

The Potential Role of Very Small Embryonic-Like Stem Cells in the Neuroinflammation Induced by Social Isolation Stress: Introduction of a New Paradigm

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Research

Keywords: social isolation stress, depression, anxiety, very small embryonic-like stem cells, neuroinflammation

Posted Date: February 25th, 2020

DOI: <https://doi.org/10.21203/rs.2.24394/v1>

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Version of Record: A version of this preprint was published at Brain Research Bulletin on October 1st, 2020. See the published version at <https://doi.org/10.1016/j.brainresbull.2020.07.006>.

Abstract

Background: Lack of social contacts could induce psychiatric features and lead to various behavioral and neurochemical abnormalities in rodents. Social isolation stress (SIS) is a valid paradigm of depressive- and anxiety-like behaviors in animals. It has demonstrated that psychiatric disorder could affect the peripheral blood population of very small embryonic-like stem cells (VSELs). The aim of the current study is to evaluate the role of VSELs in behavioral impairments induced by SIS through neuroinflammation in mice.

Methods: Behavioral experiments were evaluated by using forced swimming test (FST), open field test (OFT), and splash test in male NMRI mice. In addition, plasma and bone marrow samples, as well as hippocampus, were collected to evaluate the population of VSELs, nitrite level, and inflammatory cytokines by using flow cytometry and ELISA.

Results: Behavioral tasks showed that SIS could induce depressive- and anxiety-like behaviors in mice. Data obtained from flow cytometry showed that VSELs significantly increased in socially isolated animals in bone marrow, peripheral blood, and hippocampus. Also, TNF- α , IL-1 β , and IL-6 significantly increased in hippocampal and plasma samples in socially isolated animals. Correlation analysis indicated that mice with higher VSELs counts have better results in behavioral tasks, and lower pro-inflammatory cytokines as well as nitrite level in mice.

Conclusion: VSELs could be used as a biological marker to enhance diagnostic accuracy as well as predicting the prognosis. Also, increment in the VSELs counts might decrease the neuro-inflammation and subsequently improve the behavioral impairments induced by SIS.

1. Introduction

It has been well documented that lack of social contacts could induce psychiatric features and lead to various behavioral and neurochemical abnormalities in rodents [1]. The social isolation stress (SIS), as a chronic stressor, is a valid paradigm, which has been used as an animal model to study neurobehavioral changes [2, 3]. It has been reported that social isolation could induce depressive- and anxiety-like behavior as well as decreasing seizure threshold through various mechanism including opioid system, cannabinoid system, nitrergic system, and neuro-inflammation [3–6]. Based on clinical studies and psychiatric guidelines, the diagnosis of psychiatric disorders is limited to clinical criteria [7]. Biomarkers, as a measurable characteristic factor, have been found to increase the accuracy of both diagnosis and prognosis in various fields of medicine; however, few studies have been focused on biomarkers in the psychiatric disorders. Past studies have recommended using some biomarkers such as C-reactive protein, cytokines, neoprotein, serum S100B, and isoprostanes in the major depressive disorder and bipolar disorder [7]. Interestingly, recent studies have demonstrated that the population of stem cells in the peripheral blood could be affected in an individual diagnosed with a psychiatric disorder.

Over the past decade, Very Small Embryonic-Like Stem Cells (VSELs) have been discovered firstly in mouse bone marrow [8]. Recent clinical studies have shown that psychiatric disorder could affect the peripheral blood population of VSELs [9–11]. These studies suggested that the individuals diagnosed with the first psychotic episode had higher VSELs counts in their peripheral blood samples in comparison to control groups [12]. In addition, a related study has been shown that the population of VSELs in peripheral blood samples could be correlated with the duration of bipolar disorder [9].

It has been suggested that VSELs, with the regenerative potential ability, are 3–5 µm in size and could express pluripotent markers including OCT-4, NANOG, etc. [11, 13, 14]. Past reports demonstrated that VSEL isolated from murine are negative for lineage markers, negative for CD45, and positive for Sca-1 antigen in mice [13, 14]. Although previous studies have shown that VSELs could be detected in various adult organs in mice, the maximum numbers of VSELs have been observed in the brain. It has been well documented that stem cells are expected to egress from the relatively quiescent state under stress conditions [15]. Activator signals such as cytokine release from injured tissues could induce gene activation in these cells and subsequently initiate the cell divisions to regenerate injured tissues and thereby maintain homeostasis in the body organs [15, 16]. Interestingly, both clinical and experimental studies have reported that activated VSELs could be mobilized from the bone marrow into the peripheral blood under stress conditions such as stroke, spinal cord injury, myocardial infarction, post-cytotoxic treatments, etc. [17–23]. In addition, it has been suggested that chronic inflammation could significantly increase the process of mobilization of VSELs from the bone marrow to peripheral blood [11].

Although these studies suggested that VSELs could be used to evaluate the clinical progress of psychiatric diseases, no further studies have been established to investigate the exact role of VSELs in such diseases. In this regard, assessing the role of VSELs or its underlying mechanisms in psychiatric disorders is prior to use of these cell as a prognostic or as a diagnostic factor. Since understanding the role of VSELs in psychiatric diseases could present the potential application of VSELs as a new novel cell therapy for treating psychiatry disorders.

Based on previous publications, we hypothesized that chronic neuro-inflammation induced by SIS could be a trigger to provoke cell division of VSELs and increase the VSELs counts. Also, in this study, we hypothesized that increasing VSELs counts could have a protective role against neuroinflammation and improve behavioral impairments induced by SIS. In the current project, we used SIS as a valid model of depressive- and anxiety-like behavior in mice to evaluate the alteration of VSELs levels in bone marrow, peripheral blood, and hippocampus samples in socially isolated animals compared to normal conditioned animals. Also, we assessed the levels of pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 as well as nitrite levels in both plasma and hippocampus and their correlations with VSELs in socially isolated animals. Taken all together, the aim of the current study is to evaluate the role of VSELs in behavioral impairments induced by SIS through neuroinflammation in mice.

2. Material And Methods

2.1. Animals and housing conditions

Total 60 male NMRI mice aged 21–25 days and weighing 10–12 g were used in this study. Animals were housed in two different conditions including social condition (SC, n = 30) and isolated condition (IC, n = 30). All animals were kept under standard laboratory conditions i.e. temperature: 22 ± 2 °C, humidity: 50 ± 10%, 12-h light-dark cycle, and ad-libitum access to food and water for a period of 5 weeks. Socially conditioned mice were placed in Plexiglas boxes (25 cm × 25 cm × 15 cm) (6 mice per cage) and IC animals were placed individually in Plexiglas boxes (24 cm × 17 cm × 12 cm) [4, 24]. In order to diminish handling and social interaction cages of IC animals were cleaned weekly by the same experimenter. All behavioral tasks were carried out between 10:00 a.m. and 02:00 p.m.

