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# Bone marrow mononuclear cell transplant prevents rat depression and modulates inflammatory and neurogenic molecules

Zaquer Suzana Munhoz Costa-Ferro [a], Pedro Antônio Schmidt do Prado-Lima [a], Guilherme Ary Onsten [a], Gutierre Neves Oliveira [a], Guilherme Camargo Brito [a], Isadora Machado Ghilardi [a], Paula Gabrielli dos Santos [a], Ricardo Jean Bertinatto [a], Daniele Vieira da Silva [a], Simone Denise Salamoni [a], Denise Cantarelli Machado [a], Ivana Beatrice Mânica da Cruz [e], Bruno Solano de Freitas Souza [b,c,d], Jaderson Costa da Costa [a,*]

[a] Brain Institute (BraIns), Pontificia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Center for Biotechnology and Cell Therapy, São Rafael Hospital, Bahia, Brazil
[c] D'Or Institute for Research and Education (IDOR), Rio de Janeiro, Brazil
[d] Gonçalo Moniz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Bahia, Brazil
[e] Graduate Program in Pharmacology, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

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ABSTRACT

*Introduction:* Major depressive disorder is associated with chronic inflammation and deficient production of brain-derived neurotrophic factor (BDNF). Bone marrow mononuclear cell (BMMC) transplantation has an anti-inflammatory effect and has been proven effective in restoring non-depressive behavior. This study investigated whether BMMC transplantation can prevent the development of depression or anxiety in chronic mild stress (CMS), as well as its effect on inflammatory and neurogenic molecules.

*Method:* Three groups of animals were compared: BMMC-transplanted animals subjected to CMS for 45 days, CMS non-transplanted rats, and control animals. After the CMS period, the three groups underwent the following behavioral tests: sucrose preference test (SPT), eating-related depression test (ERDT), social avoidance test (SAT), social interaction test (SIT), and elevated plus maze test (EPMT). Transplanted cell tracking and measurement of the expression of high-mobility group box 1 (HMGB1), interleukin-1β (IL-1β), tumor necrosis factor (TNFα), and BDNF were performed on brain and spleen tissues.

*Results:* BMMC transplantation prevented the effects of CMS in the SPT, ERDT, SAT, and SIT, while prevention was less pronounced in the EPMT. It was found to prevent increased HMGB-1 expression induced by CMS in the hippocampus and spleen, increase BDNF expression in both tissues, and prevent increased IL-1β expression in the hippocampus alone, while no effect of the transplant was observed in the TNFα expression. In addition, no transplanted cells were found in either the brain or spleen.

*Conclusions:* BMMC transplantation prevents the development of depression and anxiety-like behavior triggered by CMS. It could prevent increased HMGB-1 and IL-1β expression in the hippocampus and increased BDNF expression in the same tissue. Cell treatment represents a further perspective in the research and treatment of depression and possible mood disorders.

## 1. Introduction

Major depressive disorder (MDD) is a chronic condition that usually progresses in recurrent episodes, with a 12-month prevalence of 5.5%–5.9% in different countries (Malhi and Mann, 2018). Evidence suggests that MDD is associated with a chronic inflammatory state and inadequate neuroregeneration due to a deficiency in the production of some critical neurogenic molecules, such as the brain-derived neurotrophic factor (BDNF) (Malhi and Mann, 2018; Miller and Raison, 2016). Currently, MDD treatment mainly involves the use of selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors. However, the efficacy of these drugs is limited in most patients (Malhi

* Corresponding author at: Brain Institute, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga 6690, Zip Code: 90610-000 Porto Alegre, RS, Brazil.
*E-mail address:* jcc@pucrs.br (J.C. da Costa).

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and Mann, 2018).

Emerging studies using experimental models suggest that bone marrow mononuclear cells (BMMCs) may have a therapeutic effect on MDD symptoms due to their neurogenic and anti-inflammatory properties (Costa-Ferro et al., 2012; Leal et al., 2014). In a previous study by our research team, BMMC transplantation was effective in restoring non-depressive behavior in rats such as sucrose preference and in decreasing the levels of proinflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNFα), and interferon-γ in the brain, spleen, and blood (do Prado-Lima et al., 2019).

Given these results, we investigated whether this same procedure involving transplantation of BMMCs could prevent states of depression in Wistar rats exposed to chronic mild stress (CMS). In addition to depression and anxiety behavior tests, we conducted histological analysis in the hippocampus and spleen, as well as gene expression analysis of key molecules involved in chronic inflammation, such as high-mobility group box 1 (HMGB1), proinflammatory cytokines (IL-1β and TNFα), and neurogenesis alteration (BDNF).

