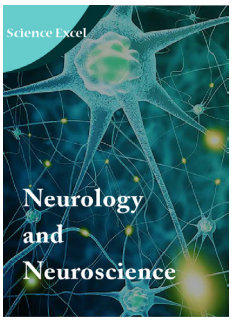
Neurology & Neuroscience

Original



Inhibition of long-term kindled seizures induced alterations in the function of bone marrow cells by AC-31B (essential oil) from *Allium cepa*

Shabana U Simjee[1,2*], Uzair Nisar[1], Raheela Khan[1], Warda Ainuddin[1], Iqra Mukhtar[1], Muhammad Jamal[1], Muhammad Azam[2], Nighat Shamim Rizvi[1], Asmat Salim[2] and Shazia Anjum[1,3]

[1]*H.E.J. Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi, Pakistan*
[2]*Dr. Panjwani Center for Molecular Medicine and Drug Research, International Centre for Chemical and Biological Sciences, Pakistan*
[3]*Department of Chemistry, The Islamia University of Bahawalpur, Pakistan*

Correspondence

Shabana Usman Simjee

H.E.J. Research Institute of Chemistry, Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences

University of Karachi, Karachi-75270, Pakistan

Tel: +92-213 4824925

Fax: (+92-213) 4819018, 4819019

E-mail: shabana.simjee@iccs.edu,

sh01us@hotmail.com

- Received Date: 03 Feb 2020;
- Accepted Date: 02 Mar 2020;
- Publication Date: 14 Mar 2020.

Keywords:

Epilepsy, CD44, CD90, seizures, PTZ-kindling

Abstract

Background: The therapeutic goal of epilepsy is to reduce the frequency of seizure. Available antiepileptic drugs only manage symptoms and are associated with hematological and immunological dysfunctions. Since the bone marrow (BM) provides a suitable environment for proliferation and differentiation of hematopoietic stem cells, any change in the BM environment could have an impact on the immune system. Here we investigated the effects of AC-31B on BM stroma cells (BMSCs) functions following kindling. Methods and Findings: The present study used CD44 and CD90 as recognizing markers. Once score five develops, animals were sacrificed and tested for the aforementioned markers in the BMSCs. Colony forming units-granulocyte/macrophage for PBMNCs and BMSCs was also performed. We observed highest expression of CD44 and CD90 in BMCs progenitor cells and in CFU-GM cultures from PTZ-control and diazepam treated mice. In contrast, AC-31B significantly lowered the expression of these markers. The BM cellularity and clonogenic assays from AC-31B treated kindled animals were comparable to the normal group. Conclusions: Data indicates that there is an immune response magnification in PTZ-kindled mice cells and AC-31B not only suppresses the kindling but also moderately reduced the expression of the CD markers. We suggest that unlike diazepam, AC-31B does not affect the BM cellularity and its associated functions. Further studies on isolation of active lead molecule from AC-31B may lead to discovery of naturally occurring AEDs.

Introduction

Epilepsy is a condition which is characterized by recurrent seizures as a result of abnormal excitability of the nerve cells. In this condition there is a loss in the balance between the excitatory and inhibitory pathways of the nervous system [1,2]. It is a universal disease; affecting approximately 70 million people of all ages worldwide [3] and nearly 80% of people with epilepsy are found in developing countries [4].

Apart from damaging the neurons in the brain, epilepsy is often associated with alterations in the cellularity of the bone marrow which leads to alteration in the immune functions [5]. Several studies have reported a linkage between changes in immune function and seizure activity [6-9]. The CNS is known to affect hematopoietic and immune systems through sympathetic nerve fibers [10, 11] and this mechanism is important for normal hematopoiesis and for the controlled release or retention of cells within the bone marrow. The bone marrow provides a microenvironment for self-renewal, proliferation, and differentiation of hematopoietic stem cells into peripheral blood cells and also supports B cell development and development of early stages of T cell progenitors [12]. Thus, any change in the bone marrow microenvironment could have an impact on the development of the components of the immune system. Since bone marrow is the source of immune cells in adults, it is possible that immune alterations during epilepsy could be partly due to disruptions of hematopoietic functions by abnormal activity of CNS neurons. Studies conducted in experimental animals have suggested a linkage between seizure activity and alterations in immune functions. In addition, there are some antiepileptic drugs (AEDs) which are associated with adverse effects on bone marrow cellularity and therefore on the immune system [13-16]. These unfavorable responses to the presently available drugs indicate a need for the development of new drugs as alternatives.

Like other diseases that have been cured by the medicines that are based on natural products, there are several plants used in folk medicine for curing the epilepsy such as compound isolated from the *Piper methysticum* [17-19]. Keeping these facts in

Citation: Simjee US, Nisar U, Khan R, et al. Inhibition of long-term kindled seizures induced alterations in the function of bone marrow cells by AC-31B (essential oil) from Allium cepa. Neurol Neurosci. 2020;1(1):1-8.

mind i.e., the limitations of current antiepileptic drug therapy and prehistoric role of natural products based drug discovery, this study was designed to test essential oils AC-31B isolated from *Allium cepa*. The reason for selecting AC-31B is purely based on the fact that it has shown potent anticonvulsant and anti-epileptic activity in our earlier studies [20]. The objectives of this study were (a) to evaluate the effect of chronic seizures-induced changes in BMSCs , (b) to characterize the anticonvulsant effects and safety of AC-31B in standard seizure models in rodents, (c) antiepileptogenic activity of the AC-31B tested in chemically-induced kindling model in mice, since this kindling permits investigations into the convulsive component of epilepsy, epilepsy-related alterations in behavior as well as neuro-morphological, neurophysiological, and neurochemical determinants of this complex disease, and (d) to explore whether changes in hematopoietic functions following kindling are likely to be controlled by AC-31B isolated from natural products. The recognizing markers of used for this study are CD44 and CD90.

The CD44 and CD90 molecules are intercellular adhesion molecules that bind to a number of ligands such as fibronectin, vascular cell adhesion molecule-1 (VCAM-1) and hyaluronic acid and are responsible for homing and proliferation of the hematopoietic progenitor cells (HPCs) [21-25]. Thus, the adhesive interaction between the HPCs and stroma leads to their proliferation and homing in the bone marrow and supports hematopoiesis [23]. Keeping in mind, the role of these CD molecules in the proliferation, homing and maturation of the immune components, it is important to consider their expression level in order to determine the immune status of an individual.