2.2. Behavioral tasks

2.2.1. Forced swimming test

The FST was conducted based on the previously described method [25–27]. Mice were separately placed in an open cylinder-shaped bottle (diameter 10 cm, height 25 cm), containing 19 cm water (23 ± 1 °C). Mice were permitted to swim for 6 minutes and the period of immobility was recorded during the last 4 min of the test. Each mouse was judged to be immobile when it terminated struggling and stayed floating immobile, making only those activities necessary to keep its head above the water.

2.2.2. Splash test

In order to evaluate the self-care and motivational behaviors splash test was performed. In this paradigm, grooming activity time, as an indirect measure of palatable solution intake, was evaluated. 10% sucrose solution was sprayed on the dorsal coat of animals. The total grooming activity time was recorded during 5 min after the sucrose vaporization [28, 29]. Grooming activity consists of nose/face grooming, head washing, and body grooming.

2.2.3. Open field test

Open-field test was done just before the FST to assess the locomotor activity of animals [2, 30]. OFT was used to exclude the possibility that changes in immobility time are not the result of modifications in motor activity and also to interpret the locomotor activity in response to SIS [31]. The apparatus of OFT was made of white opaque Plexiglas (50 cm × 50 cm × 30 cm) and was softly illuminated. Each mouse was placed gently on the central area (30 cm × 30 cm) and behaviors were documented by a camera in a 5 min period and were analyzed by Ethovision software version 8 (Noldus, Netherlands). The apparatus was cleaned with 70% ethanol after each experiment. The distance moved (horizontal activity) and the numbers of rearing (vertical activity) and time spent in the central zone (central activity) were evaluated.

2.2.4. Hole-board test

Hole-board test is considered as a reliable trial to assess the anxiety-like behaviors in the rodents [32]. The apparatus was made of a white Plexiglas square (50 cm × 50 cm with 16 equally sized holes (3 cm in

diameter) and was located 50 cm above the floor. Mice were placed in the center of the board, and the number of head-dips was calculated in a 5-min period by an experimenter.

2.3. Assessment of inflammatory cytokines

In this study, for evaluating the molecular assessment in target tissue we applied the following steps. In hippocampus samples, after sacrificing animals in all our experimental groups by decapitation under ether anesthesia, the hippocampal formation was separated and then homogenized based on our previous studies [33]. To measure circulating cytokine levels, the mixed venous-arterial blood was collected in heparinized containers and centrifuged. Pooled plasma from socially isolated and normal animals was stored at -80 degrees C until assayed for cytokine concentrations. After homogenization of the samples, samples were centrifuged at $1.466 \times g$ for 15 min at 4 °C [34]. Enzyme-linked immunosorbent assay (ELISA; Abcam, Cambridge, MA, USA) was used to determine the levels of TNF- α , IL-6, and IL-1 β . The levels of TNF α , IL-6, and IL-1 β were measured following the manufacturer's instructions. TNF α , IL-6, and IL-1 β concentration were determined using a standard curve.

2.4. Nitrite Assay

Nitrite levels of the hippocampus and plasma were measured according to our previous studies [35, 36]. Based on the Griess reaction, the colorimetric assay was done to assess the nitrite concentration. Briefly, 100 μL of samples were mixed with 100 μL Griess reagent following 10 min of incubation at room temperature, absorbance was measured at 540 nm in an automated plate reader. Nitrite concentration was evaluated by reference to a standard curve of sodium nitrite (Sigma, USA) and normalized to the mg protein of each sample.

2.5. Assessment of Very Small Embryonic-Like Stem Cells (VSELs)

2.5.1. Preparation of tissues

In hippocampus samples, after sacrificing animals in all our experimental groups, the hippocampal formation was separated and then homogenized based on our previous studies [4, 37]. The homogenized samples immediately were frozen by liquid nitrogen and stored in -80 degrees C. Samples for bone marrow cell analysis were obtained by removing the tibiae and femurs from mice and then flushing out from these bones with RPMI 1640 medium (serum-free) under sterile condition. Then, we crashed the bones, and the remaining bone tissue specimens were washed twice with the solution as mentioned earlier. All extracted solutions were labeled and collected in a falcon for each mouse. In this step, 70 μm nylon cell strainer were used to remove debris. Then the samples were centrifuged at $500 \times g$ for 10 min at 4 °C, and the supernatants were discarded. Finally, the cells were resuspended in 3 ml RBC lysis buffer (for each mouse) and incubated for 10 min at room temperature, and then washed cells by adding 47 ml of RPMI 1640 medium with 2% FBS. In the final step, we centrifuged cells again at $500 \times g$ for 10 min at 4 °C, and then discarded the supernatant. The cell pellet resuspended with 0.5 ml of RPMI 1640 medium with 2% FBS. Finally, the cells were resuspended in 1 mL of PBS- containing 2% FBS [13]. In the peripheral

blood samples, mouse mixed arterial and venous blood was collected in heparinized tubes. Isolation of mononuclear cells (MNCs) was performed in an analogous way as bone marrow-isolated cells. Finally, all samples were stained with antibodies, as described below [23].

2.5.2. Fluorescence-activated cell sorting-based analysis of Very Small Embryonic-Like Stem Cells (VSELs) in mice bone marrow, peripheral blood, and hippocampus samples

Total nucleated cells, which were obtained from the hippocampus, bone marrow, and peripheral blood, were subsequently stained for CD45, hematopoietic lineage markers (Lin) and Sca-1 antigen for 30 min. in medium containing 2% FBS. In this study, based on previously published studies we used the following antimouse antibodies (BD Pharmingen, San Diego, CA, USA) for staining: rat anti-CD45 (allophycocyanin-Cy7, clone 30F11), anti-CD45R/B220 (PE, clone RA-6B2), anti-Gr-1 (PE, clone RB6-8 C5), anti-T-cell receptor- $\alpha\beta$ (PE, clone H57-5970, anti-T-cell receptor- $\gamma\delta$ (PE, clone GL3), anti-CD11b (PE, clone M1/70), anti-Ter119 (PE, clone TER-119) and anti-Ly-6A/E (also known as Sca-1, biotin, clone E13-161.7, with streptavidin conjugated to PE-Cy5) [13, 23]. Cells were then washed, resuspended in RPMI-medium with 2% FBS, and analyzed with BD FACSCalibur™ flow cytometer device and then obtained data were analyzed with FlowJo software (TreeStar inc.).

