Differentiation of Human Bone Marrow Mesenchymal Stem Cells into Neuron-like Cells in Vitro

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Running title: Neuronal differentiation in hBMSCs

Key words: mesenchymal stem cells; neuronal differentiation; functional neurons; electrophysiology; spinal cord injury

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ABSTRACT

Study Design

Responses of human mesenchymal stem cells from bone marrow (hBMSCs) was analyzed under chemical conditions, and then characterization of ion channels was evaluated by whole-cell patch clamp.

Objective

To explore the possibility of differentiation of human bone marrow-derived mesenchymal stem cells into neuron-like cells in vitro under different conditions

Summary of Background Data

The generation of MSCs into neuron-like cells has been studied. However, few of these studies characterized functional properties of the differentiated hBMSCs

Methods

hBMSCs (passage 2) were expanded and cultured in vitro. When subcultured passage 5, the cells were induced by cytokines and antioxidants. Morphologic observation, immunocytochemistry, western blot and patch-clamp techniques were performed to evaluate properties of treated and control groups

Results

The differentiated neuronal cells from hBMSCs not only expressed neuron phenotype and membrane channel protein including Nav1.6, Kv1.2, Kv1.3 and Cav1.2, but also exhibited
functional ion currents. Both hBMSCs and differentiated cells expressed Nav1.6, Kv1.2, Kv1.3 and Cav1.2 and voltage-activated potassium currents, including delayed rectifier, noise-like and transient outward currents. However, expression of channel proteins, such as sodium channel Nav1.6 and potassium channels Kv1.2 and Kv1.3, were up-regulated. Consistently, their potassium currents were also enhanced in the differentiated cells.

Conclusion

hBMSCs possess of great potential to differentiate into functional neurons, indicating that hBMSCs may be an ideal cell source in managing a variety of clinical diseases such as spinal cord injury.

Mini Abstract

Mesenchymal stem cells (MSCs) from bone marrow are capable of self-renewal and multipotential. In this report we investigated the potential of human mesenchymal stem cells from the bone marrow (hBMSCs) to differentiate into excitable neurons. Our results demonstrate that hBMSCs possess of great potentialities for differentiating into functional neurons.

Key points:

- The differentiated neuronal cells from hBMSCs not only expressed neuron phenotype and membrane channel protein including Nav1.6, Kv1.2, Kv1.3 and Cav1.2, but also exhibited functional ion currents
- Expression of channel proteins, including sodium channel Nav1.6 and potassium channels Kv1.2 and Kv1.3, were up-regulated
hBMSCs possess of great potential to differentiate into functional neurons, indicating that hBMSCs may be an ideal cell source in managing a variety of clinical diseases such as spinal cord injury

Abbreviations:

MSCs: Mesenchymal stem cells
HBMSCs: human Mesenchymal stem cells from bone marrow
BME: Beta-mercaptoethanol
bFGF: Basic fibroblast growth factor
EGF: Epidermal Growth Factor
BDNF: Brain derived neurotrophic factor
NGF: Nerve growth factor
DMEM: Dulbecco's modified Eagle's medium
FBS: Fetal bovine serum
MAP2: Microtubule Associated Protein 2
NSE: Neuron specific enolase
GFAP: Glial fibrillary acidic protein
GAPDH: Glyceraldehyde phosphate dehydrogenase
TEA: Tetraethylammonium
4-AP: 4-Amino-pyridine
TTX: Tetrodotoxin
PBS: Phosphate buffer solution
PMSF: Phenylmethanesulfonyl fluoride
DTT:  Dithiothreitol
TBS:  Tris buffer solution
DAB:  diaminobenzidine

INTRODUCTION

Mesenchymal stem cells (MSCs) from bone marrow exhibit multiple traits of a stem cell population [1-7]. These cells have the capacity to differentiate mainly into mesoderm-type cells such as osteoblasts, chondrocytes, myocyte and adipocyte and possibly but not proven to non-mesodermal cell types under certain conditions [8-11], which provide a novel approach to treat a variety of related clinical diseases [12-17]. Theoretically, MSCs can also be used across allogeneic barrier because of their unique immune suppressive properties [18]. This property of MSCs is demonstrated by the cell's ability to facilitate bone marrow transplantation [19-23]. MSCs have been shown to transdifferentiate into cells of other germ layers [24, 25]. Furthermore, MSCs are easily obtained from adult bone marrow and can be expanded simply in vitro [26].