Material and methods

Reagents

The Collagen Type-I, diazepam, Ficoll-Hypaque, pentylenetetrazole (PTZ), and trypan blue were purchased from Sigma Chemicals (USA). The cell culture reagents were purchased from Invitrogen Corp. (CA, USA). For cell washing, physiological buffer i.e., 1x phosphate saline buffer (PBS) was used.

For immunostaining, monoclonal antibodies to rat H-CAM; CD44H (BD Pharmingen Company, Franklin Lakes, NJ, USA), and rat CD90 (Thy 1.1) (Cedarlane Laboratories Ltd. Canada) were used. Alexa flour® 488 goat anti-mouse IgG (H + L)*2 (Invitrogen Corporation Carlsbad, CA, USA) was used as secondary antibody. For RT-PCR analysis of CD markers, RNeasy Mini Kit (Qiagen Inc. Valencia, CA, USA), Superscriptase III First-Strand synthesis system for RT-PCR (Invitrogen Corporation Carlsbad, CA, USA) and Omniscript RT kit (Qiagen Inc. Valencia, CA) were used. For Agarose gel electrophoresis; Agarose, was purchased from MP Biomedicals. Inc, France and DNA ladder; Gene Ruler™, 100bp DNA ladder was purchased from Fermentas. Inc. (Glen Burnie, MD, USA). The GAPDH was used as an internal standard (house keeping gene). The primer sequence of the mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, CD44 and CD90 are shown in table 1.

In vivo anti-epileptogenic activity screening of AC-31B

Animals: The NMRI male mice of 20-25 kg weight were used. The animals were maintained under standard laboratory conditions with a 12 hr light: dark cycle, at 21 ± 1°C temperature. All experiments were carried out following Standard International Guidelines for Animal Care and Use and regulated by Advisory Committee on Animal Care, Use, and Standards of International Center for Chemical and Biological Sciences, University of Karachi. Study was commenced once the protocol was approved by the institutional advisory committee (Protocol Approval No.: 2015-0007).

scPTZ-induced kindling in mice (model of epileptogenesis)

Each treatment group (table 2) consists of 18 male NMRI mice. Kindling was induced according to the modified method of De Sarro et al. (2000). Except normal control, all other groups were given sub-convulsive doses of PTZ i.e., 50 mg /kg subcutaneously once every second day between 9:30 - 11:00 am. The normal, drug control and test groups received daily dose of their saline, diazepam and AC-1B respectively. On the day of PTZ administration, the treatment of saline, diazepam (7.5 mg/kg) or AC-31B (80 mg/kg) was given 45-50 minutes before the PTZ. After each PTZ injection, animals were placed in observation chambers for 1 hour, and behavioral seizure activity was rated. Animals were scored according to a pre-validated scoring scale for the severity of the seizure activity. Seizure patterns during the gradual development of kindling are classified into five distinct behavioral stages: Stage 0 no response, stage 1 ear and facial twitching, stage 2 convulsive wave through the body, stage 3 myoclonic jerks, stage 4 clonic-tonic convulsions, turnover into side position, stage 5 generalized clonic-tonic seizures, loss of postural control. The cumulative kindling score was then calculated i.e., average of the individual behavioral scores and then dividing them with the number of the animals in order to plot against the number of treatments. The experiments were terminated once the PTZ-control group reached the score 5. The samples were collected and processed as per requirement.

Culturing of BMSCs and determination of BMS cellularity

Mice were sacrificed under aseptic condition and both femurs were immediately removed, ends were cut and the cells were dislodged with a 22 G needle attached to a syringe containing complete DMEM cell culture media with 5% FBS, and 1% streptomycin/ penicillin. A single cell suspension was produced by repeatedly flushing the bone marrow cells through a syringe. The total number of viable cells was enumerated by Trypan Blue Exclusion test. The viability of the cells used in this study varied from 70-90%.

To determine the stromal cellularity; the cell suspension was checked by counting the number of cells on hemacytometer. The total number of cells was then multiplied by the factor 104 in order to get number of cells/ml. For BMS culturing, the cells were washed with 1X phosphate buffer saline (PBS) and then resuspended at $2x10^6$ cells /ml in complete DMEM culture medium and placed in 75-cm^2 tissue culture flasks. Cultures were incubated at 37°C in 5% CO_2. The cultures received weekly replacement of 50% culture media until cells attained confluence.

Isolation of mononuclear cells from bone marrow and peripheral blood

Mononuclear cells from peripheral blood or bone marrow were isolated by a standard Ficoll density gradient centrifugation method. The ring of mononuclear cells at the Hypaque–plasma interface was taken in a separate tube and washed twice with PBS.

Clonogenic assays

To assess the total number of progenitor cells in BM and periphery, BMNCs (bone marrow mononuclear cells) and PBMCs (peripheral blood mononuclear cells) were cultured in methylcellulose for colony-forming units' i.e., granulocyte/macrophage (CFU-GM). BMNCs (105/ml) and PBMCs (10^6/ml) were plated with methylcellulose in 33-mm culture dishes/75-cc tissue culture flask. Lymphocyte conditioned medium was provided to the cultures to support the growth of CFU-GM. To determine cells/clone, clones were observed at 40× under an inverted Nikon microscope. Clusters of ≥20 cells were scored as a single clone.

Immunostaining for CD44 and CD90

Once the cells attain confluence; they were trypsinized and centrifuged to get the cell palate. The palate was re-suspended in the DMEM media and allowed to grow overnight in the 4-valve slide and kept in an incubator maintained at 37°C with 5%CO_2. Next day, the cells were fixed in 4% paraformaldehyde for 30 minutes, washed with phosphate-buffered saline (PBS) and blocked in PBS containing 2% bovine serum albumin, 2% normal goat serum and 0.2% Tween20 (blocking solution) for 1 hour at 37°C. Following the incubation, the cells were washed with PBS and re-incubated overnight at 4°C with the respective primary

antibodies (CD44 or CD90) at 1:50 dilutions in blocking solution. Next day, cells were gently washed with 1x PBS and were then incubated with secondary antibody Alexa flour® 488 goat anti-mouse IgG at 1:200 dilution in PBS for 1 hr at room temperature. Before observing the cells, DAPI was added for the staining of the cell nuclei and cells were examined under fluorescence microscope. The intensity of CD44 and CD90 immunofluorescence was measured and analyzed by image processing program ImageJ (National Institutes of Health, MD, USA). This software helps in multiple imaging system data comparisons taking density (densitometry) in consideration. For each image, background density was determined and subtracted; the remaining particles were considered to represent CD44 and CD90 expression. Data were obtained from 3-4 images and presented as means ± S.E.M.

