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Protective Effects of Mouse Bone Marrow Mesenchymal Stem Cell Soup on Staurosporine Induced Cell Death in PC12 and U87 Cell Lines

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ABSTRACT

Mouse bone marrow mesenchymal stem cells (mBMSCs) soup is promising tool for the treatment of neurodegenerative diseases. mBMSCs soup is easily obtained and is capable of transplantation without rejection. We investigated the effects of mBMSC soup on staurosporine-induced cell death in PC12 and U87 cells lines. The percentage of cell viability, cell death, NO concentration, total neurite length (TNL) and fraction of cell differentiation (f%) were assessed. Viability assay showed that mBM soup (24 and 48h) in time dependent were increased cell viability ($p<0.05$) and also cell death assay showed that cell death in time dependent were decreased, respectively ($p<0.05$). TNL and fraction of cell differentiation significantly were increased compared with treatment1 ($p<0.05$). Our data showed that mBM Soup protects cells, increases cell viability, suppresses cell death and improvement the neurite elongation. We concluded that Mouse bone marrow mesenchymal stem cell soup plays an important protective role in staurosporine-induced cell death in PC12 and U87 cell lines.

Key words: mBMSC soup, Cell death, Neurite outgrowth, PC12, U87

INTRODUCTION

Studies of bone marrow stem cell-based therapy for improve organ functions have been investigated for the past 10–15 years. Initial reports proposed the ability of adult bone marrow mesenchymal stem cells (BMSCs) can differentiate into tissue lineages [1-3]. Many studies showed that BMSCs could differentiate into various non-mesenchymal tissue lineages under appropriate experimental conditions *in vitro* and *in vivo*, such as hepatocytes [4, 5], cardiomyocytes [6,7], lung alveolar epithelium [8), even neuron and glia [9-13]. The amazing neuro-differentiation potential of BMSCs attracts intense interest in the possible applications of BMSCs in cell and gene therapy for neurological disease, because BMSCs can be obtained from bone marrow easily and expanded rapidly *in vitro* [14,15]. It has been reported that BMSCs could induce neuro-differentiation through many ways *in vitro*, such as chemical inducers (9), cytokines [4, 16, 17], co-culture with neural cells [18,19], chemical inducers plus cytokines [10,20] and transfect plus cytokines [21], etc.

Previous studies had been suggested that mesenchymal stem cell transplantation improved neurological functional recovery, decreased apoptosis, increased endogenous cell proliferation, promoted angiogenesis and reduced lesion size [22] in central nervous system (CNS) injuries including stroke and spinal cord injury in animal models. It showed that transplanted BMSCs might exert beneficial effects in CNS injury include their ability to the production of growth factors by BMSCs [22-24]. However, BMSCs can secrete a variety of bioactive molecules such as trophic factors and anti-apoptotic molecules, which may provide the main mechanism responsible for their therapeutic effects [25]. In addition, BMSCs can inhibit the release of proinflammatory cytokines and promote the survival of damaged cells [26]. For example, the therapeutic benefit of BMSC cytokines has been observed in acute lung injury [27, 28], myocardial infarction [29], acute renal failure [30], cerebral ischemia [31, 32] and Alzheimer's disease [33]. On the other side, the most recent mechanism of action is that BMSC cells provide a local paracrine effect [34-36]. In summary, tissue regeneration and improvements have been proposed as paracrine effect of stem cell action [35-37]. However at closer investigation, could not fully explain organ improvement. Meanwhile, several studies have shown that stem cells release soluble factors that acting in a paracrine fashion, contribute to organ repair and

regeneration. These factors are cytokines; growth and other factors induce cytoprotection, neovascularization, and mediums endogenous tissue regeneration via activation of resident tissue stem cells. In addition, tissue remodeling and organ function is affected by these paracrine factors [35]. Recent study showed that BMSC soup by paracrine mechanism is the main cause behind the reported improvement of salivary organ function [38]. However, how they survive and differentiate into distinct cell types is still not clear.

The aim of this study is to test the efficiency of mBMSC soup in suppressing the staurospurine-induced cell death in PC12 and U87 cell lines. The hypothesis is that mBMSC soup protects cells, increases cell viability and improvement the neurite elongation.

