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EXPERIMENTAL STUDY

The effect of hydrostatic pressure on staurosporine-induced neural differentiation in mouse bone marrow-derived mesenchymal stem cells

Javanmard F, Azadbakht M, Pourmoradi M

Department of Biology, Faculty of Basic Sciences, Razi University, Kermanshah, Iran.
azadbakhtm_tmu@yahoo.com

ABSTRACT
In this study, the role of hydrostatic pressure on staurosporine-induced neural differentiation in mouse bone marrow mesenchymal stem cells were investigated. The cells were cultured in treatment medium containing 100 nM of staurosporine for 4 hours; then the cells were affected by hydrostatic pressure (0, 25,50, 100 mmHg). The percentage of cell viability by trypan blue staining and the percentage of cell death by Hoechst/PI differential staining were assessed. We obtained the total neurite length. Expression of β-tubulin III and GFAP (Glial fibrillary acidic protein) proteins were also analyzed by immunocytochemistry. The percentage of cell viability in treatments decreased relative to the increase in hydrostatic pressure and time ($p < 0.05$). The results revealed that total neurite length increased in cells treated with 25 mmHg hydrostatic pressure and decreased in those treated with 100 mmHg hydrostatic pressure ($p<0.05$). Low hydrostatic pressure as a mechanical force can improve neural differentiation in mMSCs (mouse mesenchymal stem cells) *(Tab. 3, Fig. 7, Ref. 30)*. Text in PDF *www.elis.sk.*
KEY WORDS: bone marrow mesenchymal stem cell, hydrostatic pressure, immunocytochemistry, neural differentiation, neurite length, cell differentiation.

Introduction

Mesenchymal stem cells derived from the bone marrow stroma are multipotent (Le et al, 2003; Ryan et al, 2005) and proliferate freely *in vitro* to undergo self-renewal and differentiation into many cell lineages such as chondrocytes, osteoblasts, adipocytes (Petrenko et al, 2008), myoblasts, cardiomyocytes (Pittenger et al, 1999) and neuronal cells (Hermann et al, 2006; Scintu et al, 2006; Qi et al, 2010). This is attractive because, for example, unlike extraction of the neural precursors that would require surgery, mMSCs are relatively easy to isolate and differentiate to neuronal cells, and therefore it may be useful for tissue engineering strategies and cell therapy (Titushkin et al, 2010).

At least three different techniques have been used to induce mMSC differentiation. Namely biological and pharmacological techniques (1), mechanical cues (2), and external mechanical and electrical forces (3) have been used to regulate mMSCs differentiation (Titushkin et al, 2010). Brown (2000) suggested that a significant factor in nerve regeneration is the mechanical environment. It has been shown that mechanical forces could induce proliferation and differentiation of mMSCs (Song et al, 2007).

It also has been shown that the cells in the body are exposed to a complex mechanical environment. Investigations by Guanbin Song and coworkers (2007) have shown that mechanical stimulations are important to morphological, developmental and functional conditions of living cells. It has been shown that multiple pathways are involved in mechanical stress signal transduction while activating a variety of molecules known to function in signal transduction, including ion channel, G proteins, inositol trisphosphate (IP3), and many protein kinases (Katz et al, 2006). Mechanical forces involved in development (Mammoto et al, 2010) are spring forces (Shin et al, 2007), osmotic pressure (Horner and Wolfner, 2008), surface tension (Krieg et al, 2008; Foty and Steinberg, 2005), tensional forces (Ingber, 2006), and shear stress (North et al, 2009; Adamo et al, 2009). Hydrostatic pressure, as a mechanical force, plays a key role in regulating the neural differentiation in many types of cell lines.

The aim of the present study was to evaluate the effect of hydrostatic pressure in the presence of staurosporine on neurite outgrowth in mouse bone marrow-derived mesenchymal stem cells.