Determination of expression of CD44 and CD90 mRNA

Total cellular RNA was extracted from the cells according to manufacture's protocol provided along with the RNeasy Mini Kit and was then reverse transcribed into cDNA using Superscriptase III First-Strand synthesis system for RT-PCR. The final product i.e., cDNA was then amplified using Omniscript RT kit and oligonucleotide primers corresponding to transcripts of the murine GAPDH, CD44 and CD90 genes. To exclude contamination of genomic DNA, reverse transcription was also carried out for the same sample without adding the enzyme (negative control). Mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal standard. Each sample was assayed in triplicate and normalised to the expression of the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The products of reverse transcription reactions were placed in a preheated (94°C) thermal cycler and then denatured for 1 minute at 94°C, followed by 35 cycles of amplification in the following manner: 15 seconds *denaturation* at 94°C then 30 seconds *annealing* at 55-58°C and then 1 minute extension at 72°C. The final extension step was performed at 72°C for 10 minutes and upon completion the reactions were maintained on 4°C. The prepared PCR products were analyzed on 1% agarose-gel electrophoresis. Ethidium bromide (1.5 µL) was added into the gel for the detection of specific bands of DNA under UV light. DNA standard (GeneRulerTM 100 bp DNA Ladder) and 10 µL PCR products were loaded in each well. After complete run, the gel was removed and visualized under UV transluminescence and photographed using Gel-Doc system (Alpha Innotech, CA, USA). The density of each band was quantified. The density of CD44 and CD90 products were normalized to that of GAPDH. Density of each band was quantified by taking two dimensional area of each band. The integrated density value (IDV) was obtained through the corresponding pixel intensity using Spot Density Tools of the Gel-Doc system.

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS). The result is shown in mean ± SEM. Data of seizure score was analyzed by nonparametric Mann-Whitney U tests. The difference in the cellularity of the cells among various treatment groups and mRNA expression of CD44 and CD90 were analyzed using one-way analysis of variance i.e., ANOVA (using Bonferron's post hoc test). The immunostaining data was analyzed by one-way ANOVA with Dunnett's post hoc tests. Sequential differences among means were calculated at the level of $P < 0.05$ using the SPSS version 19.

Results

Seizure score in PTZ -induced kindling model

After third dose of PTZ, the animals in the control kindled group displayed a gradual increase in the seizure score and exhibited the score 5 with the average seizure score of 4.83 by the sixteenth dose (Figure 1). The seizure score of PTZ-kindled control group was significantly different from day 8 onward ($P < 0.05$) compared to the normal group. The diazepam treated kindled animals exhibited an average seizure score of 0.33. In contrast, the AC-31B (80 mg/kg) treatment exhibited average seizure score of 0.778 and protection from different seizure patterns, such as twitches, body jerks, clonus and generalized seizure.

Cellularity of the BM from kindled and unkindled mice

Studies have reported that stress conditions influence BM cellularity, affecting overall changes in progenitor cells. Accordingly, we first determined the cellularity of the cells for each group of mice. The cells from the two femurs of each animal were pooled and the total number of viable cells counted. One-way ANOVA with bonferroni's post hoc test was used to analyze the data. A comparison of the total number of cells in femurs from the six groups (Figure 2) showed significant differences where, PTZ-kindled control group was found to exhibit a significant increase in the cellularity compared to normal control ($P < 0.04$) and AC-31B ($P < 0.01$) treated groups. A post hoc analysis indicated that both the kindled and un-kindled mice treated with diazepam had reduced cellularity of the bone marrow compared to the normal ($P < 0.02$) and PTZ-kindled control ($P < 0.01$). When the diazepam treatment was compared to AC-31B kindled groups, a significantly decrease cellularity was observed ($P < 0.05$, Figure 2).

Proliferation of primitive BM progenitors

After observing increased cellularity or hyperproliferation of bone marrow progenitors in PTZ-kindled mice, we next determined if there were changes in bone marrow stroma because stroma plays an important role in providing the basis for the development of progenitor cells and supporting hyperproliferation of these cells. Our results showed an increase in stromal cells in the PTZ-kindled control seizure group ($P < 0.01$) relative to the normal control mice (Figure 3). These results indicate that the immature bone marrow progenitors in kindled mice were 'primed' to proliferate rapidly, suggesting that the defect seems to be present at the level of the primitive bone marrow progenitors. The AC-31B kindled and un-kindled group showed same proliferative pattern as the normal control group and there was no significant difference between these groups. However, diazepam treated kindled and un-kindled groups showed significantly lower ($P < 0.05$) than the normal control animals.

Clonogenic assays from peripheral blood and bone marrow

Since we observed changes in bone marrow cellularity, we next determined if the effects of seizures were extended to dysfunction in the more immature progenitors. One-way ANOVA for clonogenic assays from peripheral blood for the six groups showed that the groups differed significantly in total numbers of colonies observed. Post hoc analysis indicated that kindled mice in PTZ-control group had a significantly higher colony number ($P<0.005$, $F=4.81$) relative to normal control and kindled animals treated with AC-31B, however kindled and un-kindled mice treated with diazepam showed significantly reduced number of clones compared to other groups included in this study ($P<0.01$, Figure 4).

One-way ANOVA analysis for clonogenic assays from BMNCs for the BM progenitors showed that the kindled and un-kindled groups differed with respect to the number of colonies ($P<0.05$, $F=13.54$). Post hoc analysis showed that BMNCs from PTZ-kindled control animals displayed a significantly higher proliferation ($P<0.05$) as compared to the normal control animals or kindled mice treated with reference drug or AC-31B (Figure 5). Within the treatment groups, the diazepam treated kindled or un-kindled groups exhibited a significantly low CFU compared to normal ($P < 0.02$) and AC-31B treated kindled groups ($P < 0.04$). Microscopic observation for cluster of cells/clone showed average number of cells/clone for kindled mice were 20 ± 5 cells.