MATERIALS AND METHODS

Preparation of Mouse Bone Marrow Mesenchymal Stem Cells (mBMSCs)

Isolation and culture of mBMSCs were carried out as previously described [39]. Bone marrow was obtained from 6-8-week-old NMRI mice. For isolation of MSCs, tibias and femurs were dissected and the ends of the bones were cut. The marrow was extruded with flushing the shaft with DMEM culture medium (Gibco) completed with 10% FBS (Gibco), 1% L-glutamine (Sigma), 1% non-essential amino acids (Sigma) and 100 IU/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) using a 27-gauge syringe. Cells were disaggregated by gentle pipetting several times on 25 cm^2 plastic flask. The cell pellet was resuspended in DMEM for each 10^8 cells. Cell suspension was incubated at 37°C in humidified atmosphere containing 5% CO_2 for 72 hour. After that the nonadherent cells were removed by replacing the medium. Culture medium was replaced every 2 or 3 days about 2 weeks. When cell cultures reached to 80% confluency, they were harvested with trypsin-EDTA0.25% (Sigma) for 5 minutes, again cultured to next confluence and harvested. Expanded cells from passages three−eight were used for further testing.
The surface marker expression of mBMSC cells were assessed by flow cytometry. mBMSC cells were sub cultured at a density of 10^5 cells/well in 24-well culture plates.

Mouse Bone Marrow Mesenchymal Stem Cells Soup (mBM soup)

After confirming and harvesting the mBMSCs, condition medium as mBM soup, was pooled. Briefly, when cell cultures reached to 80% confluency, the medium were changed by fresh DMEM free serum and 0.2% BSA culture medium. The cells maintained for 24h or 48h. Then, the condition mediums were harvested and stored at -70 ^oC until uses as mBM soup (mBM soup 24 and mBM soup 48h, respectively).

Cell lines

PC12 and U87 cells were grown in a 25-cm2 tissue culture flask in DMEM culture medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco; UK), 1% NEAA (Sigma), 100 u/ml of penicillin (Sigma) and 100 mg/ml streptomycin (Sigma). The cells were maintained at 37°C in a humidified, 5% CO_2 environment.

Cell Treatment

One day after plating the cells, cells were washed with PBS, pH 7.4. There were six treatments including; treatment 1: 1µM staurospurine, treatment 2: no incubation with staurosporine, treatment 3: mBM Soup 24h, treatment 4: mBM Soup 24h together with 1µM staurospurine, treatment 5: mBM Soup 48h and treatment 6: mBM Soup 48h together with 1µM staurospurine Then, the cells were placed in the incubator at 37 °C with 5% CO2. The cells were cultured in DMEM culture medium containing 0.2% BSA.

Cell viability measurement

Trypan blue viability measurement was performed by standard methods [40]. The traditional method of performing trypan blue (0.4gr / 100ml in PBS) cell viability analysis involves manual staining and use of a hemocytometer for counting.

MTT assay

Cell viability was quantified by MTT assay. MTT measurement was performed by standard methods [41]. To perform the test, 1×10^4 PC12 and U87 cells were loaded into a 96-well plate and 200 µL of DMEM medium containing 0.2% BSA was added. After 24-hour incubation, 200 µL of treatments medium as described was added to the wells. The cells were separately incubated with different treatments medium for 24 and 48 hours.

The optical density of each well was measured using a microplate reader (EL800; USA). Reader at 570 and 630 nm. The viability of the cells for each concentration was calculated using the following formula:

Cell viability (%)=(A570, 630(sample)/A (control))×100

Quantification of cell death incidence

Hoechst/PI nuclear staining was carried out as previously described [43]. Briefly, cells were plated in 24 well culture plates with 5×10^4 cells/well density for 24h. Cells were treated with different treatment mediums for a range of times in differentiation medium (6, 12, 24 and 48h). Then cells were incubated for 30 min at 37°C with Hoechst 33342 dye (10 ng/ml in PBS), washed twice in PBS. PI (50 ng/ml in PBS) was added just before microscopy. Cells were visualized using an inverted florescence microscope (Olympus IX-71, Japan). The cell death index were calculated by the fraction of numbers of apoptotic cells on the total cell count in 100 (300 cells), respectively.

