apoptosis cascade activation and excitotoxicity [38, 39]. Takano et al. reported that treatment with calmodulin antagonist prevented neuronal cell death, elicited by excitotoxicity, in a dose-dependent manner [46]. In this study, cleaved caspase 3 level and Bax/Bcl2 ratio showed increases in the CLP group while treatment with MSC and MSC-CM caused a decrease in these proteins. Although the underlying mechanisms of CaMKII action are not fully known, CaMKII regulated by Ca^{2+} has been reported to increase cleaved caspase 3 and Bax activation in hippocampal neurons [35]. Therefore, it is plausible that caspase 3 and Bax might be downstream players of CaMKII- α -mediated excitotoxic cell death.

So far, several mechanisms have been suggested for the beneficial effects of MSCs and conditioned medium on the brain tissues. It seems that MSCs and conditioned medium act as potent regulators to protect the BBB integrity. Cheng et al. reported that MSCs by their paracrine actions reduce neutrophil infiltration, matrixmetallo-proteinase-9 function, and BBB breakdown via downregulating the endothelial intercellular adhesion molecule-1 (ICAM-1) expression. They introduced ICAM-1 as a key factor in the paracrine actions of MSCs [47]. In contrast, Tang et al. believed that MSCs exert an inhibitory function on aquaporin-4 expression in astrocytes, which ultimately preserves BBB integrity and attenuates brain edema [48]. In another study, it is reported that MSCs stabilize BBB permeability via decreasing microglial proinflammatory cytokine secretion and modulating astrocytic vascular endothelial growth factor A signaling pathway which, in turn, stabilizes expression of tight junction proteins on BBB [49]. It seems that, further studies are needed in order to determine the exact underlying regulatory action of MSC or MSC-CM.

In the present study, we tried to compare the protective effects of mesenchymal stem cells and conditioned medium therapy on some complications of sepsis. For this purpose, we intraperitoneally injected either 1×10^6 mesenchymal stem cells or the conditioned medium of this number of cells to the MSC and MSC-CM rats respectively. Some studies reported that stem cell therapy has beneficial effects in animal models of polymicrobial septic shock [50] cardiovascular disease [51] stroke [52] and many other pathological conditions. Although numerous roles have been showed for MSCs, the underlying mechanisms are not still fully reported. Early assumption was that transplanted MSCs may differentiate into the other cell types to repair organ injuries. However, this theory was proved to be unreliable by some investigators. A growing body of evidence supports the view that MSCs may function in a paracrine manner [53]. Therefore, in the present study, the biological mediators (exosomes and soluble factors) derived from MSC cultures in conditioned medium were collected and used. The results from the present study showed that MSC-CM mediates some biological functions of MSCs and has functions similar to those of MSCs, such as improvement in sepsis score and systolic blood pressure, and repairing hippocampal damage (as mentioned in novel object recognition test, p-CaMKII- α and c-fos protein expressions as well as suppressing apoptosis). However, the underlying mechanisms are only partially understood and the results are controversial. There is a possibility of considering conditioned medium as an alternative treatment for various diseases in the future. The reason is due to the higher stability of CM comparing with MSCs, absence of any risk of aneuploidy and less immunological rejection following *in vivo* allogeneic administration [13]. As was mentioned above, protective functions of the media appears earlier than the positive effects of the MSCs transplantation.

In the present study, it seems that CAMKII and c-fos are inversely involved in regulating memory processes in hippocampus. Phosphorylated form of CaMKII- α overexpression does not play a neuroprotective role and may impair the ability of object recognition. Our findings from the present study confirmed that MSC-conditioned medium mediates some biological functions of MSCs and has functions similar to those of MSCs. In addition, MSC-conditioned medium application has more advantages compared with transplanted MSCs as mentioned above and may be offered as a promising therapy for various diseases such as severe sepsis.

Authors' Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Fariba Akhondzadeh, Farzaneh Kianian, Ghorbongol Ashabi, and Kamal Abdolmohammadi. All authors read and approved the final manuscript.

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Data Availability The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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