## 2. Methods

### 2.1. Animals and experimental design

In this study, 29 adult male Wistar rats (45 days old, 220–250 g), obtained from the Centro de Modelos Biológicos Experimentais of the Pontifícia Universidade Católica do Rio Grande do Sul, were housed in cages (four rats per cage) on a ventilated rack with controlled temperature and humidity. The temperature in the room was maintained at 22 °C, with a 12 h light–dark cycle (light on at 8 a.m.). Food and water were available ad libitum, except when food and/or water deprivation was applied as a stress parameter. Before the experimental period, the animals were acclimatized for 10 days. After the baseline period, the rats were randomized into three groups: CMS previously treated with saline solution (S-CMS group) ($n$ = 10), CMS previously treated with BMMC transplant (BMMC-CMS group) (n = 10), and Control group ($n$ = 9), which were not subjected to CMS. The CMS procedure lasted for 45 days, and all experimental procedures were performed between 8 a.m. and 8 p.m. Twenty-four hours after the last stress session, the animals from the three groups (S-CMS, BMMC-CMS, and Control groups) underwent the following behavioral tests: sucrose preference test (SPT), eating-related depression test (ERDT), elevated plus maze test (EPMT), social avoidance test (SAT), and social interaction test (SIT). The tests were conducted over the subsequent 10 days. Following the behavioral tests, the rats were euthanized, and the hippocampus and spleen tissues were collected for subsequent gene expression analysis (five animals each from the S-CMS and BMMC-CMS groups, and four from the Control group), with the remaining animals (five from each group) being used in the histological analysis.

The entire study lasted for 65 days, including a 7-day room habituation period and a 3-day habituation to the baseline sucrose preference testing period. Pretreatment with BMMC was performed on the first day of CMS (1 day), which lasted for 45 days, and behavioral testing for 10 days (Fig. 1).

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the guidelines of the Brazilian Association for Laboratory Animal Sciences. The procedures were approved by the Ethics Committee for Animal Research of the PUCRS (#7943).

### 2.2. BMMC and BMMC transplantation

BMMCs were harvested from male green fluorescent protein (GFP)-transgenic mice, as previously described (Leal et al., 2014). The animals were euthanized by cervical dislocation under anesthesia with 200 μL of 8% ketamine hydrochloride (Cristália) and 2% chlorpromazine (*União Química Farmacêutica Nacional*). Transplants were performed using

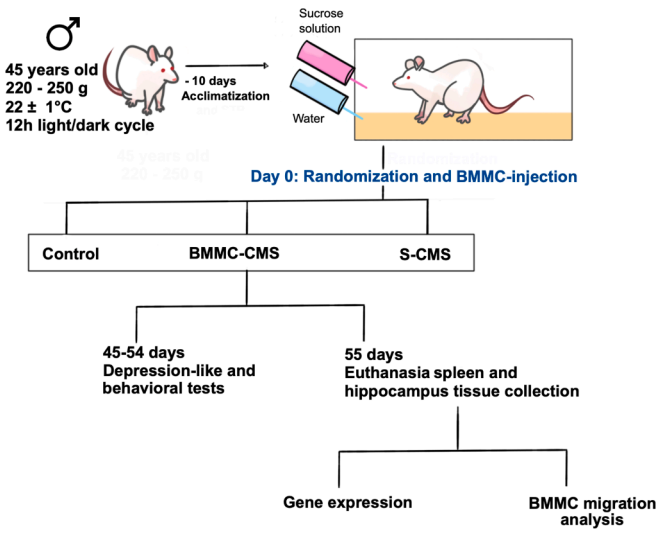


**Fig. 1.** Experimental design. STP: sucrose preference test; S-CMS: rats previously treated with saline solution ($n$ = 10) and submitted to chronic mild stress (CMS), BMMC-CMS: rats previously treated with BMMC (n = 10) and submitted to CMS, and Control group ($n$ = 9).

BMMCs harvested from male GFP-transgenic mice. Fresh bone marrow was extracted from the humerus, femur, and tibia by flushing them with phosphate-buffered saline (PBS). After centrifugation, the cell pellet was resuspended in Roswell Park Memorial Institute medium and fractionated on a density gradient generated by centrifugation over a Ficoll–Hypaque solution (Histopaque 1119 and 1077, 1:1; Sigma) at 400 × $g$ for 10 min at 25 °C. The mononuclear fraction over the Ficoll–Paque layer was collected and washed twice with PBS. In all rats from the BMMC-CMS group, injections were administered in the tail vein on day 0 of the experimental procedures. The BMMC-CMS group received 1 × $10^7$ cells/mL in a volume of 100 μL (Costa-Ferro et al., 2012).

### 2.3. Chronic mild stress protocol

After the baseline period for acclimatization, rats in the CMS and BMMC-CMS groups were subjected to the 45-day CMS procedure. To induce CMS, the animals were exposed to a sequence of different events randomly repeated during the course of the experiment: white noise (3–5 h), crowding (24 h), 45° box tilting (7–24 h), food deprivation (24 h), water deprivation (24 h), dark during light cycle (12$h$), and strobe light (7–24 h) (do Prado-Lima et al., 2019, Willner, 2016). The control group remained in their home cages. All experimental procedures were carried out between 7 a.m. and 7 p.m. Following exposure to stress, rats were characterized as anhedonic (>25% within-subject decrease in sucrose intake), and all animals were sacrificed under anesthesia with isoflurane (Cristália) through decapitation.

### 2.4. Behavioral tests for animal depression-like and anxiety-like behavior

#### 2.4.1. Sucrose preference test

The SPT was carried out performed to assess the rats' hedonic state, which is a measure of depression intensity, before and during CMS exposure. The sucrose preference test was performed as previously described (do Prado-Lima et al., 2019). Rats were allowed to consume sucrose by placing two bottles of sucrose solution (1% $w/v$) in each cage for 24 h. Subsequently, one of the bottles was replaced with water for 24 h. Then, the rats were deprived of water and food for 12 h. The rats were housed in individual cages and given free access to the two bottles that contain 100 mL of sucrose solution and 100 mL of water, respectively. After 2 h, the volumes (in mL) of consumed sucrose solution and

water were recorded, and sucrose preference was calculated based on the following formula: % sucrose preference = [sucrose consumption / (sucrose + water consumption)] × 100.