NO assay

NO was measured using the Griess staining method [42]. All wells were incubated for 15 minutes and were assessed using a microplate reader (EL800; USA) at wavelengths of 570 and 630 nm.

Measurement of Total Neurite Length

Measurement of Total Neurite Length was conducted as reported by previous study [44]. The assay is based on the measurement of total neurite length. Total neurite length (length of largest neurite on 100 cells) was assessed. Cells were treated in different treatments for certain time (6, 12, 24 and 48h) at differentiation medium, fixed, and the cell morphology was assessed by an inverted microscope (Olympus IX-71, Japan). Digital photos were taken of random fields of neurons derived from the treatments. Total neurite length was measured (Motic software; Ver.2).

The fraction of cell differentiation assessment (f (%))

Fraction of cell differentiation was carried out as previous study [45]. PC12 and U87 cells were plated at a density of 2×10^4 cells/well on 24 well plates. Cells were treatment with different treatment mediums for a range of times at differentiation medium (6, 12, 24 and 48h), fixed, and the morphology microscopically assessed (Motic software; Ver. 2). All experiments were replicated independently at least 3 times. Within each experiment, we replicated each condition 3 times.

Data analysis

Data are reported as mean ± SEM. Differences among treatments were tested using one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of BMSCs from NMRI Mice

The cells as mBMSC cells were derived from female and male NMRI mice. The 5th passage of cells had similar morphology. Most of the mBMSC cells were spindle-shaped and similar to fibroblast-like cells. The cells exhibited a rapid growth with cell clustering. For detection we were used Flow cytometry to detect the phenotype of the 5th passage cells. The results showed that about 98% cells of mBMSC CD90-positive and lack of expression of CD14, CD45 and CD34. These results were showed that mouse Bone marrow cells had the characteristics of mesenchymal stem cells (Figure1).

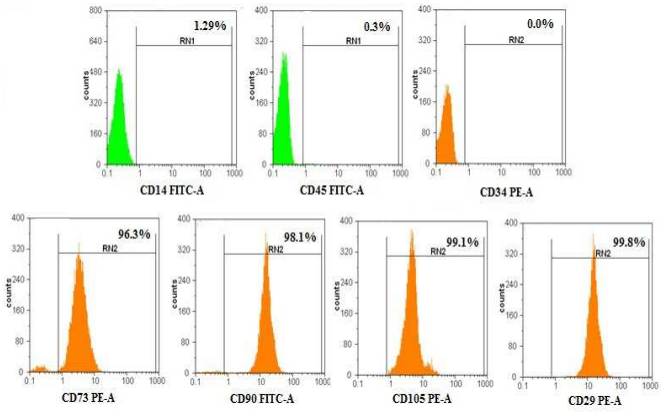

Figure 1. The characterization of mouse BMSCs by flow cytometry; BMSCs, expressed CD73 but not CD45, CD34, or CD14. F) M-SMSCs detected by flow cytometry. Of the M-SMSCs, 92% expressed CD73, CD90, CD105 and CD29 but not CD45, CD34, or CD14

Cell Viability

Comparison of the mean PC12 and U87 cell viabilities analyzed using the trypan blue method after 6, 12, 24 and 48 hours indicated a significant difference between the treatments ($p<0.05$).

After 6, 12, 24 and 48 hours the percentage of cell viability in treatments 1, 4 and 6 were decreased compared with the treatment 2 (97%), respectively ($p < 0.05$). After 6, 12, 24 and 48 hours the percentages of cell viability were not differences in treatments 3 and 5 compared with treatment 2, respectively. The percentage of cell viability was increased in treatment 3 compared with the treatment 4 ($p<0.001$, Figure 2). The percentage of cell viability was increased in treatments 4 and 6 compared with the treatment 1($p < 0.05$).