Department of Biology, Faculty of Basic Sciences, Razi University, Kermanshah, Iran

Address for correspondence: M. Azadbakht, Dr, Department of Biology, Faculty of Basic Sciences, Razi University, Kermanshah, Iran.
Phone: +98 912 661 0945, Fax:+98 833 427 4545

Materials and methods

Isolation and culture of mMSCs

Isolation and culture of mMSCs were conducted as reported by Nadri (Nadri et al, 2007). Bone marrow was obtained from 6–8-week-old NMRI mice (Naval Medical Research Institute). The animals were sacrificed by cervical dislocation and femurs and tibias were cleaned off carefully from soft tissue. The ends of each bone were cut of just below the end of the marrow cavity using rongeur. Bone marrow was flushed out using a 27-gauge needle attached to a syringe containing DMEM (Dulbecco's modified

Eagle's medium) culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin. To initiate mMSC culture, cells were plated in tissue culture flask 25 cm² (NalgeNunc; Denmark) in 5ml culture medium. The flask was incubated at 37 °C with 5% CO_2 in a humidified chamber without disturbing. After 24 hours in culture, non-adherent cells were separated from adherent ones. The adherent cells were washed with PBS (phosphate buffered saline) and cultured with culture medium for 5–7 days. At 70% confluence, adherent cells were harvested with trypsin/EDTA and plated in new dishes.

mMSCs differentiation

The proliferative stem cells were plated onto 5×10^5 cells/well in 24-well culture plates in DMEM culture medium supplemented with 10% FBS. Then the plates were kept in humidified chamber at 37 °C and 5% CO_2. After 24 hours, medium was changed with fresh DMEM culture medium supplemented with 10% FBS containing 100 nM concentrations of staurosporine. Cells cultured without staurosporine were considered to be control cells. Afterwards the culture plates were transferred to humidified chamber at 37 °C and 5% CO_2.

Experimental protocol

Both undifferentiated and differentiated mMSCs cells were exposed to hydrostatic pressure in this study, along with parallel cultures of unpressurised controls for both. In each experiment the pressure group was subjected to certain mmHg for 1 hour within the pressure chamber. The unpressurized control cell groups were treated identically within a chamber, but with no pressure applied. The cell culture pressure chamber used in this study is an established model (Agar et al, 2000; Agar et al, 2006) that allows a gas mix to be pressurized to a constant ambient hydrostatic pressure ranging from 0–200 ± 2.25 mmHg over the pressure period. After pressurization, the pressure was restored to the atmospheric value, and the culture plates were removed from the pressure chamber for analysis.

There were four experimental groups: 1) control: cells were incubated with 100 nM staurosporine and were not exposed to hydrostatic pressure; 2) treatment I: cells were incubated with 100 nM staurosporine and were exposed to 25 mmHg hydrostatic pressure for 1 hour, 3) treatment II: cells were incubated with 100 nM staurosporine and were exposed to 50 mmHg hydrostatic pressure for 1 hour, 4) treatment III: cells were incubated with 100 nM staurosporine and were exposed to 100 mmHg hydrostatic pressure for 1 hour.

Assessment of cell viability using trypan blue staining

Trypan blue viability measurement was performed by standard methods (Patel et al, 2009; Mithunand Rama, 2012). Briefly, mMSCs were plated onto 24-well culture plates with 5×10^4 cells/ml density for overnight. Then the cells were exposed to treatment medium, as previously described, for 4 hours. Following the treatment, culture plates were transferred to the hydrostatic pressure chamber for 1 hour. The cells were stained with trypan blue 0, 6, 12, 24 hours after exposing them to hydrostatic pressure. The mMSCs were washed with PBS at pH 7.4, and they were trypsinated by using trypsin-EDTA 0.25% (sigma). Then, 10 μl trypan blue solutions (0.4% w/v, Gibco) were mixed with 10 μl of cell suspension. The suspension was loaded into a Neubauer hemocytometer and scored under an inverted microscope. Cells stained blue were scored as nonviable. The cell viability was calculated by the fraction of numbers of viable cells of the total cells count in 100, respectively.

Assessment of cell death using Hoechst/PI nuclear staining

Hoechst/PI nuclear staining was carried out as previously described (Yang et al, 2009). Briefly, The mMSCs were plated onto 24-well culture plates with 5×10^4 cells/ml density and left overnight. Then the cells were exposed to treatment medium, as previously described, for 6 hours. Following the treatment, culture plates were transferred to the hydrostatic pressure chamber for 1 hour. The cells were stained with Hoechst/PI (propidium iodide) 0, 6, 12 and 24 hours after exposing them to hydrostatic pressure. The cells were incubated for 30 min at 37°C with Hoechst 33342 dye (10 mg/ml in PBS), then washed twice in PBS. PI (50 mg/ml in PBS) was added just before microscopy. Cells were visualized by using an inverted-florescence microscope (Olympus IX-71, Japan). Nuclear morphology was scored as follows: 1) viable cells had blue-stained nuclei with smooth appearance; 2) viable apoptotic cells had blue-stained nuclei with multiple bright specks of condensed chromatin; 3) nonviable apoptotic cells had red-stained nuclei with multiple bright specks of fragmented chromatin; 4) nonviable necrotic cells had red-stained, smooth and homogeneous nuclei. The cell death index was calculated by the fraction of numbers of dead cells of the total cell count in 100, respectively.