Most of previous reports used the parameters of morphology and phenotypic changes to evaluate MSC differentiation into neuron-like cells [27-30]. Few studies have been done using functional assays to determine the identity of differentiated neuronal cells. It has been reported that several types of outward currents and inward currents were found in undifferentiated mesenchymal stem cells [31-34]. MSCs can be transdifferentiated into functional neurons, resulting in functional synaptic transmission with specific induction [35-36].
However, some researches show that differentiated neuron-like cells were not true functional neurons but cellular shrinkage and adoption of pseudo neuronal morphology caused by chemical induction [37-38].

In this study, we used multiple cell differentiating factors, including edaravone, beta-mercaptoethanol (BME), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) to treat hBMSCs, followed by propagation with brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) for 10 days. We found that differentiated hBMSCs not only expressed neuronal phenotypes but showed an enhanced expression of ion channel proteins and increased potassium currents. Our data suggested that hBMSCs have substantial potential of neuronal differentiation.

MATERIALS AND METHODS

Reagents and Cytokines

Dulbecco's modified Eagle's medium (DMEM) with low glucose and fetal calf serum was purchased from Life Technologies (Invitrogen, CA, USA). BDNF and NGF were purchased from Peprotech (New Jersey, USA). Edaravone was purchased from Simcere (Nanjing, China). BME, bFGF, EGF, tetraethylammonium (TEA), 4-amino-pyridine (4-AP), tetrodotoxin (TTX), nifedipine and clotrimazole were purchased from Sigma (Santa Clara, CA, USA). UltraSensitive S-P Kit was obtained from Maxim (Fuzhou, China). DAB was purchased from Wuhan Boster Biological Technology Ltd (Wuhan, China).

Antibodies
Rabbit anti-MAP2 (microtubule associated protein 2), nestin, NSE and GFAP monoclonal antibodies and rabbit anti-Kv1.2, anti-Kv1.3, anti-Cav1.2 and anti-Nav1.6 were obtained from Chemicon (Temecula, CA, USA). Goat anti-rabbit IgG (H+L) and chemiluminescent substrate were purchased from KPL (Gaithersburg MD, USA). Rabbit anti-GAPDH mAb was obtained from Proteintech Group, Inc. (Chicago, USA).

Culture of hBMSCs

hBMSCs (passage 2) were purchased from Cyagen Biosciences Technology (Guangzhou, China) and were obtained originally from healthy male adults of 25 to 48 years old. The cells were cultured in DMEM supplemented with 20% FBS at 37°C in a humidified-atmosphere of 95% air and 5% CO2 as described previously [39]. When the cells had grown in culture flasks to 70%-80% confluence, they were detached with trypsin. After centrifugation, cells were plated at about 6000 cells/cm². At passage 5 the cells were used for neuronal induction and subsequent determination of their biological identity.

Determination of neuronal differentiation

To record ion currents, the passage 5 cells were plated in 6-well plates as described [40] plus round cover slips coated with poly-D-lysine at a density of 2500 cells/cm². The experiment was composed of five groups including control, BME, edaravone, EGF&bFGF and edaravone&EGF&bFGF groups. The control group was maintained only in DMEM/20% FBS, while the media of treated groups, twenty-four hours prior to neuronal induction, were replaced with preinduction media consisting of DMEM, 20% FBS and 10ng/ml bFGF. To initiate neuronal differentiation, the preinduction media were removed, and the cells were
washed with PBS and transferred respectively to different neuronal induction media composed of DMEM/ 10mM BME for the BME group, DMEM/ 20ug/ml edaravone for the edaravone group, DMEM/ 20ng/ml EGF&bFGF for the EGF&bFGF group and DMEM/ 20ug/ml edaravone / 20ng/ml EGF&bFGF for the rest one. After 6 hr induction, cells were studied by immunocytochemistry and western blot. To explore characterization of the functional ionic currents, the differentiated cells were continuously cultured with BDNF&NGF at a final concentration of 20ng/ml for 10 days.

Immunocytochemistry

Cultured hBMSCs were fixed with 4% paraformaldehyde, incubated with primary antibodies overnight at 4°C, and then incubated with secondary antibody for 1 hr, followed by exposure to avidin-biotin complex for 1 hr at room temperature. After washed with PBS, DAB was added to as chromagen for 3 min. The cells were counterstained with Mayer hematoxylin for 1 min, washed with PBS and immediately examined with an Olympus microscope. For oil-red staining for adipose cells, cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS, incubated with Oil-Red-O (Sigma) for 10 min. For alizarin red staining for osteoblasts, cells were fixed with 95% alcohol for 5 min, washed with PBS, incubated with 2% alizarin red solution for 5 min, checking microscopically for an orange red color, and then rinse rapidly in distilled water to shake off excessive dye.