2.6. Statistical analysis

Correlation tests and T-test analyses were used to analyze the data in the current study (GraphPad Prism version 7). P value < 0.05 was the critical criterion for statistical significance. Also, the sample size was calculated with G*Power software version 3, considering the power of the study reaching 0.8 and $\alpha = 0.05$.

3. Results

At the first part of this study, we evaluated the effect of SIS on some behavioral tasks including Forced swimming test (FST), splash test, and Open-field test (OFT), and Hole-board test (HBT). T-test analysis revealed that SIS could induce depressive-like behavior by increasing immobility time of FST ($t = 18.74$, $df = 58$, $P < 0.001$, Fig. 1A), and also decreasing grooming activity of splash test ($t = 18.67$, $df = 58$, $P < 0.001$, Fig. 1B) in comparison to social condition animals. Also, locomotor activity was assessed by using OFT. Results obtained from t-test showed that distance moved ($t = 5.57$, $df = 58$, $P < 0.001$, Fig. 1C), the number of rearings were increased ($t = 8.07$, $df = 58$, $P < 0.001$, Fig. 1D), and time spent in central zone of OFT was decreased in socially isolated animals in comparison to intact mice ($t = 5.58$, $df = 58$, $P < 0.001$, Fig. 1E). In addition, HBT showed that SIS could decrease the number of head-dips in comparison to normal animals ($t = 8.20$, $df = 58$, $P < 0.001$, Fig. 1D).

In the next step, we counted the population of very small embryonic-like stem cells (VSELs) in bone marrow, peripheral blood, and hippocampus samples in both socially isolated and normal animals. Based previous studies, VSELs were identified by flow cytometry as cells with the appropriate size of 2–10 μm

(panels A and B, Fig. 2) and lineage negative, SCA-1 positive, and CD45 negative phenotype (panels C-H, Fig. 2). In this study, we estimated that the mean population of VSELs in bone marrow was $0.062\% \pm 0.024$ in normal animals and in socially isolated animals the mean population of VSELs in bone marrow was $0.191\% \pm 0.06$. T-test analysis showed that socially isolated animals had a significantly higher level of VSELs in their bone marrow in comparison to normal mice ($t = 10.83$, $df = 58$, $P < 0.001$, Fig. 2I). In addition, data obtained from peripheral samples showed that the mean population of VSELs was $0.021\% \pm 0.014$ in normal animals and was $0.034\% \pm 0.012$ in socially isolated animals. As depicted in Fig. 2J, VSELs counts were significantly higher in socially isolated animals in peripheral blood samples in comparison to normal mice ($t = 3.62$, $df = 58$, $P < 0.001$, Fig. 2J). Also, in hippocampus samples, we observed that the mean population of VSELs was $2.05\% \pm 0.18$ and $2.46\% \pm 0.4$ in normal and socially isolated animals, respectively. T-test analysis showed that socially isolated animals had significantly higher levels of VSELs in the hippocampus in comparison to normal mice ($t = 5.08$, $df = 58$, $P < 0.001$, Fig. 2K). In addition, we used correlation study between VSELs counts in bone marrow samples and peripheral blood samples to clarify the mobilization of VSELs between bone marrow and peripheral blood. As illustrated in Fig. 3, the analysis revealed a direct correlation between the population of VSELs in the bone marrow and peripheral blood samples ($P < 0.001$, $r = 0.8384$, and 95% confidence interval = 0.6851 to 0.9206). These results strongly suggested the mobilization of VSELs between bone marrow and peripheral blood in SIS.

On the other hand, we evaluated the level of cytokines and nitrite level in both plasma and hippocampus in socially isolated and normal mice. Our results revealed that socially isolated animals had significant higher plasma levels of TNF- α ($t = 14.10$, $df = 58$, $P < 0.001$, Fig. 4A), IL-1 β ($t = 8.52$, $df = 58$, $P < 0.001$, Fig. 4C), and IL-6 ($t = 9.98$, $df = 58$, $P < 0.001$, Fig. 4E) as well as nitrite levels ($t = 18.74$, $df = 11.69$, $P < 0.001$, Fig. 4G) in comparison to normal animals. In addition, we have assessed the levels of pro-inflammatory cytokines and nitrite levels in mice hippocampus. T-test analysis revealed that TNF- α ($t = 3.04$, $df = 58$, $P < 0.01$, Fig. 4B), IL-1 β ($t = 15.54$, $df = 58$, $P < 0.001$, Fig. 4D), IL-6 ($t = 6.64$, $df = 58$, $P < 0.001$, Fig. 4F), and nitrite levels ($t = 16.75$, $df = 58$, $P < 0.001$, Fig. 4H) were significantly higher in socially isolated animals in comparison to normal animals.

Table 1 shows the correlation analysis between the VSELs population of bone marrow, peripheral blood, as well as hippocampus and other behavioral or molecular assessments in both normal and socially isolated animals. Correlation test showed that data obtained from the behavioral tasks or molecular assessments had not any significant correlation with VSELs population of bone marrow in normal mice ($P > 0.05$, Table 1). In addition, the same analysis was used to clarify the correlations between behavioral tasks or molecular assessments and VSELs counts in peripheral blood samples. As previous results, the analysis failed to show any significant correlation between VSELs counts of peripheral blood samples and all behavioral tasks or molecular assessments ($P > 0.05$, Table 1). Also, we evaluated the correlation analysis between VSELs counts of the hippocampus and other factors. As shown in Table 1, correlation analysis failed to show any significant correlation between all behavioral tasks ($P > 0.05$) and hippocampal VSELs population in normal animals. Moreover, no significant correlation was observed between hippocampal levels of VSELs and TNF- α , IL-1 β , IL-6, as well as nitrite ($P > 0.05$).

In the next step, we evaluated the correlation between VSELs population in the bone marrow and behavioral tasks as well as molecular assessments in socially isolated animals. Correlation test showed that the VSELs counts directly correlated with grooming activity of Splash test ($P < 0.01$), the number of head dips in Hole-board test ($P < 0.001$), and time in the central zone of OFT ($P < 0.01$). Also, we observed that VSELs population in bone marrow samples inversely correlated with immobility time of FST ($P < 0.001$), distance moved of OFT ($P < 0.01$) as well as the number of rearing of OFT ($P < 0.001$). Interestingly, our results revealed that the plasma level of TNF- α significantly correlated with VSELs counts ($P < 0.05$). In addition to TNF- α , we observed that the plasma levels of IL-1 β significantly correlated with VSELs counts in bone marrow samples ($P < 0.05$ and $P < 0.01$, respectively). Also, the analysis showed that nitrite levels of plasma samples inversely correlated with VSELs population in bone marrow samples of socially isolated animals ($P < 0.05$). In this study, we did not observe any significant correlation between plasma levels of IL-6 and VSELs population of bone marrow; however, this analysis suggested a marginally significant correlation ($P = 0.056$). All results and statistically reports were shown in Table 1.

