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EXPERIMENTAL STUDY

# Mesenchymal stem cell condition medium enhanced cell viability in morphine-treated cells

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**ABSTRACT**

Bone marrow mesenchymal stem cells (BM-MSC) have recently been predicted to have a major therapeutic potential due to secretion of soluble factors and the release of cytokines and growth factors, which could mediate the cellular communication to induce cell differentiation/maturity.

The aim of the present study was to determine the effect of mBM condition medium on morphine-induced cell death in PC12, U87, AGS and MCF-7 cell lines. The condition media were harvested as mBM soup (mBM soup 24 and mBM soup 48h, respectively). To investigate the effect of mBM soup on cell lines, morphological changes were studied with an inverted microscope, the viability of cells was determined with trypan blue staining and MTT assay, the type of cell death was determined using Hoescht / PI staining, and NO secretion analysis. Viability assay showed that mBM soup (24 and 48 h) in time-dependent manner increased cell viability ($p<0.05$) and cell death assay showed that cell death decreased in a time-dependent manner ($p<0.05$). Our findings suggest that mBM soup can enhance the proliferation and growth of cell lines and can suppress cell death induced by morphine *(Fig. 8, Ref. 59)*. Text in PDF *www.elis.sk.*

Key words: morphine, BM-MSC soup, cell viability, cell death, NO.

## Introduction

Morphine as an analgesic drug can induce apoptosis in proliferating cells (1–4), immunocytes (5, 6), cancer cells (7, 8), neuroblastoma cells such as: SK-N-SH, NG108-15 and PC12 cells (9–11), and neuronal cells (12, 13), as well as human microglia (14) and inhibit growth of PC-9, HL-60 and KATO III cancer cell or cancer lines (15). On the other hand, it has been showed that morphine could induce apoptosis in non-cancerous cells such as: endothelial cells (16), T lymphocytes, macrophages (17), microglia and neurons derived from rat brain (18).

On the other hand, previous studies on the amazing neurodifferential potential of MSCs attracts an intense interest in the possible applications of MSCs in cell and gene therapy for neurological disease, because MSCs can be obtained from bone marrow easily and expanded rapidly in vitro (19, 20).

On the other side, the most recent mechanism of action of BMSC cells is a local paracrine effect (21, 22). It has been showed that transplanted MSCs had the ability to produce growth factors (23–25). In summary, tissue regeneration and improvements have been proposed as paracrine effect of stem cell action (26, 27). However, MSCs 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 effect (28). Meanwhile, these factors are cytokines; growth factor and other factors induce cytoprotection, neovascularization, and medium endogenous tissue regeneration via activation of resident tissue stem cells. In addition, tissue remodeling and organ function are affected by these paracrine factors (22).

However, how they survive and differentiate into distinct cell types is still not clear. The aim of this study was to determine the effect of mBM condition medium on morphine-induced cell death in different cell lines.

## Material and methods

*Preparation of mouse bone marrow cells*

Isolation and culture of mBMSCs were carried out as previously described method (29). Bone marrow was obtained from 6–8-week-old NMRI mice. Cell suspension was incubated at 37 °C in humidified atmosphere containing 5 % $CO_2$ for 72 hours. 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 70 % confluency, they were harvested with trypsin-EDTA 0.25 % (Sigma) for 5 minutes, again cultured to next confluence and harvested. Expanded cells from passages five-eight were used for further testing.

The surface marker expression of mBMSC cells was assessed by a flow cytometry. $1\times10^6$ cells/ml mBMSC were suspended and stained with FITC- or R-phycoerythrin (PE)-conjugated monoclonal antibodies, such as: CD14 (eBioscience), CD105 (eBio-

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science), CD73 (eBioscience), CD29 (Biolegend), CD34 (Santa Cruz), CD45 (eBioscience), CD90 (Biolegend).

*mBM condition medium*

Briefly, after confirming and harvesting the mBMSC cells, cells were cultured in DMEM: F12 culture medium, when cell reached to 80 % confluency, the media were changed by fresh DMEM: F12 supplemented with 0.2 % BSA culture medium. The cells maintained for 24 h or 48 h. Then, the condition media were harvested as mBM soup 24 and mBM soup 48 h, respectively. Protein concentration of mBM Soup was 0.52 µg/ul (from $10^8$ cells).

*Cell lines*

PC12, U87, MCF-7 and AGS cells were grown in a 25-$cm^2$ tissue culture flask in DMEM culture media (Gibco), supplemented with 5 % fetal bovine serum (FBS, Gibco; UK), 100 u/ml of penicillin (Sigma) and 100 mg/ml streptomycin (Sigma). The cells were maintained in standard condition (37 °C and 5 % $CO_2$).

*Cell treatment*

One day after plating the different cell lines in DMEM culture media containing 0.2 % BSA, cells were washed with PBS, pH 7.4. There were six treatments including; treatment 1: 1mM morphine, treatment 2: culture medium, treatment 3: mBM Soup 24 h, treatment 4: mBM Soup 24 h together with 1 mM morphine, treatment 5: mBM Soup 48 h and treatment 6: mBM Soup 48 h together with 1mM morphine.