Western Blot

Western blot analysis of protein expression levels was performed on undifferentiated hBMSCs and neuronal differentiated hBMSCs. Primary antibodies of ion channels were used
in the detection of protein expression. The cultured cells were rinsed with PBS and then lysed for 30 min on ice using RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 uM Na3VO4, 1 mM DTT, and 50ug/ml PMSF). The lysate was centrifuged at 12,000 rpm for 20 min at 4°C; the pellet was discarded and the supernatant was subjected to a 4-8% gradient acrylamide gel and electrophoretically transferred to a PVDF membrane. The blots were blocked in TBS containing 5% BSA and 0.05% Tween 20. The membrane was incubated at 4°C overnight with primary antibodies, washed extensively in TBS containing 0.05% Tween 20 and then treated with a secondary antibody conjugated horseradish peroxidase for 1 hr. The blots were subsequently detected by electrochemiluminescence.

Electrophysiological Recordings

Electrophysiological recordings were conducted as described previously [31] at room temperature(20~24°C). In brief, whole-cell patch-clamp configuration was used to record electrical activity with a List EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany), a laboratory interface (CED 1401, Cambridge, UK) and EPC software (CED, Cambridge, UK). Data were filtered at 3 KHz and sampled at 10 KHz. The patch-clamp pipettes were made from standard wall borosilicate glass capillaries with an inner filament on a two-stage vertical puller and gave a resistance of 4-6 MΩ when filled with pipette solution. The junction potential between the patch pipette and the bath solutions was nulled just before forming the giga-seal by negative suction. The cell membrane was ruptured by gentle suction to establish the whole-cell configuration. Cell capacitance compensation and series resistance compensation were used to minimize voltage errors. The amplifier reading of capacitance was used as the value for whole cell membrane capacitance. The cells on the coverslips were
perfused with external solution containing (in mM) NaCl 136, KCl 5.4, CaCl2 1.8, MgCl2 1, HEPES 10, NaH2PO4 0.33 and glucose 10 (300 mosmol/L, pH set to 7.4 with 1 N NaOH). The patch electrodes were filled with the solution containing (in mM) K-Gluconate 120, KCl 20, MgCl2 1, EGTA 0.5, HEPES 10, Na2-ATP, with the pH adjusted to 7.2 by 1N KOH and the osmolarity adjusted to 300 mosmol/L with sucrose. K+ in superfusion and pipette solutions was replaced by equimolar Cs+ when K+-free conditions were applied for recording sodium currents.

Cell were held at -100 mV and stepped to +60 mV in a step increment of 10 mV. Each step lasted for 300 ms and then maintained at -30 mV for 100 ms before returning to the holding potential.

Statistical analysis

Data are presented as means ± standard error (SEM). ANOVA was used to analyze data to detect statistically significant differences and Student Newman-Keuls was employed as the post hoc identifying test. Values of P < 0.05 were considered to be statistically significant. All experiments were repeated at least 3 times.

RESULTS

Neuronal differentiation

Morphological changes of the differentiated cells were observed by phase/contrast microscopy. The untreated hBMSCs predominantly consisted of spindle-shaped cells (Fig. 1a).
Within 3 hr after the addition of induction agents, some cells showed a neuronal-like phenotypes in morphology. These cells were distinctively distinguished by highly refractive cell bodies with neuron-like processes terminating in structures that resembled growth cones (Fig. 1b). These changes suggest that the hBMSCs differentiated into neuron-like cells. However, at 6 hr, the number of differentiated cells was not increased. After more than 1 week of culture in neuronal maintenance of medium, most cells presented with neuronal morphology including a small cell body and long extensions (Fig. 1c).

Expression of neuronal markers

To confirm the differentiation of neuronal lineages, we assessed the expression of neuronal and glia markers. Nestin is classically considered as a specific marker of neural stem cells. GFAP is a glia marker, and NSE and MAP2 are neuron markers. After 6 hr induction with EGF/bFGF/edaravone, NSE-positive cells increased significantly (Tab 1). GFAP expression was low without any significant changes after induction. There was no MAP2 or Nestin expression. The untreated cells were negative for NSE, Nestin, GFAP and MAP2 (Fig. 2).