#### 2.4.2. Eating-related depression test

This test examines the motivation to eat after food deprivation and is used to measure depression-like behavior (Soubríe et al., 1975). The animals were first deprived of food for 16 h. The rats were provided access to water ad libitum. After 16 h, each rat was removed from its home cage and placed temporarily in an identical new cage with a few food pellets positioned at the center of a plexiglass arena (length × width × height: 60 × 40 × 60 cm). The animal was placed in a corner and allowed to explore for 5 min. The latency to smell the food and the latency to the first bite of food were recorded. To exclude odor-related issues, the food pellets were changed for each rat.

#### 2.4.3. Social avoidance test

The SAT is used to measure anxiety-like behavior (Peña et al., 2017). In the first session, the rat was allowed to explore an open field arena (length × width × height, 60 × 40 × 60 cm) containing a protective cage (13 × 13 cm) placed against one wall of the arena for 2.5 min (NT, no target). In the second session, a 2.5 min test (T, target), the experimental rat was immediately returned to the arena where a novel young rat was enclosed in the protective cage. The total time spent in the interaction zone was recorded and calculated offline from the video. The social preference ratio was computed as time in interaction zone/(time in interaction zone + time in corners) and analyzed as the ratio between the two test sessions (T/NT).

#### 2.4.4. Social interaction test

SIT was also used to measure anxiety-like behavior. It comprises two trials (File and Seth, 2003). In the first trial, the rat was introduced into the social interaction open field arena (length × width × height, 60 × 40 × 60 cm) alone and was allowed to explore freely for 5 min. The rats were then removed and placed back into their home cage for approximately 30 s. In the second trial, the rat was re-introduced into the arena with an unfamiliar rat. The interactions between them in the target session were recorded on a camera for 5 min and analyzed offline from the video.

#### 2.4.5. Elevated plus-maze test

The EPMT is a widely used measure of anxiety (Zhao et al., 2019). The maze consisted of four arms (30 cm × 5 cm) radiating from a central platform (5 cm × 5 cm), 40 cm above the floor. Two of the arms had no walls on any side (open), and two had a 14-cm high wall on all sides, except at the center of the platform (closed). The number of transitions between the arms and the time spent in the open arms was analyzed. This behavior assessed is based on a conflict between the animal's desire to explore and its fear of open spaces and was calculated by counting the number of risk assessment events, number of entries in the open arms, and the time spent in the open arms.

### 2.5. Tissue preparation

After completing the tests, the rats were anesthetized with isoflurane and decapitated to extract the entire brain. The hippocampus and spleen were quickly separated on ice and frozen at −80 °C for further analysis.

### 2.6. Quantitative reverse transcription-PCR (q-PCR)

RNA was extracted using an SV-Total RNA kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Total RNA was quantified using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). cDNA synthesis was performed using the SuperScript VILO Master Mix (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions, and the cDNA was quantified using a NanoDrop spectrophotometer. The samples were amplified from an initial amount of 20 ng of cDNA for each sample. The primers used in this study are listed in Table 1. For the data analysis related to gene expression, the $2^{-\Delta\Delta Ct}$ relative quantification method was used, with the endogenous *GAPDH* gene as a control.

### 2.7. Histological analysis

The tissues were fixed in 4% paraformaldehyde for 24 h, followed by dehydration and paraffin embedding. We prepared 3-μm-thick serial coronal brain paraffin sections localized between bregma −1.58 to 3.64 mm caudal from the bregma (Paxinos and Watson, 1998). Each section was collected at 30 μm intervals, and one in every 10 sections was analyzed. Six sections were observed. Spleen paraffin sections of 3-μm thickness were taken at three different levels of tissue 200 μm apart. The slices were deparaffinized, rehydrated, and antigen retrieval was performed using citrate buffer (pH = 6.0, 95–98 °C) for 20 min. Sections were incubated overnight at 4 °C with chicken anti-GFP antibody (1:400, ThermoFisher). The following day, the sections were incubated for 1 h at room temperature with anti-chicken IgY Alexa Fluor 488 (1:500, Molecular Probes, Carlsbad, CA, USA) and mounted using the VECTASHIELD mounting medium with 40,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA, USA). GFP+ BMMCs were tracked by GFP expression in six sections per animal, using a confocal microscope (A1+, Nikon, Tokyo, Japan).

### 2.8. Data analysis

The data are presented as the mean ± standard error of the mean. The results were analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni test for post-hoc comparisons. Analysis and mean ± standard deviation graph generation were performed using the GraphPad Prism software (version 7.05). Statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Effects of BMMC on sucrose preference

There was no significant difference in SPT between the Control, S-CMS, and BMMC-CMS groups before CMS procedures [one-way ANOVA, $P > 0.05$, F (2, 26) = 0.12] (Fig. 2A). However, after 45 days of CMS, a significant difference was observed [one-way ANOVA, $P < 0.001$, F (2, 26) = 10.59]. Post-hoc comparisons indicated that the BMMC-CMS group did not differ significantly from controls, but that the S-CMS group consumed significantly less fluid than the Control ($P < 0.001$) and BMMC-CMS ($P < 0.01$) groups (Fig. 2B).