*Cell viability measurement*

Trypan blue viability measurement was performed by standard methods (30).

The traditional method of performing trypan blue (0.4gr / 100ml in PBS) cell viability analysis involves a manual staining and a use of hemocytometer for counting.

*Cell proliferation assay*

To perform the test, $1\times10^4$ different cell lines were loaded into 96-well plate and 200 µL of DMEM: F12 medium containing 0.2 % BSA was added. After 24-hour incubation, 200 µL of different treatment media as described was added to the wells. The cells were separately incubated with different treatments medium for 24 and 48 hours. Cell proliferation was quantified by MTT assay. MTT cytotoxicity measurement was performed by standard methods (31). The optical density of each well was measured using a plate reader at 570 and 630 nm.

*Quantification of cell death incidence*

Hoechst/PI nuclear staining was carried out as previously described (32). Briefly, cells were plated in 24 well culture plates with $5\times10^4$ cells/well density for 24 h. Cells were treated with different treatment media for a

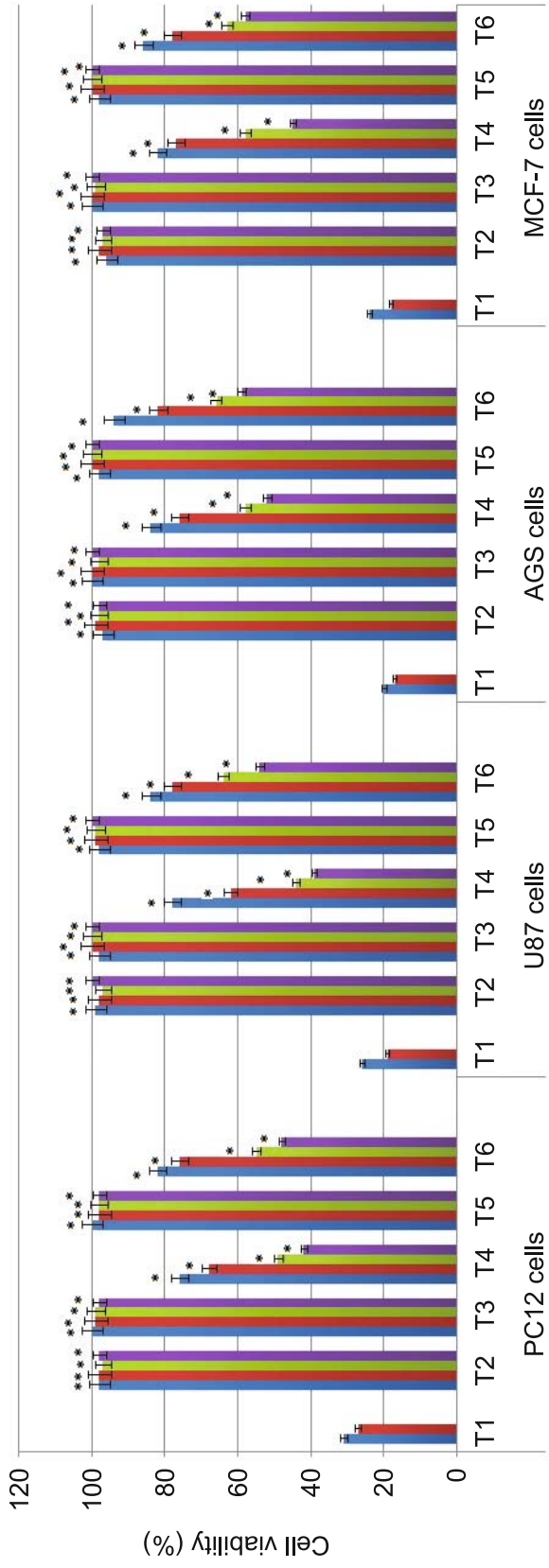


**Fig. 1. Cell viability assessed by trypan blue after exposed to different treatment media. T1: 1 mM morphine, T2: control cells, T3: mBM Soup 24 h, T4: mBM Soup 24 h together with 1 mM morphine, T5: mBM Soup 48 h and T6: mBM Soup 48 h together with 1 mM morphine. All data represented by mean ± standard. * $p < 0.05$ as evaluated by paired ANOVA.**

range of times (6, 12, 24 and 48 h). 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 apoptotic index was 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 (33). All wells were incubated for 15 minutes and were assessed using a plate Reader at wavelengths of 570 and 630 nm.

**Results**

*Characteristics of BMSCs*

The cells as mBMSCs were derived from female and male NMRI mice. The 5th passage of cells had similar morphology. The results showed that about 98 % cells of mBMSCs were CD90-positive and a lack of expression of CD14, CD45 and CD34. These results showed that mouse Bone marrow cells had the characteristics of mesenchymal stem cells, which were carried out as previously described (our published manuscript DOI: 2016, 5, 11:22-33, IJMRHS).