Measurement of neurite length

Measurement of total neurite length was conducted as previously reported (Ronn et al, 2000). Briefly, mMSCs were plated overnight onto 24-well culture plates with 5×10^4 cells/ml density. The culture medium was replaced with treatment medium 100 nM staurosporine for 6 hours. Then the culture plates were transferred to the hydrostatic pressure chamber for 1 hour. The total neurite length (TNL) was evaluated under an inverted microscope 0, 6, 12 and 24 hours after exposing them to hydrostatic pressure. TNL is the length of the largest neurite measured on individual mMSCs. More than 100 cells were analyzed in each well.

Immunocytochemistry

The mMSCs were plated overnight onto 96-well culture plates with the density of 6×10^3 cells/well. Then the cells were treated with 100 nM staurosporine for 6 hours, and then the culture plates were transferred to the hydrostatic pressure chamber for 1 hour. Six hours after exposing them to hydrostatic pressure, cells were fixed in 4% w/v para-formaldehyde for 20 min at room temperature and permeabilized in 0.1% Triton/PBS for 5 min. Cells were then incubated in PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 30 min to reduce the nonspecific binding. This is then followed by overnight incubation at 4 °C with rabbit polyclonal antibodies β-tubulin III (1:40; Sigma) and GFAP (1:80;

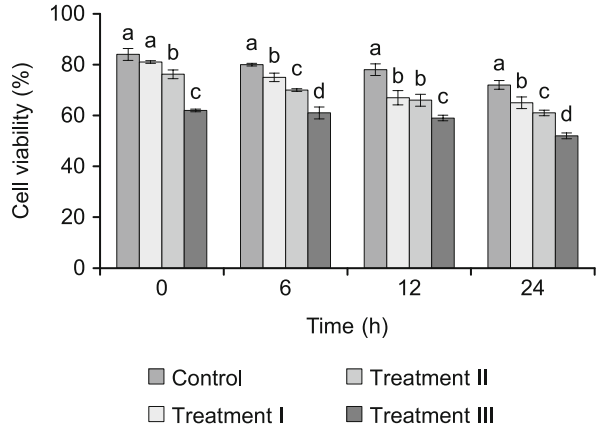

Fig. 1. The effects of hydrostatic pressure on cell viability in BMSCs. Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure; Control: cells were not exposed to hydrostatic pressure; all data represented by mean ± S.E.M ($p < 0.05$).

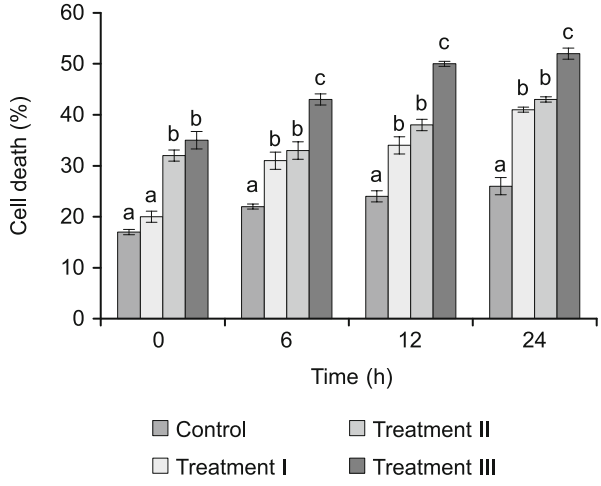

Fig. 2. The effects of hydrostatic pressure on cell death in BMSCs. Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure; Control: cells were not exposed to hydrostatic pressure; all data represented by mean ± S.E.M ($p < 0.05$).

Sigma). After washing, FITC-conjugated (green) secondary antibody (1:100; Santa Cruz Biotechnology) was applied for 1 hour at room temperature. The slides were treated with anti-fade reagent (Molecular Probes, Inc) and examined for immunofluorescence under a fluorescent microscope (Olympus AX-70).