On the other hand, we evaluated the correlations between VSELs counts of peripheral blood samples and behavioral tasks as well as molecular assessments in socially isolated animals. As depicted in Table 1, VSELs counts of peripheral blood samples had a significant inverse correlation with immobility time of FST ($P < 0.05$), distance moved of OFT ($P < 0.05$), and the number of rearing of OFT ($P < 0.01$). Also, the analysis showed a direct correlation between VSELs counts of peripheral blood samples and grooming activity of Splash test ($P < 0.01$), number of head dips in Hole-board test ($P < 0.001$), and time in the central zone of OFT ($P < 0.001$). In plasma samples, we observed that TNF- α , IL-1 β , and nitrite level had a significant inverse correlation with VSELs counts of peripheral blood samples ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). Also, analysis revealed a marginally significant inverse correlation between IL-6 and VSELs counts of peripheral blood samples in socially isolated animals ($P = 0.051$).

In the final step, the correlation study demonstrated that the hippocampal VSELs levels inversely correlated with immobility time in FST ($P < 0.001$), distance moved and the number of rearings in OFT ($P < 0.05$ and $P < 0.001$, respectively). On the other hand, direct correlations were observed between VSELs population of hippocampus and grooming activity in Splash test ($P < 0.05$), number of head dips in HBT ($P < 0.01$), and distance moved in OFT ($P < 0.05$). In addition, we observed that VSELs inversely correlated with pro-inflammatory cytokines including TNF- α ($P < 0.01$), IL-1 β ($P < 0.01$), and IL-6 ($P < 0.05$) as well as nitrite level ($P < 0.01$) in the hippocampus. All statistical analysis reports were mentioned in Table 1.

4. Discussion

The primary purpose of the current study was to evaluate the possible role of the VSELs in depressive- and anxiety-like behaviors and neuroinflammation induced by SIS. This study showed that the levels of the VSELs are correlated to mice's behavioral tasks as well as pro-inflammatory cytokines. Our results demonstrated that the mice with lower immobility time, lower locomotor activity, and higher grooming activity in the socially isolated group had higher amount of the VSELs in their bone marrow, peripheral

blood, and hippocampus. In addition, we observed that higher levels of the VSELs are correlated to lower pro-inflammatory cytokines levels in mice hippocampus and plasma. Based on these findings, we speculate the proliferation of VSELs in response to chronic stressor might act as an anti-inflammatory agent in mouse brain.

It has been well-documented that exposure to SIS could induce depressive- and anxiety-like behaviors through various mechanisms. There is strong evidence indicating that some of the adverse effects of SIS are mediated through the nitrergic system and neuroinflammation. On the other hand, our past studies showed that SIS could induce depressive- and anxiety-like behavior in some behavioral tasks such as the FST, Splash test, OFT, and HBT. In line with past studies, our results demonstrated that socially isolated animals had higher immobility time in the FST and lower grooming activity in the Splash test. Also, increased distance moved and the number of rearings, as well as the decreased time spent in central zone in the OFT, indicated the higher locomotor activity in socially isolated animals in comparison to normal animals. Also, inconsistent with past studies, we observed that socially isolated animals had a lower number of head-dips in the HBT, which indicates the anxiety-like behavior induced by SIS.

Calcia et al. reviewed the role various stressors in neuroinflammation and indicated that the early-life psychosocial stressors could increase the microglial activity especially in the hippocampus and subsequently induce neuroinflammation in the brain [38]. In addition, Ślusarczyk et al. showed that activated microglia induced by prenatal stress could release high levels of pro-inflammatory cytokines such as IL-1 β , IL-18, TNF- α , and IL-6 in the hippocampus and induce depressive-like behaviors later in life in rodents [39]. In line with the previously published articles, we observed that SIS could increase the plasma and hippocampal levels of pro-inflammatory cytokine including TNF- α , IL-1 β , and IL-6 as well as nitrite levels in comparison to normal animals.

Although numerous studies have investigated the role of stem cells in several areas of clinical medicine, psychiatry disorders were not exempted from this subject. In this regard, few studies have been established to review the properties of stem cells in the pathogenesis of psychotic disorders [40]. These studies are limited to evaluate the diagnostic applications of these cells in psychiatric disorders by investigating the number of stem cells in circulation or factors that direct their trafficking [41–45]. To support this last notion, it has been shown that in response to systemic or local inflammation due to tissue or organ injuries, stem cells could be mobilized into peripheral blood from bone marrow and other tissue-specific niches as well [46–50]. These circulating stem cells potentially would home to the damaged tissues and attempt to contribute to regeneration [51, 52]. However, blood-brain-barrier (BBB) is an obstacle for stem cells passage into the brain [53]. Due to lack of infiltration into brain, the usage of circulating stem cells is faded out in regenerative medicine to induce neurogenesis in the brain with intact BBB. In this regard, more attention was paid to neural stem cell located in subventricular zone and hippocampus [53–55]. In the previous decade, scientists identified a new population of stem cells, known as VSELs, in the bone marrow and peripheral blood [13, 19]. Recently, it has been shown that the number of these cells could increase in peripheral blood after exposing to stress and pathological conditions related to tissue damage such as stroke, myocardial infarction, and chronic obstructive pulmonary

disease. [56–58]. Interestingly, it has been shown that the absolute number of VSELs was highest in the brain in comparisons to other organs in normal animals [14].

Clinical studies have observed a higher number of circulating VSELs in patients diagnosed with the first psychotic episode in comparison to the control group [12]. In addition, a study conducted by Ferensztajn-Rochowiak et al. investigated the number of VSELs in peripheral blood of patients with bipolar disorder [9]. The recent mentioned study showed that individuals were not treated with lithium had a higher number of VSELs in comparison to the control group. Also, they found a correlation with the duration of the disorder with the number of these cell in circulation. In addition, the circulating VSELs was similar to their number in healthy subjects in individuals treated with long-term lithium; and finally, authors observed a negative correlation between the duration of lithium treatment and serum lithium concentrations [9]. In contrast to recently mentioned reports, the results of a study on VSELs in individuals with panic disorder were also presented by Jabłoński et al. This study mentioned a lower number of VSELs in individuals with panic disorder prior to initiation of the treatment in comparison to post-treatment conditions [59]. Also, it has been reported that chemoattractants such as sphingosine-1-phosphate (S1P), stromal cell-derived factor 1 (SDF-1) and the elements of the immune system, which belong to innate immunity, such Toll-like receptors could induce the mobilization of VSELs to circulation [60]; and this subject might be a connector between these cells and neuroinflammation.