### 3.2. Effects of BMMC on eating-related depression

Two parameters were examined in the ERDT: latency to smell the food and time to the first bite. Regarding the latency to smell the food, there was a difference between the groups [one-way ANOVA, F (2, 26) = 9.23, P < 0.001]. Post-hoc analysis revealed that the S-CMS rats took

**Table 1**
Primers used in the study.

| Genes names | Primers sequences |
|---|---|
| BDNF | Forward primer 5′..0.3′TTTCTAGCTCTGTGGTGCGG |
| | Reverse Primer 5′..0.3′ TCGTCAGACCTCTCGAACCT |
| HMGB1 | Forward primer 5′..0.3′TCCCCTACTAAAGACCTGAGAATG |
| | Reverse Primer 5′..0.3′ TTTATCCGCTTTCCTTGTATCTGA |
| TNF-α | Forward primer 5′AGAACAGCAACTCCAGAACACCCT3’ |
| | Reverse Primer 5′ATCTCGGATCATGCTTTCCGTGCT3’ |
| GAPDH | Forward primer 5′TGCCACTCAGAAGACTGTGGATG3’ |
| | Reverse Primer 5′GCCTGCTTCACCACCTTCTGAT3’ |

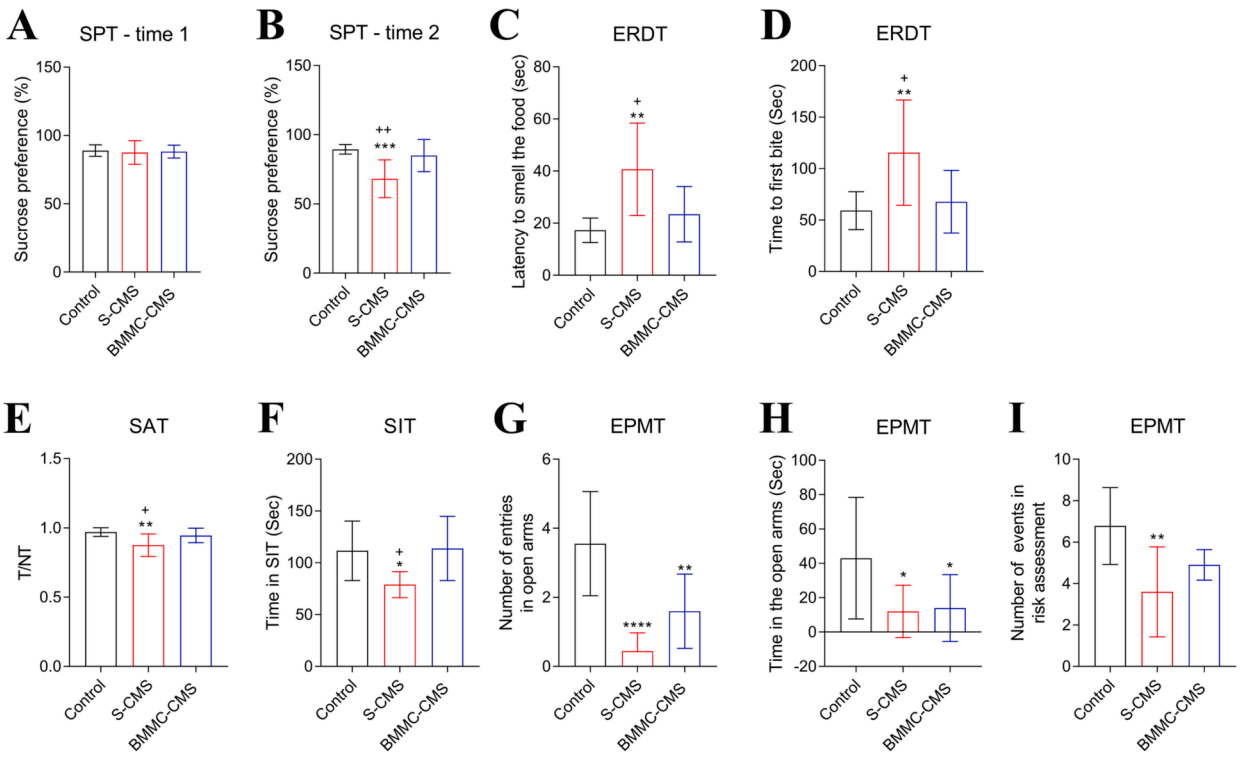


**Fig. 2.** Effects of BMMC on behavioral tests. SPT, sucrose preference test time 1 before (A) and after (B): ***$P < 0.001$, S-CMS vs. control; $^{++}P < 0.01$, S-CMS vs. CMS-BMMC. ERDT, eating related depression test. Latency to smell the food (C): **$P < 0.01$, S-CMS vs. control; $^{+}P < 0.05$, S-CMS vs. BMMC-CMS. Time to first bite (D): **$P < 0.01$, S-CMS vs. control; $^{+}P < 0.05$, S-CMS vs. BMMC-CMS). SAT, social avoidance test computed as: time in interaction zone/(time in interaction zone + time in corners), and analyzed as the ratio between the two test sessions (T/NT) (E): **$P < 0.01$, S-CMS vs. control; $^{+}P < 0.05$, S-CMS vs. BMMC-CMS. SIT, social interaction test (F): *$P < 0.05$, S-CMS vs. control; $^{+}P < 0.05$, S-CMS vs. BMMC-CMS. EPMP, elevated plus-maze test number of entries in open arms (G): ****$P < 0.0001$, S-CMS vs. control; **$P < 0.01$, BMMC-CMS vs. control. Time in the open arms (H): *$P < 0.05$, S-CMS vs. control; *$P < 0.05$, BMMC-CMS vs. control. Number of events in risk assessments (I): **$P < 0.01$, S-CMS vs. control.