Statistical analysis

Comparisons between group means were made by using one-way analysis of variance (ANOVA). When an effect was statistically significant ($p < 0.05$), mean comparisons were done by post hoc comparisons with a Tukey HSD multiple comparison test. The analysis was carried out by using SPSS version 19, and the probability of 0.05 or below was considered statistically significant.

Tab. 1. Percentage of cell viability of mBMSC after trypan blue staining 0 (A), 6 (B), 12 (C), 24 (D) hours after exposing them to hydrostatic pressure.

	0	6	12	24
Control	84%	80%	78%	72%
Treatment I	81%	75%	67%	65%
Treatment II	76.2%	70%	66%	61%
Treatment III	62%	61%	59%	52%

Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure; Control: cells were not exposed to hydrostatic pressure; all data represent mean ± S.E.M ($p < 0.05$)

Tab. 2. Percentage of cell death of mBMSC after PI/Hoechst staining 0 (A), 6 (B), 12 (C), 24 (D) hours after exposing them to hydrostatic pressure.

	0	6	12	24
Control	17%	22%	24%	26%
Treatment I	20%	31%	34%	41%
Treatment II	32%	33%	38%	43%
Treatment III	35%	43%	50%	52%

Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure; Control: cells were not exposed to hydrostatic pressure; all data represent mean ± S.E.M ($p < 0.05$)

Tab. 3. Neurite length of BMSCs treated in culture media containing 100 nM staurosporine at different values of hydrostatic pressure.

	0	6	12	24
Control	169.08±0.987	178.89±0.857	181.15±0.628	185.59±0.918
Treatment I	210.80±0.785	222.98±0.563	197.29±0.876	191.94±0.812
Treatment II	160.98±0.783	170.17±0.847	157.86±0.679	151.51±0.862
Treatment III	118.35±0.568	120.19±0.912	95.65±0.747	82.57±0.695

Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure; Control: cells were not exposed to hydrostatic pressure; All data represent mean ± S.E.M ($p < 0.05$)

Results

The percentage of cell viability in the control group and treatment groups I, II, and III are shown in Figure 1 and Table 1. After 6 hours, the highest percentage of cell viability was in control group (80 %) and lowest percentage of cell viability was in treatment group III (61 %; $p < 0.05$). The percentage of cell viability in treatment groups I, II, and III decreased compared with that in control group ($p < 0.05$). After 12 hours, the percentage of cell viability was highest in the control group (78 %) and lowest in treatment group III (59 %) ($p < 0.05$). The percentage of cell viability in treatment group I was similar to that in treatment group II. After 24 hours, the percentage of cell viability in the control group and treatment groups I, II, and III were 72 %, 65 %, 61 %, 52 %, respectively. The highest percentage of cell viability was in the control group compared to treatment groups I, II, and III ($p < 0.05$). After 6, 12 and 24 hours, the highest percentage of cell viability was in the control group and lowest in treatment group III ($p < 0.05$). In the control group and treatment groups I, II, and III, the percentage of cell viability decreased relative to the increase in hydrostatic pressure and time ($p < 0.05$).

The percentage of cell death in the control group and treatment groups I, II, and III are shown in Figures 2 and 3 and Table 2. After 6 hours, in treatment groups I, II, and III, the percentage