Immunochemical analysis of CD44 and CD90

One-way ANOVA with Dunnett's post hoc was used to analyze the immunostained cultured stromal cells harvested from the PTZ-kindled control group revealed the highest expression of CD44 (Figure. 6) and CD90 positive cells (Figure. 7). Amongst the treatment groups, the kindled mice treated with diazepam also showed high expression of these markers which was comparable to that of the PTZ-induced

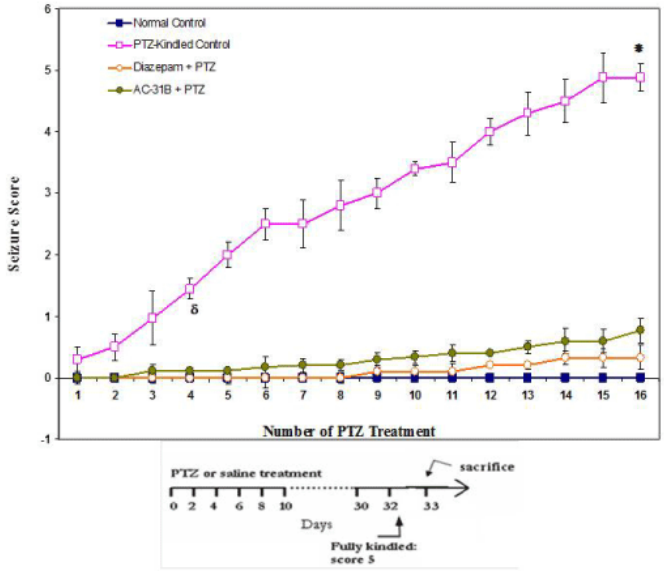


Figure 1. ***Effect of AC-31B on scPTZ-induced kindling.*** *The kindling scores are expressed as the arithmetic mean ± SEM of 18 animals in each treatment group. The treatment of 80 mg/kg of AC-31B exhibited a mean of 0.77 by the end of the experiment. The bar under the figure represents the timeline of the experiments. The rats were treated with PTZ on alternate day. A total of 16 doses of PTZ were given during the 32-days period and by this time the animals in control group were fully kindled. Starting from the 4th injection of PTZ, the average scores of PTZ-induced seizures were significantly higher in the kindled control rats (receiving no other treatment except PTZ, $^{\delta}P < 0.05$). *P < 0.03: significantly higher than normal control revealed by nonparametric Mann-Whitney U tests. The animals were sacrificed for immunohistochemistry and RT-PCR analysis after 33 days experiment.*

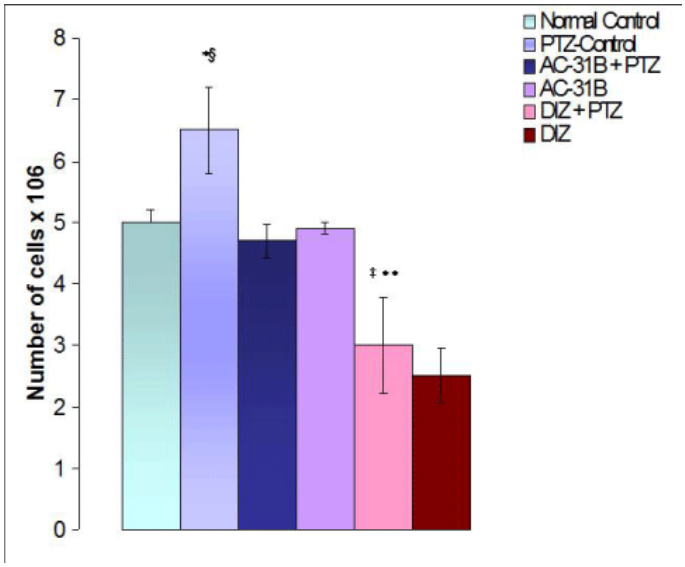


Figure 2. ***Cellularity of bone marrow harvested from kindled and un-kindled animals.*** *The results are expressed as the mean ± SEM. Graph indicates that total cellularity of bone marrow was significantly increased in control PTZ-kindled group compared to the normal control (*P < 0.04) and AC-31B or diazepam treated kindled animals (§P < 0.01 for both AC-31B and diazepam). The diazepam treated kindled mice also showed marked decrease in the bone marrow cellularity when compared to the normal control group (P < 0.01). Likewise, a significant decrease in the cellularity of diazepam treated group was also observed when compared to the AC-31B treated group (**P < 0.05).*

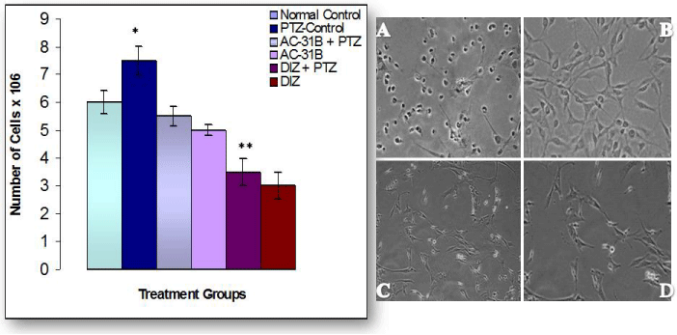


Figure 3. ***Effect of kindled seizures on bone marrow (BM) cells.*** *Control PTZ- kindled mice show significant hyperproliferation of BM progenitors (*P < 0.01) compared to normal control and kindled AC-31B treated mice. Within the treatment groups, the diazepam treated groups exhibited significantly reduced proliferation compared to normal control group (**P < 0.05). The results are expressed as the mean ± SEM (in box image) Phase contrast images of bone marrow stromal cells of the kindled and unkindled groups. The control PTZ- kindled group demonstrated marked hyperproliferation of BM progenitors compared to normal control and kindled AC-31B treated mice. Where A-D represents normal control, PTZ-control, AC-31B treated kindled group and Diazepam treated kindled group respectively.*