Nonetheless, it was suggested that an increased number of VSELs might be used as a biological marker of psychiatric disorders. In the current study, we observed that socially isolated animals had a significantly higher number of VSELs in bone marrow, peripheral blood, and hippocampus in comparison to normal mice. This observation might be due to the proliferation of VSELs after exposing to chronic stress condition. In addition, the direct correlation between the number of circulating VSELs and bone marrow in socially isolated animals suggests an active mobilization of these cells into the peripheral blood. On the other hand, we evaluated the pro-inflammatory cytokines both in hippocampus and plasma. In both hippocampus and plasma samples, higher levels of pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 as well as nitrite level were observed in socially isolated animals in comparison to normal mice. Interestingly, we observed the population of VSLEs in both circulation and bone marrow was negatively correlated with nitrite levels, TNF- α , and IL-1 β . Finally, our results demonstrated that hippocampal VSELs not only negatively correlated with hippocampal pro-inflammatory cytokines and nitrite levels but also negatively correlated with the depressive- and anxiety-like behavior in socially isolated animals. Briefly, our correlation analysis showed that higher VSELs counts might decrease the pro-inflammatory cytokines in both plasma and hippocampus. In this regard, further studies should be established to evaluate the exact underlying mechanisms of a correlation mentioned above.

Based on previously mentioned data and our correlation analysis, we hypothesized that VSELs not only could be used as a biological marker but also it could be used as diagnostic and prognostic factors; in addition, VSELs could shed more light on stem cell-based therapeutic strategies in psychiatric disorders. In this regard, further studies should be established to evaluate the effects of stereotaxic administration

of cultured VSELs on behavioral outcomes in animals. Finally, we hypothesize that VSELs have high potential to be an alternative treatment in wide ranges of neurodegenerative diseases.

5. Conclusion

In the current study, we concluded that VSELs could be proliferated in response to a chronic stressor such as SIS. Also, we observed that socially isolated animals with the higher hippocampal number of VSELs had lower levels of pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 as well as nitrite levels in hippocampus. Finally, our results showed that the hippocampal number of VSELs is negatively correlated with depressive- and anxiety-like behavior in socially isolated animals. These results suggested that VSELs could be used as a biological marker to enhance diagnostic accuracy as well as predicting the prognosis. Also, based on correlation analysis, increment in the VSELs counts might decrease the neuro-inflammation and subsequently improve the behavioral impairments induced by SIS.

6. Abbreviations

SIS

Social isolation stress

VSELs

Very small embryonic-like stem cells

FST

Forced swimming test

OFT

Open field test

HBT

Hole-board test

TNF- α

Tumour Necrosis Factor alpha

IL-1 β

Interleukin-1 Beta

IL-6

Interleukin-6

IL-18

Interleukin-18

IC

Isolation condition

SC

Social condition

NO

Nitric oxide

7. Declarations

- ***Ethics***

All of our in vivo work were in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (HHS publication 85-23, 1985), legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and our institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran).

- ***Conflicts of interest***

The authors have indicated that they have no conflicts of interests regarding the content of this article.

- ***Authors' contributions***

Dr. Arvin Haj-Mirzaian has written the most parts of the manuscript and done the most experimental tasks. Dr. Ayda Khosravi has written and edited the most parts of the manuscript. Dr. Arya Haj-Mirzaian has invented the idea of the current manuscript and done some parts of the experimental tasks. Dr. Alireza Rahba, Kiana Ramezanzadeh and Rajan Nikbakhsh have analyzed the data and provided the result section. Dr. Fardad Pirri, Bhenam Talarai, Saeed Shakib², Rambod Nikbakhsh and Abolfazl Badripour have edited and reviewed the whole manuscript. All experimental tasks and analysis were done under supervision of professor Ahmad Reza Dehpour.

- ***Consent for Publication***

Not applicable

- ***Availability of supporting data***

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

- ***Funding***

Not applicable

- ***Acknowledgments***

This study was financially supported by Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran and Iran National Science Foundation (INSF). We gratefully acknowledge the dedicated efforts of the investigators and the coordinators who participated in this study.

8. References

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Table

Table1. Correlation analysis between bone marrow, peripheral blood, and hippocampal VSELs population and behavioral experiments as well as molecular assessments in both normal and socially isolated animals. *** P<0.001, ** P<0.01, and * P<0.05.

Assessments	Group	VSELs' Samples	P value	95% of confidence interval	r
Forced swimming test	Social Condition	<i>Bone Marrow</i>	0.448	-0.4793 to 0.2282	-0.14
		<i>Peripheral Blood</i>	0.904	-0.3401 to 0.3801	0.02
		<i>Hippocampus</i>	0.985	-0.3572 to 0.3633	0.03
	Isolation Condition	<i>Bone Marrow</i>	0.000***	-0.8194 to -0.3805	-0.65
		<i>Peripheral Blood</i>	0.010*	-0.7029 to -0.1181	-0.45
		<i>Hippocampus</i>	0.000***	-0.8178 to -0.3763	-0.64
Open field test (distance moved)	Social Condition	<i>Bone Marrow</i>	0.253	-0.5339 to 0.1574	-0.21
		<i>Peripheral Blood</i>	0.822	-0.3969 to 0.3225	-0.04
		<i>Hippocampus</i>	0.577	-0.4490 to 0.2645	-0.10
	Isolation Condition	<i>Bone Marrow</i>	0.001**	0.2410 to 0.7617	0.55
		<i>Peripheral Blood</i>	0.005**	0.1612 to 0.7245	0.49
		<i>Hippocampus</i>	0.017*	-0.6855 to -0.08485	-0.43
Splash test	Social Condition	<i>Bone Marrow</i>	0.942	-0.3721 to 0.3483	-0.01
		<i>Peripheral Blood</i>	0.593	-0.2686 to 0.4455	0.10
		<i>Hippocampus</i>	0.497	-0.4673 to 0.2428	-0.12
	Isolation Condition	<i>Bone Marrow</i>	0.002**	-0.7439 to -0.2020	-0.52
		<i>Peripheral Blood</i>	0.02*	-0.6655 to -0.04819	-0.40
		<i>Hippocampus</i>	0.012*	0.1099 to 0.6987	0.45