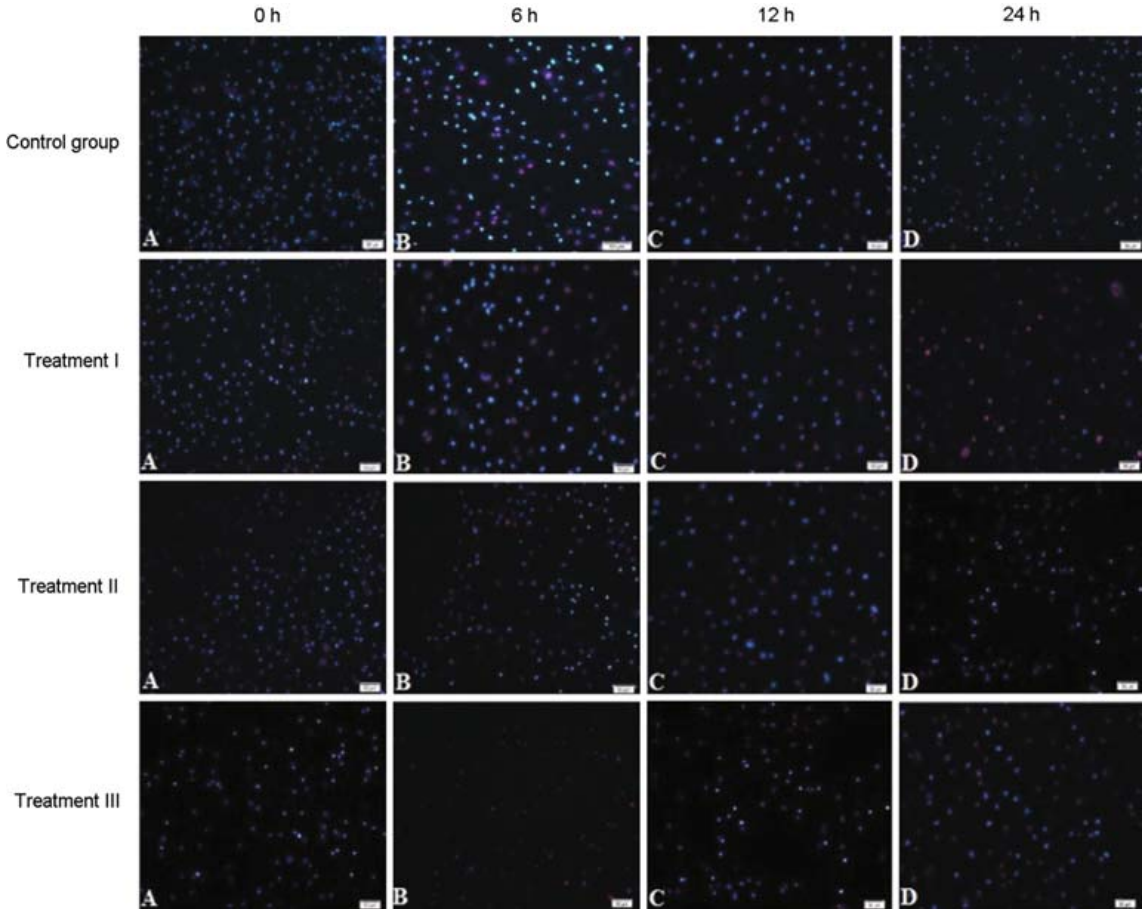

Fig. 3. Florescence images of neural cells derived of mBMSC after PI/Hoechst staining after 0(A), 6(B), 12(C), 24(D) hours exposing to hydrostatic pressure. Control group: cells were not exposed to hydrostatic pressure; Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure.

of cell death increased compared with that in the control group and the percentage of cell death in treatment groups I, II, and III, and control group were 31 %, 33 %, 43 %, 22 %, respectively ($p < 0.05$). The percentage of cell death in treatment group I was similar to that of the treatment group II. After 12 hours, the percentage of cell death in treatment groups I, II, and III increased compared with that of the control group ($p < 0.05$). The highest percentage of cell death was in the treatment group III (50 %) and the lowest percentage of cell death was in the control group (24 %). The percentage of cell death in treatment group I was similar to that in the treatment group II. After 24 hours, the percentage of cell death in treatment groups I, II, and III increased compared with that of the control group ($p < 0.05$). The lowest percentage of cell death was in the control group (26 %) compared to those in treatment groups I, II, and III ($p < 0.05$). The percentage of cell death in treatment I was similar to that in treatment group II. The highest percentage of cell death was in treatment group III (52 %; ($p < 0.05$). In the control group and treatment groups I, II, and III, the percentage of cell death increased relative to the increase in hydrostatic pressure and time ($p < 0.05$).

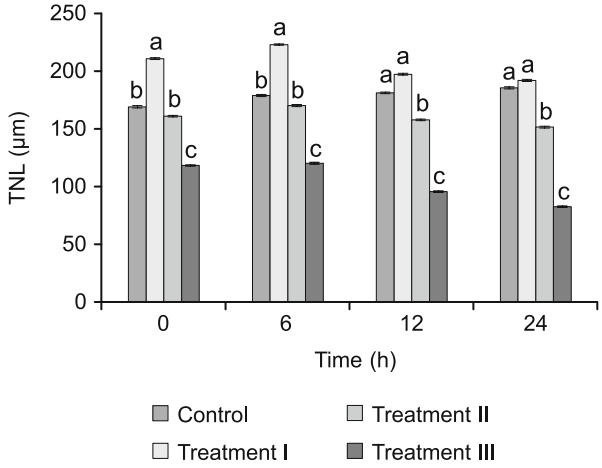

Fig. 4.The effects of hydrostatic pressure on total neurite length (TNL) in BMSCs. Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure; Control: cells were not exposed to hydrostatic pressure; All data represented by mean ± S.E.M ($p < 0.05$).