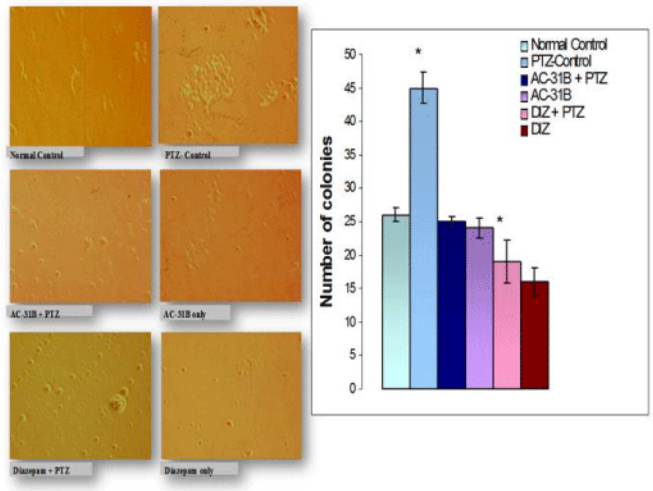


Figure 4. ***CFU-GM assays performed with peripheral blood mononuclear cells (10⁶/ml).*** *The control PTZ-kindled group indicated significantly higher numbers of colonies in PBMCs (*P < 0.01) cultures; relative to normal control and AC-31B treated kindled mice. In contrast, diazepam treated kindled mice showed significant reduction in CFU assay (**P < 0.005). The image in the figure shows the CFU of PTZ-control and Normal-control groups. The results are expressed as the mean ± SEM.*

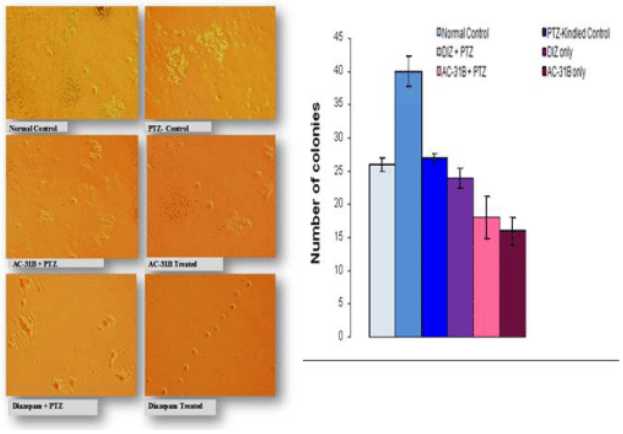


Figure 5. ***CFU-GM assay of bone marrow mononuclear cells (10^5/ml) from the kindled and un-kindled animals.*** *Significantly higher numbers of colonies was observed in the BMNCs cultures of control PTZ-kindled group (*P < 0.05); relative to normal control and AC-31B treated kindled mice. In contrast, diazepam treated kindled mice showed significant reduction in CFU assay (**P < 0.005). The results are expressed as the mean ± SEM.*

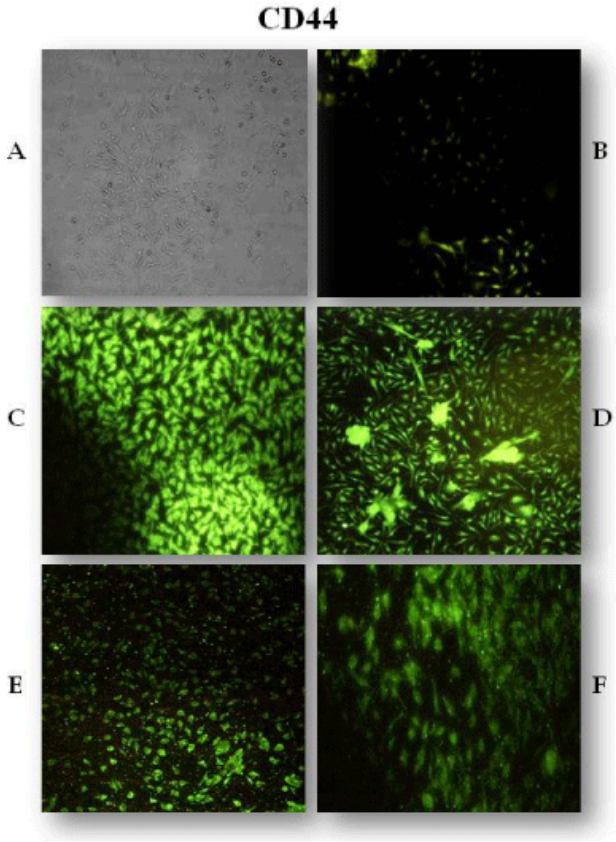


Figure 6. ***Representative photomicrograph of CD44 immunoreactivity in normal and kindled mice brains with stage 5 seizure activity.*** *Phase contrast photomicrograph of the normal control group (A) has been shown along with its fluorescence photograph (B) in order to show the population of the cells present in the culture. Note the increased immunofluorescence in the PTZ kindled control cultured cells (C) compared to normal control. The diazepam treated kindled group also demonstrated marked increase in the CD44. The AC-31B treated kindled group (E) showed a significant decrease in the expression of CD44. A baseline expression of CD44 was also detected in AC-31B treated normal group (F) however it was comparable to the normal control group.*

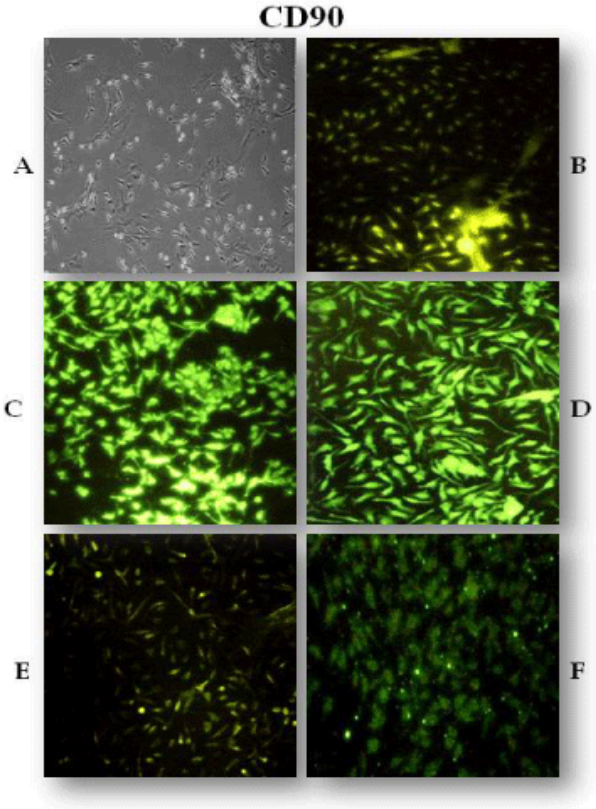


Figure 7. ***Fluorescence photomicrographs of bone marrow stromal cells showing CD90 immunoreactivity in kindled and un-kindled groups.*** *Phase contrast photomicrograph of the normal control group (A) has been shown along with its fluorescence photograph in order to show the population of the cells present in the culture (B). The baseline fluorescence was detected in the normal un-kindled group. In contrast, cells harvested and cultured from PTZ-kindled control group (C) showed marked increase in the expression of CD90 followed by diazepam treated kindled group (D). The AC-31B treated kindled group (E) showed a significant decrease in the expression of CD90, however, it was slightly higher than the normal control. A baseline expression of CD44 was also detected in AC-31B treated normal group (F).*