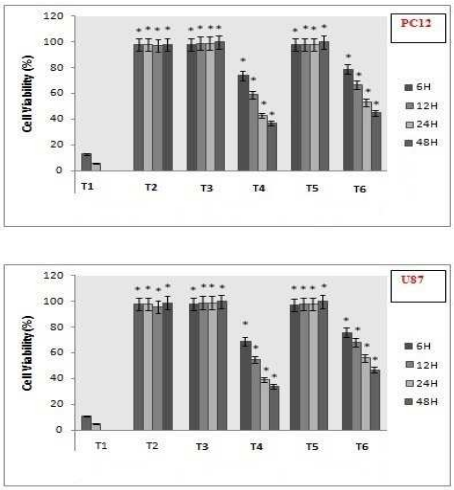

Figure 2. PC12 and U87 cells, cell viability assessed by trypan blue after exposed to different treatment mediums; T1: 1µM Staurospurine, T2: control cells, T3: mBM Soup 24h, T4: mBM Soup 24h together with 1µM Staurospurine, T5: mBM Soup 48h and T6: mBM Soup 48h together with 1µM Staurospurine. All data represented by mean ± standard.* $p<0.05$ as evaluated by paired ANOVA.

Comparison of the mean cell Viability analyzed using the MTT assay after 24 and 48 hours revealed a significant increase between the treatments compared with treatment 1, respectively ($p < 0.05$). After 6-48h, the cell viability in PC12 and U87 was again significantly decreased by increasing the time in treatment 1 compared with treatment 2, respectively ($p < 0.05$). The percentages of cell viability were increased in treatments 4 and 6 compared with treatment 1($p < 0.05$). After 6-48h, the percentage of cell viability was lowest in treatment 1 and was highest in treatments 2, 3 and 5 compared with treatment 2 ($p < 0.05$, Figure 3).

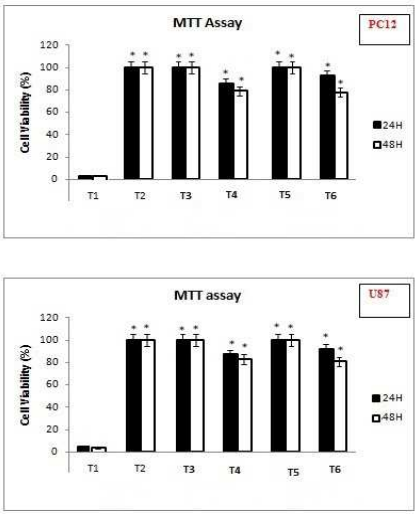

Figure 3. PC12 and U87 cell line viability (%) assessed by MTT in different treatment mediums and different culture periods; T1: 1µM Staurospurine, T2: control cells, T3: mBM Soup 24h, T4: mBM Soup 24h together with 1µM Staurospurine, T5: mBM Soup 48h and T6: mBM Soup 48h together with 1µM Staurospurine. All data represented by mean ± standard.* p<0.05 as evaluated by paired ANOVA.

NO levels

NO concentration was evaluated using the Griess method. The effect of different concentrations of staurospurine on PC12 and U87 cells after 24 and 48hours indicated a time-dependent decrease in NO secretion compared with control cells (treatment2), respectively ($p<0.05$).

Figure 4 shows NO concentration amounts (μM) in the culture medium of PC12 and U87 cells that contained staurospurine (1μM) plus different mBM soup for 24 and 48 incubation times.

After 24 and 48h incubation, in treatment 1 the NO concentration was decreased in the medium compared with the control sample (treatment 2), respectively ($p<0.05$). After the 24 and 48 h incubation, NO concentration significantly increased when cells was treated with treatments 2-6 compared with treatment 1, respectively ($P < 0.05$). Data shows the highest NO concentration was in treatment 5 and the lowest concentration was in treatment 1 in culture medium for 24 and 48h, respectively ($P < 0.05$, Figure4).