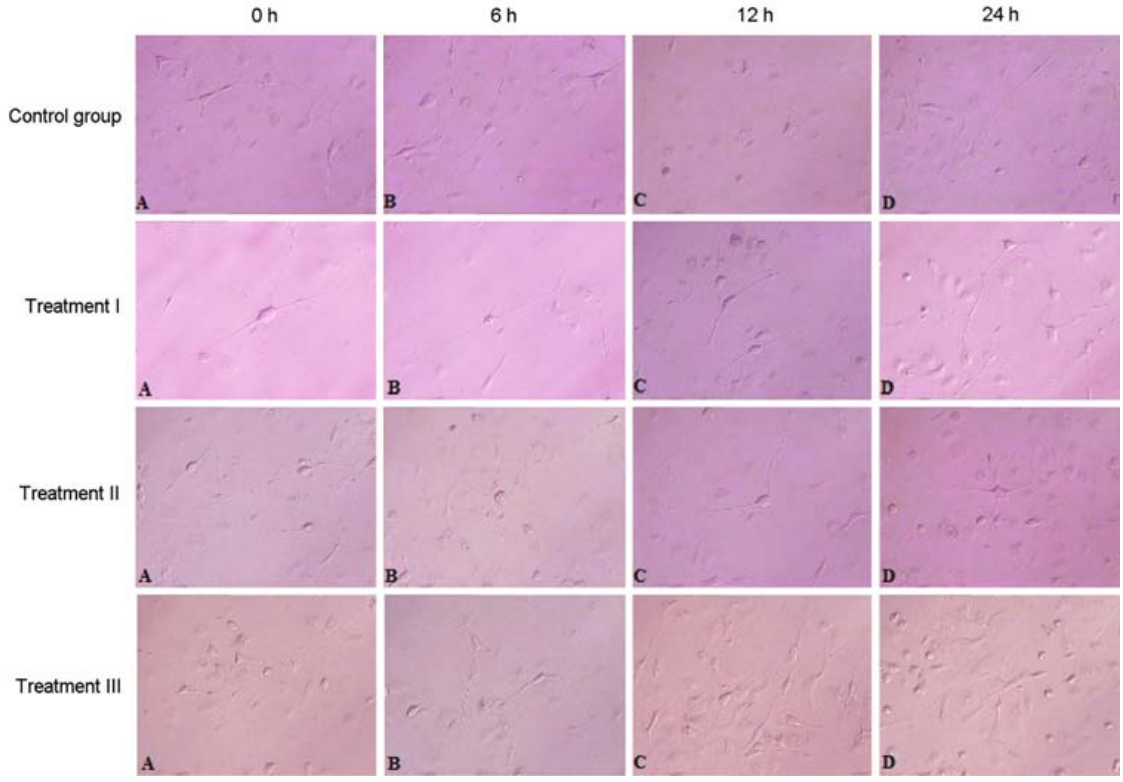

Fig. 5. The images of neural cells derived from mBMSC after 0 (A), 6 (B), 12 (C), 24 (D) hours exposing to hydrostatic pressure. Control group: cells were not exposed to hydrostatic pressure; Treatment I: 25 mmHg hydrostatic pressure; Treatment II: 50 mmHg hydrostatic pressure; Treatment III: 100 mmHg hydrostatic pressure.