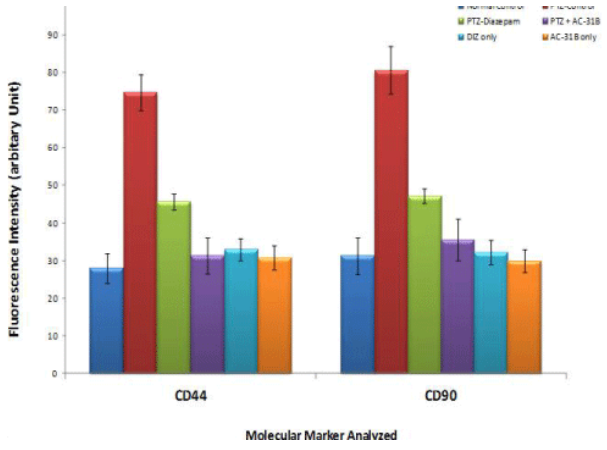


Figure 8. ***Graphic representation of the level of expression of immunochemically detected CD44 and CD90 in cultures set from kindled and unkindled animal groups and analyzed by ImageJ software.*** *In comparison to normal control group (29.47 ± 3.73), the PTZ control animals showed a significant increase in the CD44 and CD90 intensity (74.69 ± 17.3, *p < 0.024).*

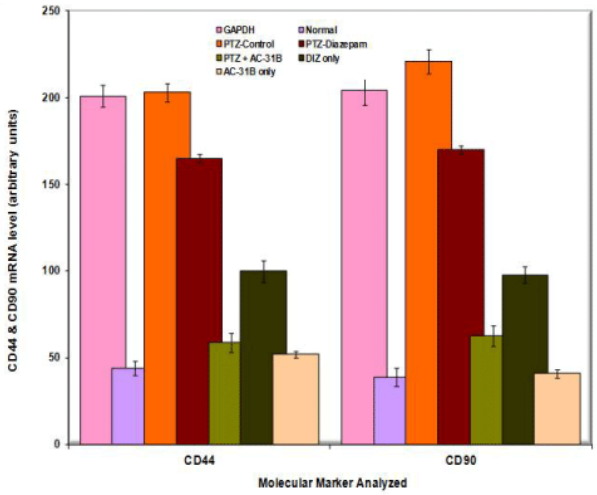


Figure 9. ***Representative for mRNA expression of CD44 and CD90 molecules on the surface of BMSCs.*** *Total RNA were extracted from BMSCs of kindled and un-kindled groups. Following the agarose gel electrophoresis, the bands were quantified by densitometry and each mRNA level was normalized to that for GAPDH mRNA. Each bar represents the mean ± S.E.M.*

control animals. In contrast, lower expression of CD44 and CD90 was observed in the kindled animals treated with AC-31B (Figure. 8). The baseline expression of these markers was also observed in the normal control and un-kindled animals given the diazepam or AC-31B.

RT-PCR analysis

Following RT-PCR reactions, CD44 and CD90 receptors' specific bands were detected in all samples. We have observed that the expression of the mRNA for these markers correlates with that of the immunostaining data. Figure 9 shows a representative agarose gel of typical RT-PCR reactions indicated significantly higher expression of CD44 and CD90 in the PTZ-kindled group compared to the normal control animals ($p < 0.02$ and $p < 0.01$ for CD44 and CD90 respectively). In contrast, the expression of both CD44 and CD90 were lower in the diazepam treated and AC-31B-treated kindled animals. However, within the treatment groups, the expression was far less in AC-31B when compared to the diazepam treated kindled animals. The baseline expression of both the CD44 and CD90 were also observed in the normal control and un-kindled animals given the diazepam or AC-31B (Fig. 9). In all experiments, the levels of housekeeper GAPDH were equal between treated and untreated samples.

Discussion

Earlier in our laboratory we observed a potent anticonvulsant activity of AC-31B both in maximal electroshock test (MEST) and scPTZ-induced acute seizure model in mice. Therefore, it was decided to evaluate its action on the process of epileptogenesis in the model of chemical kindling induced in mice. Our data demonstrated a potent anti-epileptogenic activity of AC-31B in scPTZ-induced kindling model in mice. It was observed that 80 mg/kg dose of AC-31B significantly retarded the development of seizures as compared to the kindled control group. Several lines of evidence suggest that PTZ produces its behavioral effects by blocking the postsynaptic actions of the inhibitory neurotransmitter gamma-amino butyric acid (GABA) and also block the neurotransmitter induced chloride conductance [26-30]. Furthermore, the benzodiazepines, a well known facilitator of GABAergic neurotransmission, antagonize the PTZ-induced convulsions [31] and PTZ has also been shown to inhibit the benzodiazepine binding competitively [32-34]. Gamma aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain, and the inhibition of GABAergic neurotransmission has been thought to be the major underlying factor in the development of epilepsy. The enhancement of the GABAergic neurotransmission has already been reported to antagonize seizures, while its inhibition promotes seizures. In our study, we have used diazepam (7.5 mg/kg) as a reference drug. The protection of mice against PTZ-induced seizures observed in diazepam treated kindled mice was expected, since various authors have shown that diazepam exert its anticonvulsant activity by enhancing GABA-mediated inhibition 35-36. Earlier studies have shown that the systemic administration of PTZ lead to generalized tonic-clonic seizures and antagonizes the effects of the GABA [36, 37-39]. The suggested reduction of synaptic inhibition can contribute to the convulsive effects of PTZ [40, 41]. In the light of these reports and our observations i.e., retardation of PTZ induced kindling by AC-31B, we suggest that the anti-seizure effect of AC-31B may be in part due to their ability to attenuate PTZ-induced seizures by interfering with GABAergic transmission. However, unlike diazepam, we are as yet not clear regarding the mechanism of action of the AC-31B. Studies are still ongoing to validate the mechanism of action of AC-31B at the receptor level.