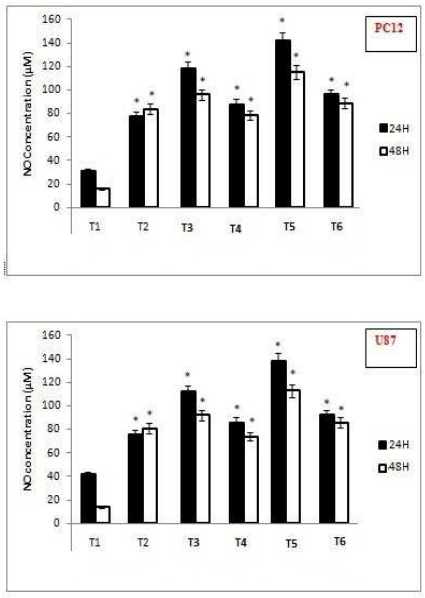

Figure 4. Nitric oxide (NO) levels in different groups and different culture periods; T1: 1μM Staurospurine, T2: control cells, T3: mBM Soup 24h, T4: mBM Soup 24h together with 1μM Staurospurine, T5: mBM Soup 48h and T6: mBM Soup 48h together with 1μM Staurospurine; All data represented by mean ± standard.* p<0.05 as evaluated by paired ANOVA.

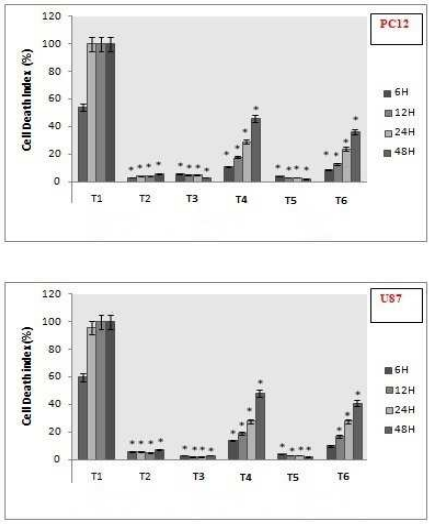

Figure 5. The effects of different treatment mediums on cell death in PC12 and U87 cells; Quantitative analysis of apoptotic cells by fluorescence microscopy in various treatments; 1μM Staurospurine, T2: control cells, T3: mBM Soup 24h, T4: mBM Soup 24h together with 1μM Staurospurine, T5: mBM Soup 48h and T6: mBM Soup 48h together with 1μM Staurospurine; All data represented by mean ± standard.* p<0.05 as evaluated by paired ANOVA.

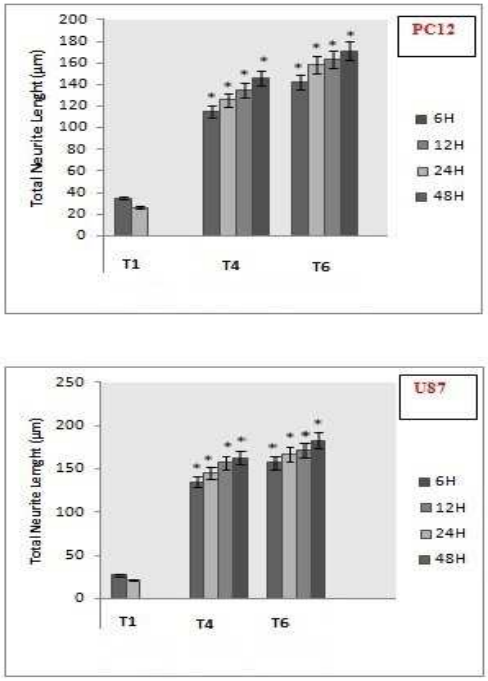

Figure 6. The effects of different treatment medium on total neurite length in PC12 and U87 cells; T1: 1µM Staurospurine, T2: control cells, T3: mBM Soup 24h, T4: mBM Soup 24h together with 1µM Staurospurine, T5: mBM Soup 48h and T6: mBM Soup 48h together with 1µM Staurospurine; All data represented by mean ± standard.* p<0.05 as evaluated by paired ANOVA.