TNL for the control group and treatment groups I, II, and III are shown in Figures 4 and 5 and Table 3. Total neurite length after 6 hours was 178.89 ± 0.857 μm in the control group and in treatment groups I, II, and III it was 222.98 ± 0.563, 170.17 ± 0.847, 120.19 ± 0.912 μm, respectively. The length of longest neurite significantly increased in treatment group I compared to those in treatment groups II, and III, and control group ($p < 0.05$). After 6 hours, the length of longest neurite in the control group was similar to that in treatment

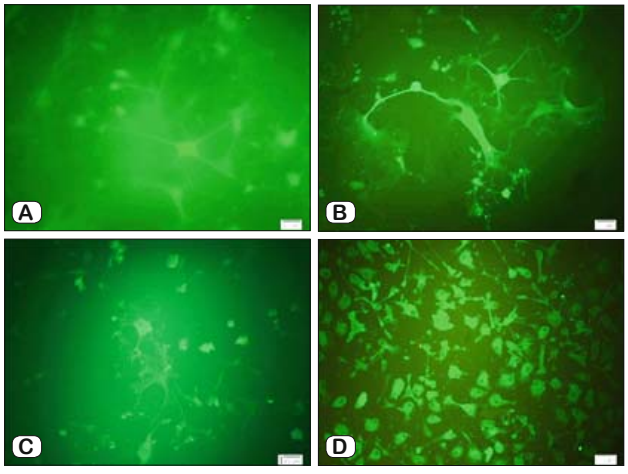

Fig. 6. β-tubulin IIIis expresion in neural cells derived from mBMSC in Control (A): cells were not exposed to hydrostatic pressure; Treatment I (B): 25 mmHg hydrostatic pressure; Treatment II (C): 50 mmHg hydrostatic pressure; Treatment III (D): 100 mmHg hydrostatic pressure.

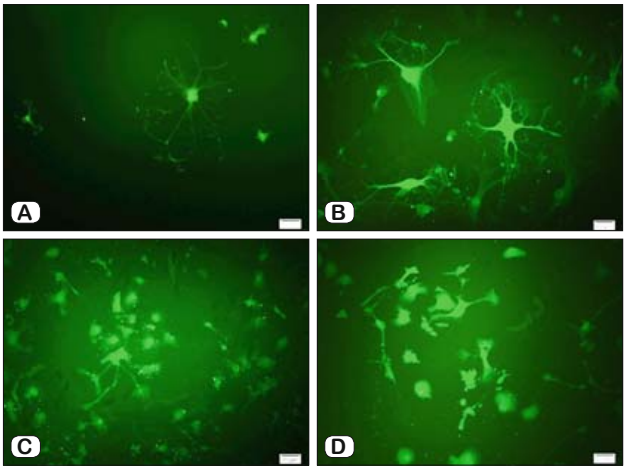

Fig. 7. GFAP is expresion in neural cells derived from mBMSC in Control (A): cells were not exposed to hydrostatic pressure; Treatment I (B): 25 mmHg hydrostatic pressure; Treatment II (C): 50 mmHg hydrostatic pressure; Treatment III (D): 100 mmHg hydrostatic pressure.

group II. After 12 hours, TNL was 181.15 ± 0.628, 197.29 ± 0.876, 157.86 ± 0.679, 95.65 ± 0.747 μm in the control group and treatment groups I, II, and III, respectively. The length of longest neurite in the control group was similar to that in the treatment group I ($p < 0.05$). After 24 hours, TNL was 185.59 ± 0.918, 191.94 ± 0.812, 151.51 ± 0.862, 82.57 ± 0.695 μm in the control group and treatment groups I, II, and III, respectively. The length of longest neurite in the control group was similar to that in the treatment group I ($p < 0.05$). Therefore, after 6, 12, and 24 hours, the longest neurite was in the treatment group I, and the shortest neurite was in the treatment group III ($p < 0.05$). In addition, the largest increase in the length of neurite in mMSCs was seen in the control group, after 6, 12, and 24 hours. TNL in treatment groups I, II, and III was increasing up to 6th hour, but after 6 hours TNL decreased ($p < 0.05$).

The state of the effects of hydrostatic pressure on staurosporine-induced differentiation in BMSC cells was characterized by immunocytochemistry 6 hours after exposure.

For the immunocytochemistry experiments, cultures were stained with β-tubulin III (mature neural markers), and GFAP (glial progenitor marker) antibodies. To further examine the possible differentiation in cells, cultured cells were exposed to hydrostatic pressure for 6 hours. Then they were fixed and stained and the percentage of stained cells was mounted. The cell bodies of stained cells were visually identified, and the number of stained cells to total cells (stained and unstained cells) in 100 was counted.

There were no β-tubulin III-stained cells among the undifferentiated mMSC cells, whereas in treatment group I, HP-induced mMSC cells show good staining with antibodies (Figs 6 and 7). In treatment group I compared with treatment group III, the density of Tubulin Beta III-stained cells was increased ($p < 0.05$). In treatment group II, it was significantly increased compared with treatment group III ($p < 0.05$) (Fig. 6).