longer to smell the food compared to the BMMC-CMS ($P < 0.05$) and Control ($P < 0.01$) rats, and there was no difference in the latency to smell the food between the BMMC-CMS and Control groups (Fig. 2C). In the time to the first bite, we also observed a difference between the groups [one-way ANOVA, F (2, 26) = 6.74, $P < 0.01$]. Post-hoc comparisons indicated that the S-CMS rats showed a significant increase in the time of eating compared to the BMMC-CMS ($P < 0.05$) and Control ($P < 0.01$) groups, with a difference also being found between the BMMC-CMS and control groups (Fig. 2D).

### 3.3. Effects of BMMC on social avoidance

One-way ANOVA revealed a difference in the time spent in the social preference/avoidance ratio between groups [F (2, 26) = 6.57, $P < 0.01$]. Post-hoc comparisons showed that social preference/avoidance ratios were significantly decreased in the S-CMS group compared to that in the Control ($P < 0.01$) and BMMC-CMS ($P < 0.05$) groups. In contrast, the CMS-BMMC rats showed a similar social preference to that of the control rats (Fig. 2E).

### 3.4. Effects of BMMC on social interaction

There was a difference in the time spent interacting with an unfamiliar rat between the groups [one-way ANOVA, F (2, 26) = 5.87, P < 0.01]. Post-hoc analysis indicated that S-CMS rats spent less time interacting with an unfamiliar partner than the Control (P < 0.05) and BMMC-CMS ($P < 0.05$) rats. BMMC-CMS rats did not differ from the control rats when interacting with an unfamiliar partner (Fig. 2F).

### 3.5. Effects of BMMC on the elevated plus-maze test

To assess the anxiety behavior in rats, the EPMT was measured in terms of the percentage of time spent exploring the open arms, open arm entries, and risk assessment. There were significant differences in the elevated plus maze for the total time spent in the open arms [one-way ANOVA, F (2, 26) = 4.71, $P < 0.05$], entries in open arms [one-way ANOVA, F (2, 25) = 18.07, $P < 0.0001$], and number of risk assessment events [one-way ANOVA, F (2, 26) = 8.35; $P < 0.001$]. Post-hoc comparisons confirmed a significant difference in the number of entries in the open arms from Control group compared to the S-CMS (P < 0.0001) and BMMC-CMS ($P < 0.01$) groups (Fig. 2G). The same was observed in the time spent in the open arms for the S-CMS ($P < 0.05$) and BMMC-CMS ($P < 0.05$) groups compared to the Control group (Fig. 2H). In the number of risk assessment events, post-hoc analysis showed a difference between the S-CMS and Control ($P < 0.01$) groups (Fig. 2I).

### 3.6. Effects of BMMC on HMGB1, IL-1β, TNFα, and BDNF expression in the hippocampus and spleen

In the hippocampus, overexpression induced by S-CMS treatment of all pro-inflammatory genes did not occur in the BMMC-CMS and control groups (Fig. 3 A, C and E).

In the spleen, only the *HMGB1* gene was overexpressed in the S-CMS in relation to that in the BMMC-CMS and Control groups, and there was no protective effect of the transplant in relation to IL-1β and TNFα expression (Fig. 3 B, D and F). BDNF was overexpressed in the hippocampus ($P < 0.05$) and spleen ($P < 0.01$) in the BMMC-CMS group compared to that in the S-CMS and Control groups (Fig. 3 G and H).