<i>Open field test</i> <i>(number of rearings)</i>	Social Condition	<i>Bone Marrow</i>	0.08	-0.04293 to 0.6116	0.322
		<i>Peripheral Blood</i>	0.962	-0.3681 to 0.3524	-0.00
		<i>Hippocampus</i>	0.741	-0.3043 to 0.4137	0.06
	Isolation Condition	<i>Bone Marrow</i>	0.000***	-0.8105 to -0.3577	-0.63
		<i>Peripheral Blood</i>	0.006**	-0.7201 to -0.1523	-0.48
		<i>Hippocampus</i>	0.000***	-0.8094 to -0.3549	-0.63
<i>Open field test</i> <i>(time in central zone)</i>	Social Condition	<i>Bone Marrow</i>	0.847	-0.3917 to 0.3280	-0.03
		<i>Peripheral Blood</i>	0.888	-0.3369 to 0.3832	0.02
		<i>Hippocampus</i>	0.632	-0.4369 to 0.2785	-0.09
	Isolation Condition	<i>Bone Marrow</i>	0.002**	0.2107 to 0.7480	0.53
		<i>Peripheral Blood</i>	0.000***	0.2696 to 0.7743	0.57
		<i>Hippocampus</i>	0.024*	0.05704 to 0.6704	0.40
<i>Hole-board test</i>	Social Condition	<i>Bone Marrow</i>	0.294	-0.1747 to 0.5211	0.19
		<i>Peripheral Blood</i>	0.414	-0.4879 to 0.2176	-0.15
		<i>Hippocampus</i>	0.107	-0.06744 to 0.5960	0.30
	Isolation Condition	<i>Bone Marrow</i>	0.000***	0.3642 to 0.8131	0.64
		<i>Peripheral Blood</i>	0.000***	0.3143 to 0.7931	0.60
		<i>Hippocampus</i>	0.007***	-0.8178 to -0.3763	0.476
<i>TNF-α Level</i>	<i>Plasma</i>	Social Condition	<i>Bone Marrow</i>	0.6011	-0.2705 to 0.4438

		<i>Peripheral Blood</i>	0.862	-0.3887 to 0.3311	-0.03
Isolation Condition		<i>Bone Marrow</i>	0.017*	-0.6847 to -0.08332	-0.43
		<i>Peripheral Blood</i>	0.012*	-0.6974 to -0.1073	-0.45
<i>Hippocampus</i>	Social Condition	<i>Hippocampus</i>	0.595	-0.2691 to 0.4450	0.10
	Isolation Condition	<i>Hippocampus</i>	0.008***	-0.7127 to -0.1375	-0.4742
<i>IL-1β Level</i>	<i>Plasma</i>	Social Condition	<i>Bone Marrow</i>	0.954	-0.3698 to 0.3507
			<i>Peripheral Blood</i>	0.595	-0.2692 to 0.4450
		Isolation Condition	<i>Bone Marrow</i>	0.045*	-0.6429 to -0.008639
			<i>Peripheral Blood</i>	0.01**	-0.7055 to -0.1232
<i>IL-6 Level</i>	<i>Hippocampus</i>	Social Condition	<i>Hippocampus</i>	0.790	-0.4035 to 0.3154
		Isolation Condition	<i>Hippocampus</i>	0.008***	-0.7131 to -0.1383
		Social Condition	<i>Bone Marrow</i>	0.729	-0.3016 to 0.4163
			<i>Peripheral Blood</i>	0.483	-0.4707 to 0.2387
		Isolation Condition	<i>Bone Marrow</i>	0.056	-0.6324 to 0.009047
			<i>Peripheral Blood</i>	0.051	-0.6368 to 0.001627
		Social Condition	<i>Hippocampus</i>	0.576	-0.2642 to 0.4492
		Isolation Condition	<i>Hippocampus</i>	0.02*	-0.6760 to -0.06715
<i>Nitrite Level</i>	<i>Plasma</i>	Social Condition	<i>Bone Marrow</i>	0.682	-0.2906 to 0.4262
			<i>Peripheral Blood</i>	0.912	-0.3785 to 0.3418
	Isolation Condition	<i>Bone Marrow</i>	0.032*	-0.6589 to -0.03646	-0.39

		<i>Peripheral Blood</i>	0.000***	-0.7265 to -0.1653	-0.49
<i>Hippocampus</i>	Social Condition	<i>Hippocampus</i>	0.752	-0.3067 to 0.4116	0.06
	Isolation Condition	<i>Hippocampus</i>	0.004***	-0.7354 to -0.1839	-0.51

Figures

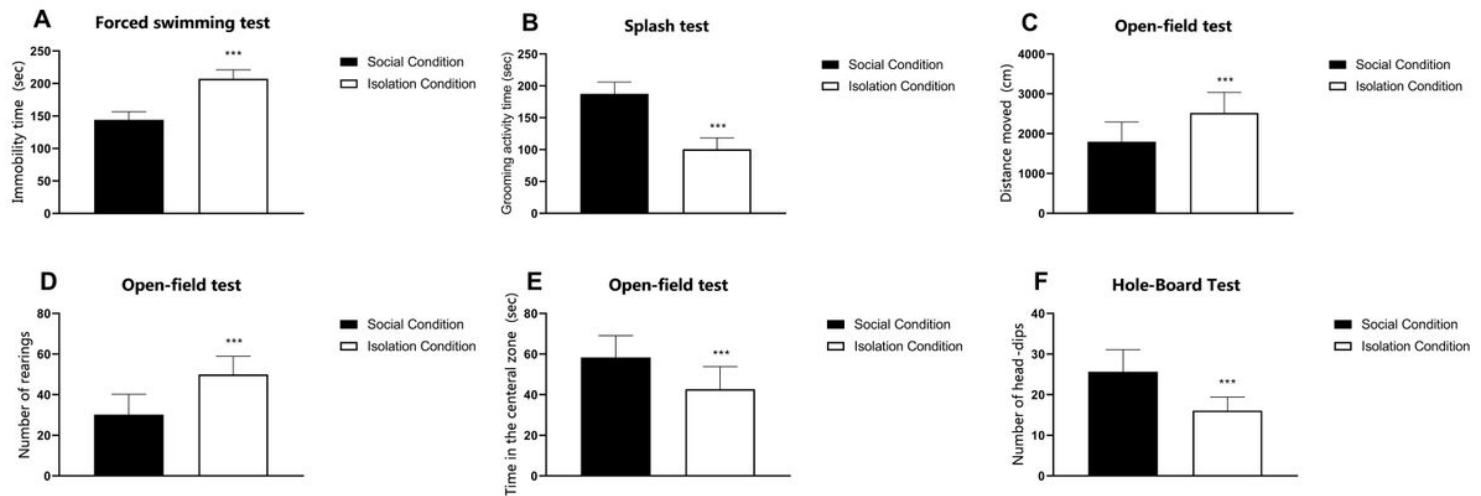


Figure 2

Effects of SIS on immobility time of FST (A), grooming activity of Splash test (B), distance moved (C), number of rearing (D), time in the central zone (E) of OFT, and number of head-dips in Hole-board test (F). Values are expressed as the mean \pm SD from 30 animals in each group and were analyzed using the unpaired t-test. *** P<0.001 compared with normal animals.