We have observed that the kindling-induced seizures resulted in marked increases in cellularity of the bone marrow. In case of PTZ-kindling, our concerned issue was to see if changes in the cellularity observed were caused due to the functional defects in bone marrow cells by the kindled seizures. In answer to this question, we observed an increase in both the proliferation of progenitors in CFU-GM assays and in stromal cells culture in untreated PTZ-kindled mice. This proliferation could be due to either an intrinsic defect in the stem cells, which programs hyperproliferation, or change in microenvironmental factors, forcing these cells to hyperproliferate. Interesting observations were made in case of kindled mice treated with diazepam. We have seen a significant reduction in the cellularity of both the unkindled-and-kindled diazepam treated mice as compared to both the normal control and PTZ kindled control groups. Studies has reported that the ligand of the peripheral benzodiazepine receptors expressed on BMSCs / MSCs tends to inhibit the cellular functions including the proliferation and also induces the apoptosis and this could account for the observations made in our study using diazepam [42-44]. The other speculated reason at the time was that the reduced cellularity in the bone marrow might be due to the redistribution of cells to the periphery since the stem cells have high migratory potential that allows them to leave and re-enter the marrow under normal conditions and also the redistribution of bone marrow cells during any kind of stress is not an unusual phenomenon [45,46,5]. However, this speculation was ruled out because the clonogenic assays of BMCs and PBMCs also showed reduction in the number of the clones in normal and kindled diazepam treated mice. In contrast, the data of BM cellularity and clonogenic assays from AC-31B treated kindled animals were comparable to the normal group.

Another major finding of the present study was the observation that the effects of long-term kindled seizures also induces the up-regulation of cluster differentiation molecules CD44 and CD90 on bone marrow cells. There are a variety of cell surface glycoproteins but we focused on the ones i.e., CD44 and CD90 that are reported to facilitate close interactions between hematopoietic progenitors (HPC) and the microenvironment provided by the stroma (BMSCs). The adhesive interaction between the HPC and stroma leads to the proliferation and differentiation in the bone marrow supports hematopoiesis and regulation of the homing of hematopoietic progenitor cells [47-49]. The higher expression of CD44 and CD90 in the PTZ-kindled group supported the results seen as hypercellularity and high number of colonies in PTZ-kindled control group. Compared to PTZ kindled control group, the immunostaining of the cultured BMSCs from diazepam treated kindled and un-kindled groups revealed a noticeable decrease in the expression of cell surface CD44 and CD90 markers. These observations were supported by the RT-PCR results which showed a reduced expression of both the CD44 and CD90 mRNA in diazepam treated groups. In contrast, the expression of these markers was increased on the surface of the cells

harvested from PTZ-kindled animals. In comparison to diazepam, the AC-31B treated groups showed comparable results to that of the normal groups.

The present study has provided evidence suggesting that immune alterations in epilepsy could be due, in part, to changes in bone marrow microenvironment and it can be modulated by appropriate anticonvulsant use which has minimum or no effect on normal bone marrow functions. Despite the fact that AC-31B has shown a remarkable activity against epileptogenesis in our kindling model developed in mice, its' underlying mechanism of action is yet to be established. The immunomodulatory effects of AC-31B has lead us to further explore it's mechanism by which it modulates the immune functions. Currently the ongoing study of our laboratory focuses at exploring the mechanisms underlying the linkage between immune dysfunction and epileptic disorders. Further studies are needed to explore the potential of this essential oil in the development of neuronal cells from pluripotent bone marrow stromal cells and its possible role in preventing / treating epileptic activity in the neurons. Moreover, we are also interested to work out in isolating its' active principle which is actually inhibiting the development of epileptogenesis in our model. Once the active principle is isolated, we will also explore to see its' activity on the specific ion-channels and we are expecting that it will give us an idea of what specific receptor–types it is acting in order to show anti-convulsant and anti-epileptogenic activity.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

We express our appreciation to the late Dr. Siddiqua Jamall, whose contribution to this work was of great significance.