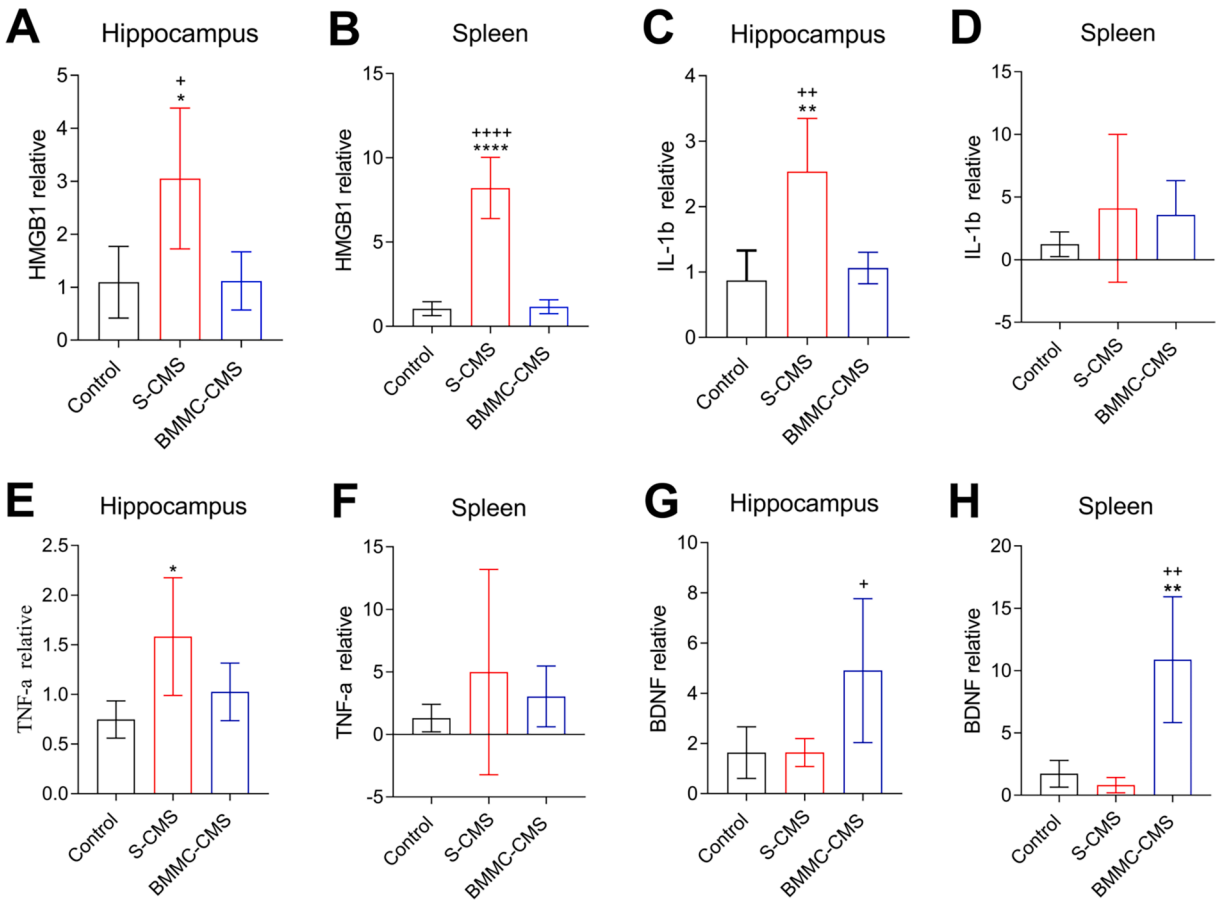


**Fig. 3.** HMGB1, IL-1β, TNFα and BDNF expression in the hippocampus and spleen. Control vs. S-CMS (A, B C and E), Control vs. BMMC-CMS (H): *P < 0.05, **P < 0.01, ****$P < 0.0001$; S-CMS vs. BMMC-CMS (A, B, C, G, and H) $^{+}$P < 0.05, $^{++}$P < 0.01, $^{++++}P < 0.0001$.

### 3.7. BMMC migration to the brain and spleen

Cell tracking was performed in the brain hemispheres and spleen sections 55 days after cell infusion by searching for GFP+ cells through confocal microscopy. At the evaluated time point, no GFP+ cells were found in either the brain or spleen, suggesting a very low or no tissue engraftment of the transplanted cells.

## 4. Discussion

We previously demonstrated that BMMC transplantation restored sucrose preference in rats subjected to a chronic stress (CS) protocol (do Prado-Lima et al., 2019). In that study, after the 4th week of the CS protocol, when sucrose preference was diminished, the rats were transplanted with BMMCs. After one week, sucrose preference was restored in stressed rats, despite the maintenance of the CS protocol.

In the present study, rather than exploring the role of BMMCs in restoring sucrose preference decreased by CS in rats, we have investigated the effects of these transplanted cells in preventing the development of behavioral changes in rats subjected to CMS for 45 days. From a translational point of view, our first study demonstrated the effect of BMMC transplantation in established depression, and in the present study, we have demonstrated that BMMC transplantation can prevent stress from triggering depression. To explore the preventive effect of BMMC transplantation, we performed five behavioral tests that are discussed separately below.

### 4.1. Sucrose preference test

The SPT is used to measure anhedonia, which is a typical depressive symptom. BMMC transplantation prevented the characteristic decrease in sucrose preference observed in the S-CMS group. Chronic stress may diminish sucrose preference through neuroinflammation, since both HMGB1 and IL-1β (Franklin et al., 2018, Zhang et al., 2018), and NLRP3 inflammasome (which is activated by HMGB1 and TNFα, among others) could diminish sucrose preference (Zhu et al., 2017). The preventive effect of BMMC transplantation could be due to its effect in preventing neuroinflammation, as discussed below. However, BDNF could also be involved, since BMMC transplantation provoked a significant increase in BDNF mRNA in relation to the Control and CMS groups. Piperine also increased BDNF expression and restored sucrose preference in CMS rats. K252a, an inhibitor of the TrkB receptor, blocked the effect of piperine in SPT (Mao et al., 2014).

### 4.2. Eating related depression test

The ERDT is used to measure the motivation to eat food after 24 h of deprivation (Upadhya et al., 2016). It is considered a measure of depression because it was designed based on the "novelty suppressed feeding test" to avoid anxiety interference in the animal's performance (Upadhya et al., 2016). BMMC transplantation prevented the increased latency to smell the food and time to the first bite observed in the S-CMS group, because the BMMC-CMS rats obtained the same scores as those of the Control rats. These results were consistent with those of the SPT.