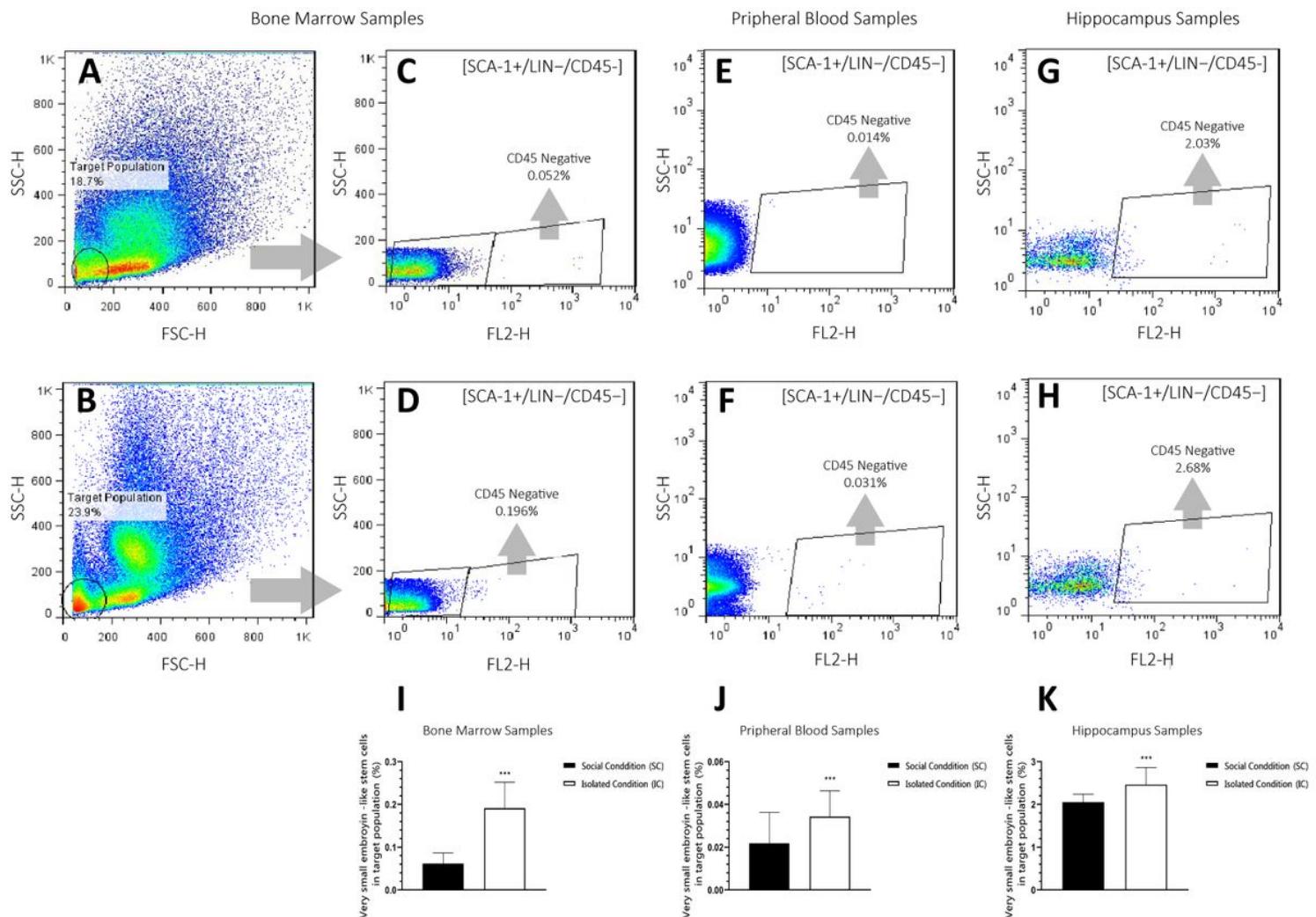


Figure 4

Evaluation of VSELs counts obtained from hippocampus, bone marrow, and peripheral blood in normal and socially isolated animals. A and B panels show the target population of VSELs based on the size of bone marrow samples. C and D panels show the final results of VSELs population percentage (SCA1+, LIN-, and CD45-) in bone marrow. E and F panels show the final results of VSELs population percentage in peripheral blood. G and H panels show the final results of VSELs population percentage in the hippocampus. I, J, and K panel show the unpaired t-test analysis of data mentioned above. Values are expressed as the mean \pm SD from 30 animals in each group. *** P<0.001 compared with normal animals.