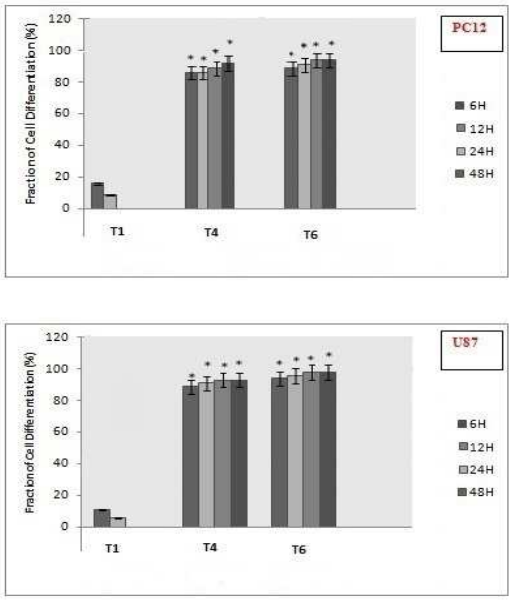

Figure 7. The effects of different treatment medium on fraction of cell differentiation in PC12 and U87 cells; T1: 1µM Staurospurine, T2: control cells, T3: mBM Soup 24h, T4: mBM Soup 24h together with 1µM Staurospurine, T5: mBM Soup 48h and T6: mBM Soup 48h together with 1µM Staurospurine; All data represented by mean ± standard.* p<0.05 as evaluated by paired ANOVA.

Cell Death Indexes

The cell death index of PC12 and U87 cells treated with different treatments showed an increase from treatment 1 to other treatments in order of the time ($p < 0.05$). After 6, 12, 24 and 48h, the cell death index was highest in treatment 1 and were lowest in treatments 3 and 5, respectively ($p<0.05$). The cell death index was increased in treatment 1 compared with treatment 2 for 6-48h incubation, respectively ($p < 0.05$). After 6-48h, the cell death index of PC12 and U87 cells in treatments 3 was decreased compared with treatment 4, respectively ($p < 0.05$). After 6-48h, the cell death index of cells in treatment 5 was decreased compared with treatment 6, respectively ($p < 0.05$).

The cell death index of PC12 and U87 cells were decreased in treatments 4 and 6 compared with treatment 1 for 6-48h incubation, respectively ($p < 0.05$, Figures 5, 6 and 7).

Total Neurite Length measurement

Data show that mBM soup together with staurospurine suppresses the cell death in cells and induces the neurite elongation. The average of total neurite length for PC12 and U87 Cells was assessed. The total neurite length (TNL) was calculated. For treatment 1, our data showed that saturospurine in 1µM induced cell death and low neurite elongation in PC12 and U87 cells. Long Neurite elongation was not seen in treatment 1. The effect of different concentrations of mBM soups on PC12 and U87 cells indicated a time-dependent increase in neurite elongation ($P<0.05$). TNL were increased after 6, 12, 24 and 48 hours, TNL significantly were increased in treatments 4 and 6 compared with treatment 1, respectively ($p<0.05$). After 48h, TNL was lowest in treatment 1 and was highest in treatment 6 ($p < 0.05$, Figure 8). TNL were increased in time dependent in treatment 6 compared with treatment 4 ($p<0.05$).

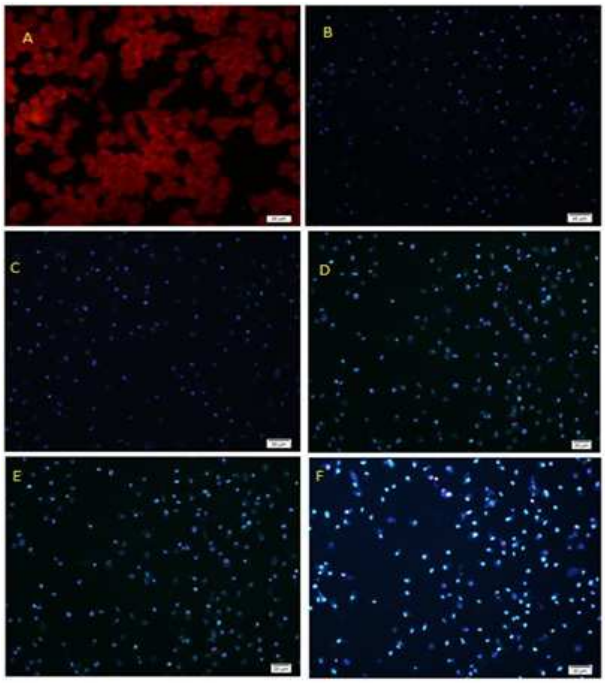

Figure 8. Morphology of PC12 cells in examined by florescence microscopy after 48 hours; A: 1µM Staurospurine, B:control cells, C: mBM Soup24h, D: mBM Soup24h together with 1µM Staurospurine, E: mBM Soup 48h and F: mBM Soup 48h together with 1µM Staurospurine.