### 4.3. Social avoidance test and social interaction test

In both tests, the results obtained corresponded with those in the SPT and ERDT. The S-CMS group was different from the control and BMMC-CMS groups, which were comparable in the control and BMMC-CMS

groups. Because the SAT and SIT are meant to measure anxiety in social interaction (Peña et al., 2017; Templer et al., 2018), it is interesting to point out that they disagreed with what we observed in the EPMT. There are many interferences in the results of such tests; one would be anhedonia, which could diminish the curiosity of the animal in interacting with others.

### 4.4. Elevated plus maze test

The EPMT is a widely used measure of anxiety without a social component (Kraeuter et al., 2019). The time spent and the number of entries into the open arms decreased in the BMMC-CMS group when compared to the control group, revealing a failure to prevent the effect of CMS in this anxiety behavior. However, regarding the number of risk assessment events, there was no difference between the BMMC-CMS and Control groups. These findings demonstrate that the CMS protocol increased anxiety despite the BMMC effect. The relationship between anxiety and depression is complex, as anxiety may trigger depressive episodes and be part of the depression. As BMMC transplantation was effective in other depressive components, as measured by the other tests we performed, we speculate that in the BMMC-treated group, CMS was unable to induce depression even when anxiety was increased.

In recent years, there has been a growing interest in the role of inflammation as a mechanism underlying depression (Slavich and Irwin, 2014; Miller and Raison, 2016). Studies on this subject can be divided into two fields: research into the behavior of the hippocampal-pituitary-adrenal axis in depression, with numerous studies performed in the last 40 years that have provided considerable detail; and research into the inflammatory cascades themselves, which has attracted increasing attention in the last 15 years. However, the number and complexity of the different study targets (cells, mediators, receptors, among others) have made it difficult to discern the influence of stress on the various systems that make up the inflammation itself and what is required for this inflammation to translate into depression.

Psychosocial stress triggers inflammatory cascades. Although the mechanisms involved are not fully understood, it is now clear that danger-associated molecular patterns (DAMPs) are released after psychosocial stress (Fleshner et al., 2017). HMGB1, a DAMP and nuclear protein present in all cell types, is particularly interesting in psychosocial and depression research. Upon release, it activates the TLR4 and RAGE receptors, inflammatory events, including the activation of the NLRP3 inflammasome and subsequent caspase-1 activation, which in turn provokes cleavage of pro-inflammatory IL-1β and IL-18 cytokines (Fleshner, 2013; Yang et al., 2020), and NF-κB pathway activation with subsequent IL-6 release (Fleshner et al., 2017). It also activates the kynurenine pathway, which is associated with the development of depression (Wang et al., 2018). A recent systematic review identified six controlled studies in which CMS in rats was associated with increased levels of HMGB1 (Zhang et al., 2019). Direct infusion of HMGB1 into the hippocampus caused anhedonic behavior in rats, while CMS was unable to provoke anhedonia in RAGE knockout mice (Franklin et al., 2018). In our study, we identified augmented levels of HMGB1 mRNA in the hippocampus and spleen of rats subjected to CMS compared to the controls not subjected to CMS. BMMC transplantation performed prior to the CMS protocol prevented HMGB1 expression, as its levels were equal to those of the controls. This suggests that the anti-inflammatory effect of BMMC transplantation is manifested in the early stages of inflammatory cascades. Similarly, it was recently demonstrated that glycyrrhizic acid, which binds to HMGB1 and inhibits its effect, was effective as an adjunctive treatment in MDD in a randomized, double-blind, placebo-controlled trial (Cao et al., 2020). The use of glycyrrhizic acid was associated with a considerable reduction in IL-1β and TNFα, but not IL-6 (Cao et al., 2020).

HMGB1 activates inflammasomes via TLR4. This inflammasome assembly results in caspase-1 activation that leads to the cleavage of pro-IL-1β and pro-IL-18 (inactive) into IL-1β and IL-18 (active form), which are then released (Fleshner, 2013; Fleshner et al., 2017). In our study, we found increased IL-1β expression in the hippocampus and spleen of rats subjected to CMS in comparison to the Controls not subjected to CMS. Interestingly, the effect of BMMC transplantation prior to the CMS protocol differs between these organs, exerting a protective effect in the hippocampus, but not in the spleen. A previous study by our group demonstrated that BMMC cells were able to normalize IL-1β levels in the hippocampus of epileptic rats, an effect not seen in rats subjected to splenectomy (Silva et al., 2021). This study suggests that a change in immunology occurring in the spleen is responsible for the changes observed in the brain. If this is true, IL-1β is not a mediator. However, this does not detract from the importance of IL-1β in the brain as a mediator for the development of depression.

The role of peripheral IL-1β in depression remains controversial. A meta-analysis of nine studies participated by major depressive patients and controls showed that peripheral IL-1β levels were similar between patients and controls, although two of these nine studies demonstrated a difference (Dowlati et al., 2010). In contrast, another meta-analysis showed that psychological stress promotes the acute release of IL-1β in humans (Marsland et al., 2017). However, peripheral levels of IL-1β do not reflect levels inside the different brain circuits and structures, and we cannot discard the importance of this cytokine in underpinning depression. One example of the central effect of IL-1β is that this cytokine induction of MAPK has been shown to increase the expression and function of the serotonin transporter, diminishing the availability of serotonin in the synaptic cleft and provoking depressive-like behaviors in an animal model (Zhu et al., 2010).