Expression of ion channel proteins

We then examined the protein expression of membrane channels, such as Nav1.6, Kv1.2, Kv1.3 and Cav1.2 after neuronal differentiation. As shown in Fig. 3, expression of Nav1.6, Kv1.2 and Kv1.3 channels was dramatically increased after addition of differentiating agents compared to the untreated cells. However, Cav1.2 expression was basically unchanged. The quantitative data of band densities were summarized in Table 2. These results suggest that a
neuronal differentiation was induced, which is consistent with the immunocytochemical results.

Functional changes of ion channels during hBMSC differentiation

To assess the functional changes of ion channels, whole-cell currents in hBMSCs were recorded with the patch-clamp technique. Cells were held at -100 mV and stepped to +60 mV in a step increment of 10 mV. Each step lasted for 300 ms and then maintained at -30 mV for 100 ms before returning to the holding potential (Fig 4A). In the undifferentiated hBMSCs, three types of voltage-activated currents were recorded, including a delayed rectifier current, a noise-like current and a transient outward current (Fig 4B and D). The delayed rectified current was usually activated at potentials between -10 and +20 mV. The noise-like current was activated rapidly at potentials between +30 and +60 mV. The transient outward current was activated rapidly when stepped to the potentials between -80 mV and +60 mV, but inactivated immediately when the peak current was reached.

At the regular culture condition, the mean current in undifferentiated hBMSCs cells was 18.4±3.6 pA/pF. Addition of potassium channel blockers, tetraethylammonium (TEA, Fig 4C) and clotrimazole (Fig 4E), to cell culture media or removal of potassium from the recording pipettes (data not shown) suppressed the current. These results indicate that the currents are carried out by potassium.

Next, we analyzed the voltage-activated potassium currents in differentiated hBMSCs induced by different reagents. Similar to those recorded in undifferentiated hBMSCs,
voltage-activated currents were detected in differentiated hBMSCs. However, the levels of potassium currents in differentiated cells were significantly higher than those in undifferentiated hBMSCs, as shown in Fig. 5. These results are consistent with the findings of protein expression levels of ion channels (Fig 3), further confirming that differentiation was induced in hBMSCs.

Finally, we determined if the increased voltage-activated current in differentiated hBMSCs could be blocked by potassium channel blockers. The hBMSCs were treated with a combination of edaravone/EGF/bFGF to induce neuronal differentiation. Cells were subsequently subjected to measurements of voltage-activated currents. Compared to the control (Fig 6A&C), addition of TEA or clotrimazole in the media (Fig 6B&D) dramatically suppressed voltage-activated currents. Similarly, depletion of intracellular potassium also eliminated the currents (Fig 6G). These results demonstrated that the increased voltage-activated currents in differentiated hBMSCs are potassium-dependent. However, 4-Amino-pyridine (4-AP), a reagent that is usually used to inhibit transient outward potassium currents, did not inhibit the voltage-activated currents but activated a noise-like current (Fig 6E&F).

DISCUSSION

Repair of central neuronal damage and neuronal regeneration has been a hot spot for neuroscience. Recently, studies have shown that embryonic stem cells (ESCs), neural stem cells (NSCs) and MSCs have the potential to differentiate into neurons and can improve
neural function after being transplanted in animal models of nerve injury \([41,42,43]\). However, obstacles to directional differentiation and clone purification, as well as ethical and legal issues are presented in ESCs. In addition, there are limited resources for NSCs. Therefore, hBMSCs are increasingly used as seed cells for treatments of neuron-related diseases, due to their high potential of self-renewal, easy to obtain, capable for multi-directional differentiation, low immunogenicity and no ethical restriction.

In this study, we first determined the characteristics of hBMSCs. We demonstrated that the cells exhibited multiple traits of a stem cell population, which indicate that hBMSCs retain the capacity to differentiate into mesenchymal and non-mesenchymal derivatives, and are capable of self-renewing and possess multipotency, thereby fulfilling the criteria for a stem cell population. Sequentially, we used a free radical scavenger, edaravone, together with growth factors of EGF and bFGF to induce neuronal differentiation in hBMSCs. Edaravone (3-methyl-1-phenyl-pyrazolin-5-one), a newly developed radical scavenging agent, as an antioxidant, has been widely used for protection against ischemia-reperfusion (I-R) injury in patients with cerebral infarction, exerting neuroprotective effects against ischemic insults \([44,45]\). It has been shown that antioxidants and cytokines can induce MSCs to differentiate into neuron-like cells. We speculate that edaravone might be function by inducing hBMSCs to neuron-like cells. The study of the roles of edaravone in cell differentiation may benefit our understanding of the action mechanisms of edaravone in the clinical treatment of spinal cord injury. In this study, we found that hBMSCs could be differentiated into neuron-like cells with edaravone treatments. The percentage of NSE (a neuron maker)-positive cells from edaravone/EGF/bFGF group was higher than that of any other groups with individual reagent.
Our results indicate that edaravone has the potential to initiate neuronal differentiation.