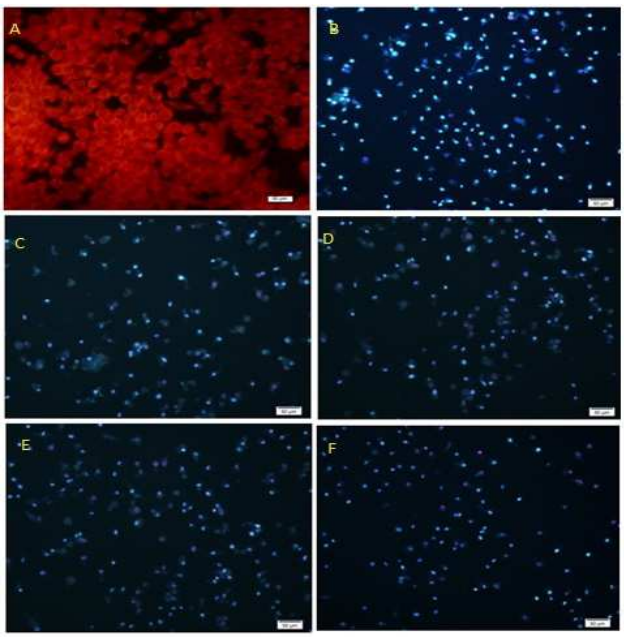

Figure 9. Morphology of U87 cells in examined by florescence microscopy 48 hours; A: 1µM Staurospurine, B: Control cells, C: mBM Soup24h, D: mBM Soup24h together with 1µM Staurospurine, E: mBM Soup 48h and F: mBM Soup 48h together with 1µM Staurospurine.

Fraction of cell differentiation assessment (f (%))

The evaluation of the fraction of cell differentiation for PC12 and U87 cells was assessed. After 48 hours, f (%) significantly were increased in treatments 4 and 6 compared treatment 1($p<0.05$). After 48h, f (%) was lowest in treatment 1 and was highest in treatments 4 and 6 ($p < 0.05$, Figure 9). After 48 h, were not significantly difference in treatment 4 compared with treatment 6.

DISCUSSION

Here, for the first time, we showed that administrations of mBM Soup were effective for prevention of Staurospurine-induced cell death in neuronal (PC12) and glioblastoma (U87) cells. Our data showed that application of mouse adult bone marrow stromal cells (BMSC) Soup treatment is mediumted by enhanced trophic support of the neurons and glioblastoma cells.

On the other side, Staurosporine, as we know, is a potent inhibitor of a number of kinases including: PKC, PKA, tyrosine protein kinase, phosphorylase kinase, and Ca^{2+}/calmodulan-dependent protein kinase [46-51]. It has been showed that Staurosporine induces cell death [46] in 1μM concentrations. Inhibition of these intracellular kinases by staurosporine was showed to lead to the induction of apoptosis [52,53].