There were no GFAP-stained cells among the undifferentiated mMSC cells, whereas in treatment grop I, the HP-induced mMSC cells show heavy staining with antibodies (Fig. 6). In treatment group I compared with treatment group III, the density of GFAP-stained cells was increased ($p < 0.05$). In treatment group II, it was significantly increased compared with treatment group III ($p < 0.05$). In treatment groups I and II, at presence of staurosporine, HP result in heavy glial-like or GFAP-positive with expansion cell body and few neuron-like morphology or β-tubulin III-positive with long neurite outgrowth in culture dish compared with treatment group III ($p < 0.05$). In treatment group I compared with treatment group II it was likely to be postsynaptic and resulted in colonization with disperse neuroglial network (GFAP-positive cells were connected to β-tubulin III-positive cells) in cultured cells after 6 hours in the culture (Fig. 7). Immunocytochemistry analysis revealed that the expression of β-tubulin III and GFAP proteins was strongly increased in 25 mmHg hydrostatic pressure-treated cells.

Discussion

Differentiation of mesenchymal stem cells into neural lineage *in vitro* could be a good means in the treatment of neurological diseases. It has been shown that different agents such as retinoic acid, nerve growth factors, growth factors, and neurotrophins can promote neural differentiation in mesenchymal stem cells (Jiang et al, 2002; Kabos et al, 2002). Investigations by Woodbury and his colleagues (2000) have shown that simple chemical complexes such as β-mercaptoethanol and dimethylsulfoxide can induce neural differentiation in mesenchymal stem cells. In recent years, many studies have been done to improve methods of neural differentiation with the aim of using differentiated cells in the treatment of neurological damage. Efforts to find proper methods for differentiation using a variety of cells in the presence of various compounds had been partly successful. The cells in the body are continuously exposed to a complex mechanical environment. Hydrostatic pressure as a mechanical force, plays a key role in regulating the neural differentiation in many types of cell lines. The protective or disruptive effects of hydrostatic pressure on organisms are not fully understood, nevertheless progress is being made on this subject. Previous studies have shown that hydrostatic pressure, as a source of cell stress, acts as a stimulus for apoptosis in neuronal cell cultures (Agar et al, 2000). Yet, the effects of hydrostatic pressure on cell death and neurite outgrowth in neuronal cells remains unexplained. This study was undertaken to evaluate the effect of hydrostatic pressure on staurosporine-induced neural differentiation in mouse bone marrow-derived mesenchymal stem cells. Previous studies have shown that pressure alone may act as a stimulus for apoptosis in neuronal cell cultures (Agar et al, 2000; Agar et al, 2006). Hydrostatic pressure induces apoptosis in differentiated PC12 cells as identified by DNA fragmentation, chromatin condensation, retraction of neuronal process and cell shrinkage (Sadri et al, 2008). In this study, our results showed that the viability of cells in treatment reduced relative to the increase in hydrostatic pressure and time and resulted in an increase in cell death. Therefore, hydrostatic pressure as a cell death inducer (Agar et al, 2006) increased the percentage of cell deaths and decreased the percentage of cell viability in mMSCs. According to our results, it seems that hydrostatic pressure can improve length of neurite in mouse bone marrow-derived mesenchymal stem cells in an amount-dependent manner. Total neurite length increased in 25 mmHg hydrostatic pressure-treated cells and decreased in 100 mmHg hydrostatic pressure-treated cells compared to control group.

However, the HP-induced increase in the density of neurite-like structure can be caused by enhanced growth of new spines. Our results indicate that staurosporine mainly affects the induction and stabilization of the formation and maturation of neuroglial network in cultured cells. Furthermore, these morphological changes are accompanied by functional changes consistent with such morphological changes (Figs 6 and 7).

In addition, low hydrostatic pressure treatment profoundly altered the glial-neuronal protein marker expression in mMSC cells. Data showed that low hydrostatic pressure administration increased the density of GFAP-positive cells in mMSC cells in comparison with other treated cells. Therefore, hydrostatic pressure as a mechanical force in low amounts can be used as a means of improving the method of neuronal differentiation in bone marrow-derived mesenchymal stem cells resulting in neurite elongation.

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