References

1. Engelboghs S, D'Hooge R, De Deyn PP. Pathophysiology of epilepsy. Acta Neurol Belg. 2000;100: 210-213.
2. Jembrek MJ, Vlainic J. GABA Receptors: Pharmacological Potential and Pitfalls. Curr Pharm Des. 2015; 21:4943-59.
3. Ngugi AK, Bottomley C, Kleinschmidt I, et al. Estimation of the burden of active and life-time epilepsy: a metaanalytic approach. Epilepsia. 2010; 51:883–890.
4. World Health Organization. Epilepsy: aetiology, epidemiology and prognosis. WHO Fact Sheet No. 999. 2009.
5. Bhatt R, Rameshwar P, Goldstein K, et al. Effects of kindled seizures upon hematopoiesis in rats. Epilepsy Res. 2003;54:209-219.
6. Eeg-Olofsson O, Prchal JF, Andermann F. Abnormalities of T-lymphocyte subsets in epileptic patients. Acta Neurol Scand. 1985;72:140–144.
7. Duse M, Tiberti S, Plebani A, et al. IgG2 deficiency and intractable epilepsy of childhood. Monographs in Allergy. 1986;20:128–134.
8. Bostantjopoulou S, Hatzizisi O, Argyropoulou O, et al. Immunological parameters in patients with epilepsy. Funct Neurol. 1994; 9:11-5.
9. Goldstein K, Bhatt R, Barton B, et al. Effects of hemispheric lateralization and site specificity on immune alterations induced by kindled temporal lode seizures. Brain Behav Immun, 2002;16:706–719.
10. Madden KS, Felten SY, Felten L, et al. Sympathetic nervous system modulation of the immune system. II. Induction of lymphocyte proliferation and migration in vivo by chemical sympathectomy. J Neuroimmunol. 1994;49:67–75.
11. Maestroni GJ. Adrenergic regulation of haematopoiesis. Pharmacolo Res. 1995;32:249–253.
12. Zhao M, Li L. Regulation of hematopoietic stem cells in the niche. Sci China Life Sci. 2015;58: 1209-1215.
13. Dessypris EN, Redline S, Hariis JW, et al. Diphenylhydantoin-induced pure red cell aplasia. Blood. 1985;65:789-794.
14. Franceschi M, Ciboddo G, Truci G, et al. Fatal aplastic anemia in a patient treated with carbamazepine. Epilepsia. 1988;29:582-583.
15. Betticher DC, Wolfisberg HP, Krapf R. Aplastic anemia in carbamazepine therapy. Schweiz Med Wochenschr. 1991;121:583-588.
16. Aarli JA. Immunological aspects of epilepsy. Brain and Development. 1999;15: 41-49.
17. Backhauss C, Krieglstein J. Extract of kava (Piper methysticum) and its methysticin constituents protect brain tissue against ischemic damage in rodents. J Pharmacol. 1992;215:265-269.
18. Singh YN, Singh NN. Therapeutic potential of kava in the treatment of anxiety disorders. CNS Drugs. 2002;16:731-743.
19. Gounder R. Kava consumption and its health effects. Pac Health Dialoug. 2006;13:131-135.
20. Shahid M, Perveen H, Anjum S, et al. AC-31B (essential oil) from Allium cepa regulate the expression of CD44 cell surface marker in PTZ-induced epileptic mice brain: A target to control epileptogenesis. J Pharmacol Toxicol Methods. 2019;99:106595.
21. Vermeulen M, Le Pesteur F, Gagnerault MC, et al. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. Blood. 1998;92:894-900.
22. Chen TY, Xie Y. Role of adhesion molecules in mobilization of hematopoietic cells. Chin Med J. 2003;116:273-277.
23. Avigdor A, Goichberg P, Shivtiel S, et al. CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. Blood. 2004;103:2981-2989.
24. Sahin AO, Buitenhuis M. Molecular mechanisms underlying bone marrow homing of hematopoietic stem cells, In: Rosana Pelayo, editor. Advances in Hematopoietic Stem Cell Research., InTech Publishers. 2012;185-204.
25. DeSarro G, Palma E, Costa N, et al. Effects of compounds acting on GABA(B) receptors in the pentylenetetrazole kindling model of epilepsy in mice. Neuropharmacol. 2000;39:2147-2161.
26. Pellmar TC, Wilson WA. Synaptic mechanism of pentylenetetrazole sensitivity for chloride conductance. Science. 1977;197:912-914.
27. Macdonald RL, Barker JL. Pentylenetetrazole and penicillin are selective antagonists of GABA-mediated postsynaptic inhibition in cultured mammalian neurons. Nature. 1977;267:720.
28. Simmonds MA. Classification of some GABA antagonists with regard to site of action and potency in slices of rat cuneate nucleus. Eur J Pharmacol. 1982;80:347-358.
29. Rocha L, Briones M, Ackermann RF. Pentylenetetrazole-induced kindling: early involvement of excitatory and inhibitory systems. Epilepsy Res. 1996;26:105–113.
30. Tallman JF, Paul SM, Skolnick P, et al. Receptors for the age of anxiety: Pharmacology of the benzodiazepines. Science. 1980;207:274- 281.
31. Muller WE, Schlafer U, Wollert U. Benzodiazepine receptor binding: The interaction of some non-benzodiazepine drugs with specific [3 H] diazepam binding to rat brain synaptosomal membranes. N S Arch Pharmacol. 1978;305:23-26.
32. Marangos PJ, Paul SM, Parma AM, et al. Purinergic inhibition of diazepam binding to rat brain in vitro. Life Sci. 1979;24:851-857.
33. Rehavi M, Skolnick P, Paul SM. Effects of tetrazole derivatives on [3H] diazepam binding in vitro: correlation with convulsant potency. Eur J Pharmacol. 1982;78:353-356.
34. Huang RQ, Bell- Horner CL, Dibas MI. Pentylenetetrazole-induced inhibition of recombinant gamma-aminobutyric acid type A (GABA(A)) receptors: mechanism and site of action. J Pharmacol Exp Ther. 2001;298:986-995.
35. Duffy S, Nguyen PV, Baker GB. Phenylethylidenehydrazine, a novel GABA-transaminase inhibitor, reduces epileptiform activity in rat hippocampal slices. Neurosci 2004;126:423-432.
36. Corda MG, Orlandi M, Lecca D, et al. Pentylenetetrazol-induced kindling in rats: Effect of GABA function inhibitors. Pharmacol Biochem and Beh. 1991;40 (2):329-33.
37. Kubova H. Pharmacology of seizure drugs. In: Schwartzkroin P (ed) Encyclopedia of Basic Epilepsy Research, vol 2, Academic Press, Oxford, 2009; 780-786.
38. Naydenov AV, Horne EA, Cheah CS. ABHD6 blockade exerts antiepilepstic activity in PTZ-induced seizures and in spontaneous seizures in R6/2 mice. Neuron 2014;83:361-371.
39. Corda MG, Giorgi O, Longoni B, et al. Decrease in the function of the gamma-aminobutyric acid-coupled chloride channel produced by the repeated administration of pentylenetetrazol to rats. J Neurochem. 1990; 55:1216-1221.
40. Omrani A, Fathollahi Y. Reversal of pentylenetetrazol-induced potentiation phenomenon by theta pulse stimulation in the CA1 region of rat hippocampal slices. Synapse. 2003;50:83-94.
41. Larkin JG, McKee PJ, Thompson GG, et al. Peripheral benzodiazepine receptors in platelets of epileptic patients. Bri J Clin Pharmacol. 1993;36:71–74.
42. Lee DH, Kang SK, Lee RH, et al. Effects of peripheral benzodiazepine receptor ligands on proliferation and differentiation of human mesenchymal stem cells. J Cell Physiol. 2004;198:91-99
43. Zhang T, Shao H, Xu KQ, et al. Midazolam suppresses osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. Eur J Med Pharmacol Sci. 2014; 18:1411-1418.
44. Cline MJ, Golde DW. Mobilization of hematopoietic stem cells (CFU-C) into the peripheral blood of man by endotoxin. Exp Hematol. 1977;5:86–190.

45. Barrett AJ, Longhurst P, Sneath P, et al. Mobilization of CFU-C by exercise and ACTH induced stress in man. Exp Hematol. 1978;6: 590–594.

46. Miyake K, Weissman IL, Greenberger JS, et al. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J Exp Med. 1991;173:599-607.

47. Miyake K, Medina K, Ishihara K, et al. A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. J Cell Biol. 1991;114:557-565.

48. Dittel BN, McCarthy JB, Wayner EA, et al. Regulation of human B-cell precursor adhesion to bone marrow stromal cells by cytokines that exert opposing effects on the expression of vascular cell adhesion molecule-1 (VCAM-1). Blood. 1993;81:2272-2282.