To determine the properties of the differentiated hBMSCs, we assessed the neuronal marker NSE using immunocytochemical approach. We found that NSE was positive in differentiating reagents-treated cells but was negative in untreated cells, indicating that the hBMSCs were successfully differentiated into neuron-like cells. Nestin, a marker of neural stem cells, was negative in both treated and untreated cells, suggesting that the hBMSCs were not differentiated to neuronal precursor cells. The differentiated cells were MAP2 negative. This implied that neuron-like cells were immature\[46\].

To define if the differentiated cells are functional, the expression of ion channel proteins, which are the base of neuron function, was evaluated first at the protein level by Western blot. The results indicated that sodium channels (Nav1.6) and potassium channels (Kv1.2 and Kv1.3) but not the calcium channels (Cav1.2) were up-regulated in the differentiated hBMSCs. Sodium and potassium channels are the fundamental proteins that are responsible for the signal conduction in neurons. These results suggest that hBMSCs have been directed to differentiate into functional neurons.

To further clarify the function of the differentiated cells, voltage-activated ion currents were recorded in the presence or absence of potassium channel blockers. We found that the voltage-activated potassium currents, including the delayed rectifier, the noise-like and the transient outward K+ currents, were dramatically increased in the differentiated cells compared to that in undifferentiated hBMSCs. Previous report from others suggests that a rise
in delayed rectifier potassium current was associated with the maturation of cell excitability and neuronal differentiation\cite{47}. After 10 days continuously cultured with BDNF and NGF that could promote neuron-like cells mature reported previously\cite{48}, K+ current amplitude including delayed rectifier potassium currents were significantly higher than that in control hBMSCs, suggesting that the excitable potential was increased. However, TTX-sensitive rapid sodium currents could not be detected in the differentiated cells, although sodium channel proteins were expressed in the cells. There are two explanations for the contradictory results. First, the differentiated neuronal cells are not fully maturated neurons but rather to be in a process of maturation and need further administration by increasing concentration of BDNF and NGF, prolonging culture period or other ways \cite{49}. Second, sodium channels are expressed and functioned in the treated cell population, but not all the treated cells differentiate into functional neurons. The recorded cells are not fully functional.

In conclusion, to the authors aware, our study is the first report that hBMSCs can differentiate into neuron-like cells in vitro with edaravone. Our results demonstrate that hBMSCs has substantial potential of neuronal differentiation. Further studies are needed, however, to ensure the long-term safety and efficacy of seed cells for therapeutically cell treatment.

REFERENCES


Figure Legends

Fig. 1 Neuronal differentiation of hBMSCs. a: The untreated cells of passage 5 were similar to spindle-shaped cells. ×40. b: Cell bodies were highly retracted and transparent. Neuron-like processes terminating in structures resembled growth cones after 6 hours induction. ×200. c: Cell bodies became smaller and neuron-like processes were extensive after 10 days of maintenance of culture with BDNF and NGF at a final concentration of 20ng/ml. ×200.
Fig. 2  Immunostaining of cellular makers of NSE, GFAP, MAP2 and Nestin.