A meta-analysis of 13 studies with patients suffering from major depression and controls showed that peripheral TNFα levels were higher in patients than in controls (Dowlati et al., 2010). Another recent meta-analysis showed that cerebrospinal-fluid TNFα levels were also elevated in patients with major depression compared to controls (Enache et al., 2019). In our study, we found that hippocampal BMMC transplantation prior to CMS prevented augmented TNFα expression. In the spleen, there was no difference among the three groups. In our previous study, when BMMCs were used as a treatment for depression provoked by CS, TNFα levels were normalized both in the periphery (blood and spleen) and in different brain regions (do Prado-Lima et al., 2019). This difference could be due to the different stress protocols used, CS in the former, and CMS in the present study, with CS provoking a more intense inflammatory response.

As with IL-1β, peripheral TNFα does not seem to mediate the effect of BMMC transplantation. However, the effect of brain TNFα underpinning depression must be considered. TNFα also induces MAPK, which increases the expression and function of the serotonin transporter (Zhu et al., 2010). Moreover, TNFα is involved in microglial activation, which in turn promotes NF-κB activation, leading to TNFα overexpression in a positive feedback loop (Brás et al., 2020).

BDNF is considered a key neurotrophic factor for hippocampal shrinkage in depression and neurogenesis during depression recovery (Martinowich et al., 2007). In our study, we found a robust increase in BDNF mRNA (both hippocampal and spleen) in the BMMC-CMS group when compared to Control and CMS groups. Previously, our group demonstrated that BMMC transplantation in epileptic rats increased BDNF levels and expression (Zanirati et al., 2015). BDNF expression was also found to be increased in rats subjected to bilateral common carotid ligation and in their normal controls, both injected with BMMCs (Gubert et al., 2013).

Increased pro-inflammatory signaling leads to a reduction in BDNF mRNA levels in different brain structures, mainly the cortical areas (Giacobbo et al., 2019). The increase in IL-1β, IL-6, and TNFα (pro-inflammatory cytokines) leads to a decrease in BDNF and TrκB gene expression in the hippocampus and frontal cortex of rats (Gibney et al., 2013). The behavior effect (sickness behavior) linked to inflammation provoked by systemic injection of lipopolysaccharide in mice was counteracted by a TrκB agonist, 7,8-dihydroxyflavone (Zhang et al.,

2014).

BDNF and TrκB are expressed in tissues other than the brain, such as the liver and spleen, but their functions in these tissues are not well understood (Yang et al., 2017). Post-mortem human samples of depressed and bipolar patients showed low levels of pro-BDNF, which, after cleavage, were transformed into active BDNF, and normal levels of active BDNF in the spleen and liver (Yang et al., 2017). In our study, the CMS and Control groups showed the same expression of BDNF in the spleen, but the BMMC-CMS group showed higher BDNF mRNA levels.

Interestingly, the prolonged effects observed in this study were not associated with long-term persistence of the transplanted cells in the analyzed tissues (brain and spleen). This is compatible with a "hit-and-run" paracrine effect that was previously described in other experimental models, where transplanted BMMCs were only detected for a brief period shortly after cell infusion (Ng et al., 2015). However, we have previously reported that few of these cells migrate and remain within the brain (Costa-Ferro et al., 2010; Venturin et al., 2011; Costa-Ferro et al., 2012; Leal et al., 2014).

Our study has a limitation. It would be preferable to have two or more groups, one receiving an established antidepressant treatment, escitalopram, and another BMMC transplanted group not submitted to CMS. In a previous study, we included a group treated with antidepressants, which showed that a similar effect was produced by BMMC transplantation (do Prado-Lima et al., 2019).

Future research dedicated to understanding why BMMC transplantation robustly increased BDNF levels in the hippocampus would be interesting. Nevertheless, this would be challenging because BMMC culture is composed of mesenchymal stem/stromal cells, hematopoietic progenitor cells, and their exosomes, thus involving a large number of variables. It is plausible to suppose that mesenchymal stem cells are the best candidates because their anti-inflammatory effects have been demonstrated in several studies (Huang et al., 2020). One of the mechanisms that should be explored were those involved in the "hit-and-run" phenomenon. Moreover, the findings from this study highlight the development of treatments that act on inflammatory and immunological systems in depression.

In conclusion, BMMC transplantation is an innovative tool for research into the control of inflammatory mechanisms involved in chronic stress and depression. It could also provide the basis for a new treatment strategy (autologous BMMC systemic injection) for very refractory patients with major depressive or bipolar disorder, where these inflammatory mechanisms are involved.

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## CRediT authorship contribution statement

**Zaquer Suzana Munhoz Costa-Ferro:** Conceptualization, methodology, investigation, validation, formal analysis, writing - original draft, project administration. **Pedro Antônio Schmidt do Prado-Lima:** Writing - Review & Editing. **Guilherme Ary Onsten:** Investigation. **Gutierre Neves Oliveira:** Investigation. **Guilherme Camargo Brito:** Investigation. **Isadora Machado Ghilardi:** Investigation. **Paula Gabrielli dos Santos:** Investigation. **Ricardo Jean Bertinatto:** Investigation. **Daniele Vieira da Silva:** Investigation. **Simone Denise Salamoni:** Investigation. **Ivana Beatrice Mânica da Cruz:** Writing - original draft. **Bruno Solano de Freitas Souza:** Investigation, formal analysis, Writing - original draft. **Jaderson Costa da Costa:** Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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