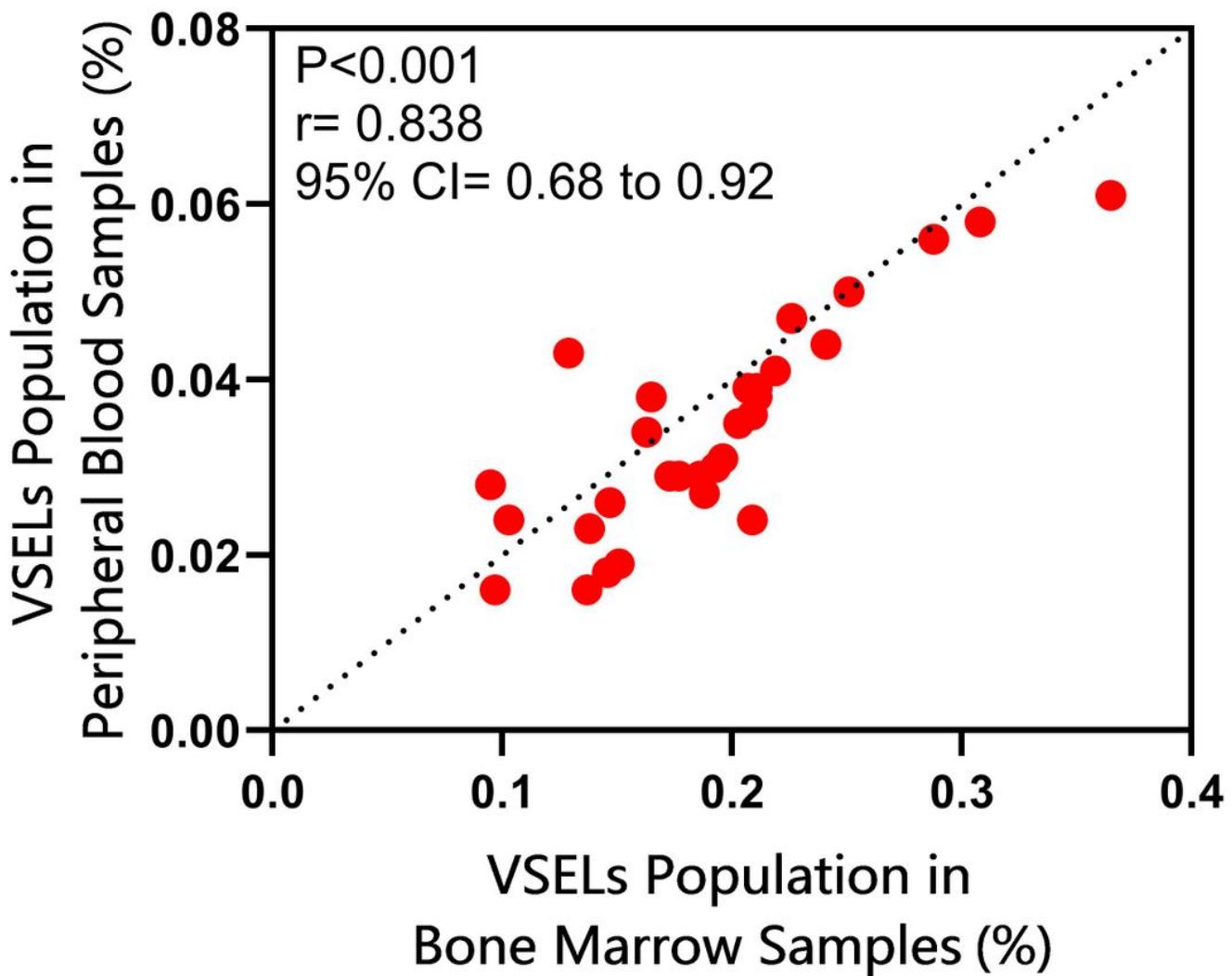


Figure 6

Correlation analysis between the VSELs counts of bone marrow and peripheral blood for clarifying the mobilization of VSELs from bone marrow to peripheral blood.

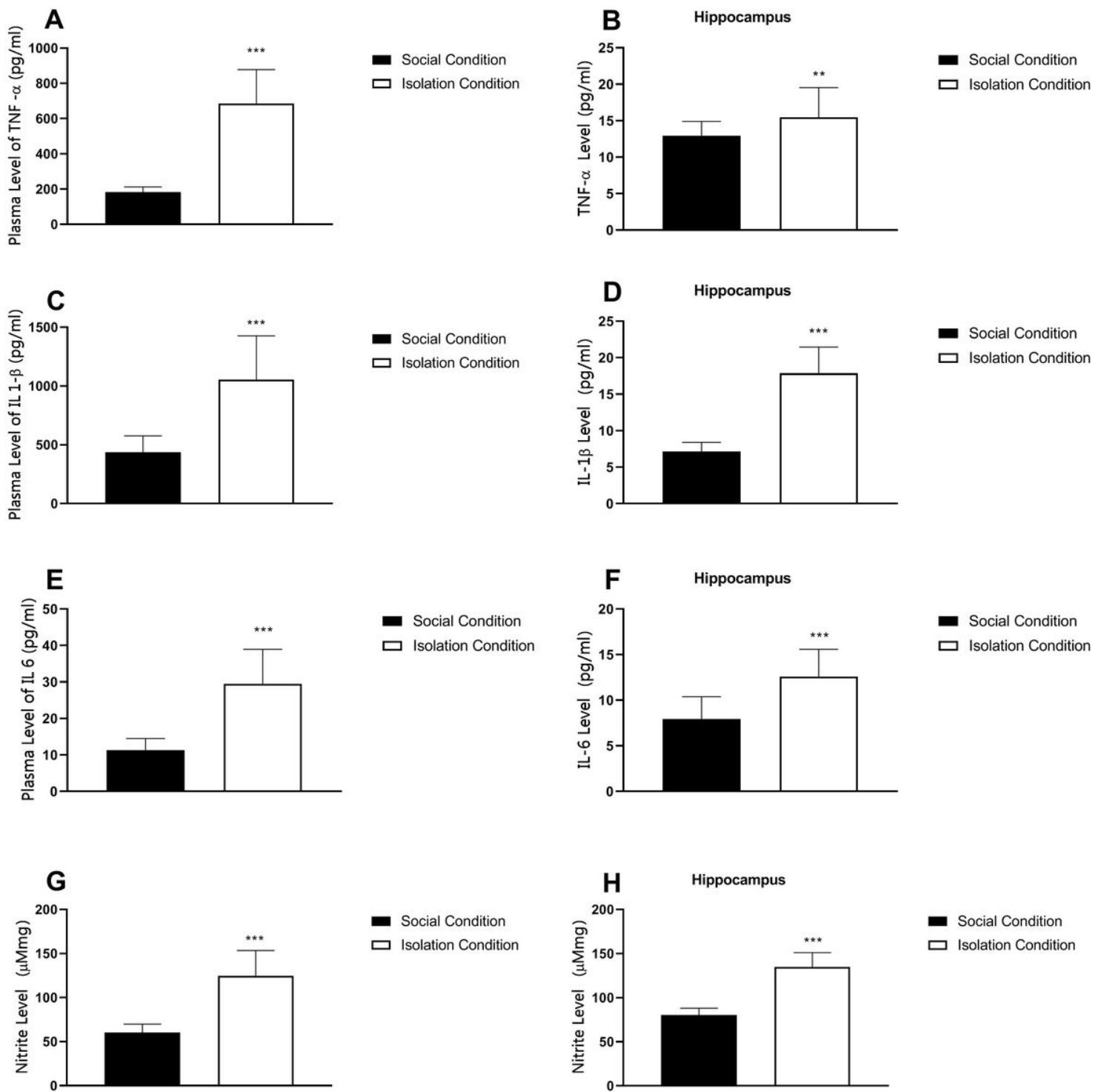


Figure 8

Evaluation of pro-inflammatory cytokines including TNF- α (A and B), IL-1 β (C and D), and IL-6 (E and F), as well as nitrite level (G and H) in plasma (A, C, E, and G) and hippocampus (B, D, F, and H) in both normal and socially isolated animals. Values are expressed as the mean \pm SD from 30 animals in each group and were analyzed using the unpaired t-test. *** P < 0.001, ** P < 0.01 compared with normal animals.