In another study it demonstrated that mitogen-activated protein kinase (MAPK) activation provides cell type-specific signals important for cellular differentiation, proliferation, and survival. MAPK activation is an important survival signal in the neurons studied, and may mediumte the pro-survival effects for cAMP in neurons [54]. Previous study showed that MSC can be expressing a number of glial cell markers such asS100 and GFAP and that these cells promote neurite outgrowth [55]. It has been showed that MSC significantly enhanced neurite outgrowth of DRG neurons. Data showed that mRNA transcripts for NGF, GDNF, NT3, BDNF, TGFβ and VEGF expressed in undifferentiated MSC [56]. This result is consistent with another study in rat MSC [57]. It showed that BDNF levels correlate with enhancement of SH-SY5Y [56] and DRG [58] neurite outgrowth in response to MSC. Meanwhile, Tyrosine kinase receptor signaling by MSC soup can induced MAPK activation and increased cell viability and cell proliferation and decreased cell death in the cells. Other side, it has been showed that TGF-β has been implicated in the migration of mesenchymal-like cells toward wounds [59]. TGF-β is produced and secreted by many types of tumors, including 30–70% gliomas, and is implicated in many tumor related functions [60-62]. It showed that TGF-β contributes to the self-renewal and tumorigenesis, angiogenesis and cell proliferation of Glioma Stem Cells "GSCs" [63,64]. Meanwhile, Tyrosine kinase receptor signaling activation like TGF-β or NGF by BM-MSC soup can induced MAPK activation and increased cell viability and cell proliferation and decreased cell death in the cells. It confirmed by recent study. It has been showed that Condition Medium from MSCs, particularly from genetically modified MSCs overexpressing Akt-1 (Akt-MSCs), exerts cardiomyocyte protection [65,66]. It has been showed that injected MSCs act *via* a paracrine mechanism and secrete trophic factors which to enhance angiogenesis, synaptogenesis, and neurogenesis [67]. Meanwhile, MSC secrete trophic inhibit scar formation (mainly caused by astrocytosis) as well as stimulating neural progenitor cells (NPCs) proliferation, migration and differentiation [68,69]. Hung, et al (2007) has been shown that conditioned medium of BM-MSCs can activate the PI3K/Akt pathway in hypoxic endothelial cells resulting in an inhibition of cell death, an increased cell survival, and a stimulation of angiogenesis. Data has been showed that BM-MSC Soup having a higher content of anti-apoptotic and angiogenic factors, such as IL-6, VEGF, and monocyte chemoattractant protein (MCP)-1 [70].

Cell survival factor inhibits cell apoptosis through activating specific signaling pathway(s), including the PI3K/Akt pathway. It has been shown that, transfection of constitutively active Akt prevents cell apoptosis while a dominant negative Akt induces cell apoptosis [71]. In the other side, recent studies shown that inhibitors of the PI3K/Akt pathway can sensitize cells to apoptotic stimuli [71,72]. For example, Osaki et al, have showed that inhibition of PI3K caused inhibition of cell proliferation without induction of cell apoptosis and that inhibition of the PI3K-Akt signaling pathway significantly increased the sensitivity of cell apoptosis [73].

This paper highlights the importance of the paracrine effects of mBM soup on neuroglial cells, and that intact mBMSC cells may not be necessary. In previuose studies shown that paracrine cross-talking between salivary gland (SG) cells and other cell populations (such as MSC or amniotic epithelial cells) was demonstrated by using a culture system that physically separated the cell populations [74]. These findings on the paracrine effects of MSC have been showed that organ repair was due to the secretion of cytokines, chemokines, and growth factors [75]. It had been showed that injecting conditioned medium from MSC cultures exerted cardiomyocyte protection and improved cardiac function in mouse infarcted hearts [76]. Administrating BM Soup does not require the injection of cells, which carry the risk of differentiating into unwanted/tumorigenic cell types in organs and is not patient-specific.

Study showed that BMSC Soup includes all cell types of whole BM and consequently numerous proteins, cytokines and paracrine factors [77]. Overall, our data demonstrate that mBM Soup trigger endogenous survival signaling pathways such as increasing the NO concentration in neuron and glioblastoma that medium protection against staurospurine-induced cell death insults. Moreover, the interaction between stressed neurons and mBM Soup further amplifies the observed neuroprotective effect.

Our results provide evidence that mBMSC soup is capable to suppress cell death and induce neurite elongation in PC12 and U87 cells. Studying the paracrine factors that are differentially produced in mBMSC should eventually allow us to design new therapeutic approaches for neuroglial cells.

CONCLUSION

According to the results of present study, application of mBMSC Soup lead to enhance of cell viability, NO and neurite outgrowth and decreasing in cell death. However, more key factors need to be investigated in these effects.

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