hBMSCs-derived neurons (long arrow) stain dark brown for NSE expression and display condensed cell bodies and highly branched processes. Transitional cells (short arrow) exhibit intermediate neuronal morphologies, with partially retracted cell bodies and light brown NSE staining. GFAP expression is positive in a very low level in hBMSCs-derived glia. The stained cell is large and round and lack of highly branched processes. MAP2 and Nestin expression are negative for all cells. Magnification ×100.
Fig. 3 Western blotting analysis for detecting ion channels expressed in treated and untreated hBMSCs. Ionic channel proteins Cav1.2, Kv1.2, Kv1.3 and Nav1.6 were detected by Western blotting at 249 (Cav1.2), 75 (Kv1.2), 58 (Kv1.3) and 225kDa (Nav1.6) in different groups of hBMSCs compared with GAPDH (37 kDa). Nav1.6, Kv1.2, Kv1.3 and Cav1.2 were all expressed in treated and untreated cells. Kv1.2, Kv1.3 and Nav1.6 expression were higher in treated cells than that of control and Cav1.2 expression was nearly unchanged.
Fig. 4. Whole-cell recordings of voltage-activated currents in undifferentiated hBMSCs. As shown in A, cells were held at -100 mV and stepped to +60 mV in a step increment of 10 mV. Each step lasted for 300 ms and then maintained at -30 mV for 100 ms before returning to the holding potential. B and C show the inhibitory effect of 10 mM tetraethylammonium (TEA) on a typical cell with a noise-like current activated at potentials between +30 and +60 mV and a delayed rectified current activated at potentials between -40 and +20 mV. D and E show the effect of 20 M clotrimazole on a typical cell with a noise-like current, a delayed rectified current and a transient outward current that was activated rapidly when stepped to potentials between -60 mV and +60 mV, but inactivated once the peak was reached.
Fig. 5. Voltage-activated currents in differentiated hBMSCs. Cells were kept untreated or treated with differentiating reagents as indicated. The voltage-activated currents were recorded as described above in the legend of figure 4. Data represent the mean ± standard error from 6 to 19 cells. The asterisk indicates a significant difference (P < 0.05, Student t-test).
Fig. 6. Inhibition of voltage-activated currents by potassium channel blockers in differentiated hBMSCs. The hBMSCs were treated with a combination of edaravone/EGF/bFGF to induce differentiation. Whole-cell currents were recorded before (Control; A, C and E) and after treated with 10 mM TEA (B), 20 M clotrimazole (D) or 3 mM 4-AP (F) using the protocol described in Fig 4. A measurement was also carried out with the recording pipette containing the potassium-free solution (G).
### Tab1. The percentage of cells with positive expression of nestin/NSE/GFAP/MAP2 in hBMSCs (Mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nestin</th>
<th>NSE</th>
<th>GFAP</th>
<th>MAP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BME</td>
<td>0</td>
<td>59.20 ± 4.61*</td>
<td>8.02 ± 1.20</td>
<td>0</td>
</tr>
<tr>
<td>Edaravone</td>
<td>0</td>
<td>61.41 ± 3.80*</td>
<td>5.01 ± 2.51</td>
<td>0</td>
</tr>
<tr>
<td>EGF&amp;bFGF</td>
<td>0</td>
<td>54.72 ± 1.92*</td>
<td>11.20 ± 3.00</td>
<td>0</td>
</tr>
<tr>
<td>EGF&amp;bFGF&amp;Edaravone</td>
<td>0</td>
<td>81.91 ± 4.32**</td>
<td>6.00 ± 2.04</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* indicates that NSE expression was significantly higher than GFAP expression in the treatment groups.

** indicates that the percentage of NSE-positive cells in EGF&bFGF&Edaravone was significantly higher than that of any other groups.

### Tab2. Quantitative data of band density of western blot in hBMSCs (Mean ± SD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nav1.6/GAPDH</th>
<th>Kv1.2/GAPDH*</th>
<th>Kv1.3/GAPDH*</th>
<th>Cav1.2/GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BME</td>
<td>0.60 ± 0.15</td>
<td>4.77 ± 0.75</td>
<td>0.75 ± 0.25</td>
<td>1.29 ± 0.38</td>
</tr>
<tr>
<td>Edaravone</td>
<td>0.60 ± 0.20</td>
<td>2.96 ± 0.74</td>
<td>0.75 ± 0.34</td>
<td>1.29 ± 0.42</td>
</tr>
<tr>
<td>EGF&amp;bFGF</td>
<td>0.76 ± 0.17</td>
<td>4.58 ± 0.45</td>
<td>0.75 ± 0.30</td>
<td>1.29 ± 0.35</td>
</tr>
<tr>
<td>EGF&amp;bFGF&amp;Edaravone</td>
<td>0.75 ± 0.21</td>
<td>3.14 ± 0.86</td>
<td>0.69 ± 0.35</td>
<td>1.29 ± 0.40</td>
</tr>
<tr>
<td>Control</td>
<td>0.49 ± 0.12</td>
<td>1.46 ± 0.35</td>
<td>0.20 ± 0.15</td>
<td>1.29 ± 0.36</td>
</tr>
</tbody>
</table>

* Indicates a significant difference (p < 0.05) between the control and treatment groups.