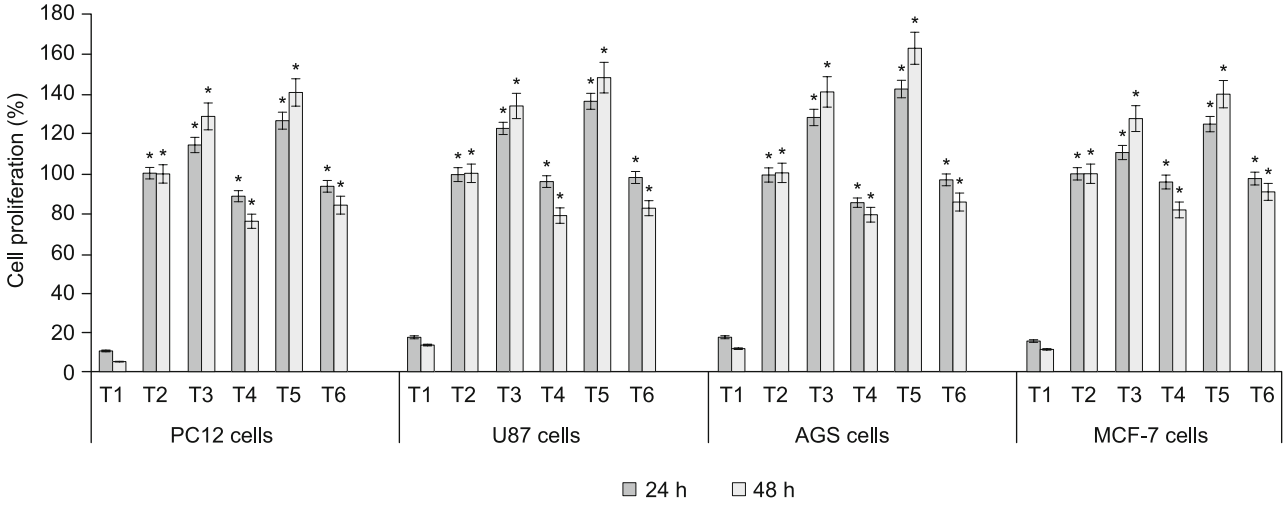


**Fig. 2. Cell prolifration (%) assessed by MTT in different treatment media and different culture periods. T1: 1 mM morphine, T2: control cells, T3: mBM Soup 24 h, T4: mBM Soup 24 h together with 1 mM morphine, T5: mBM Soup 48 h and T6: mBM Soup 48 h together with 1 mM morphine. All data represented by mean ± standard. * p < 0.05 as evaluated by paired ANOVA.**

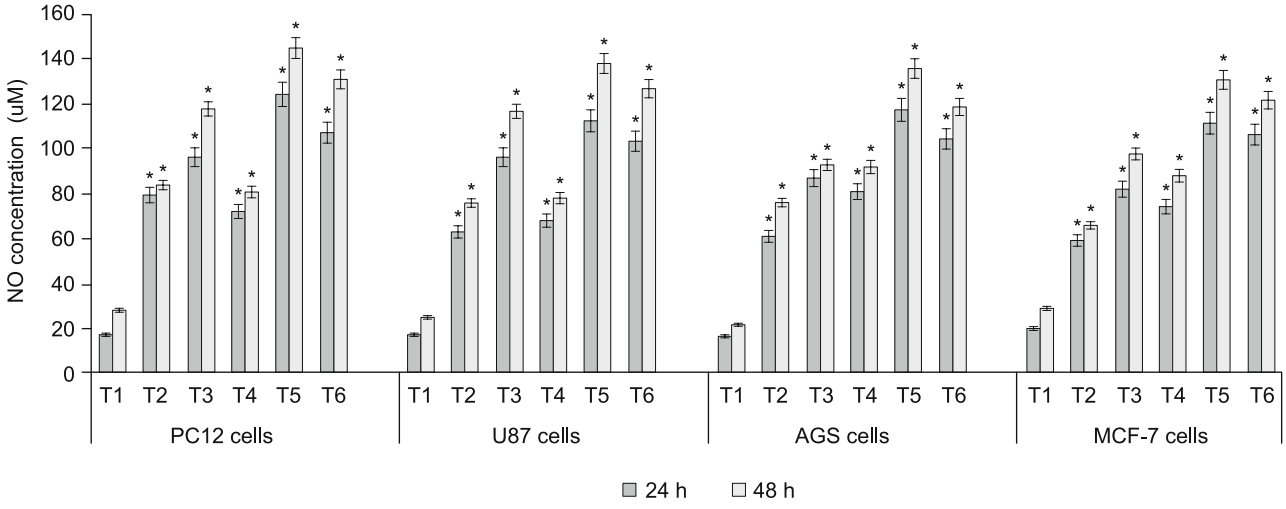


**Fig. 3. Nitric oxide (NO) levels in different treatments and different culture periods. T1: 1 mM morphine, T2: control cells, T3: mBM Soup 24 h, T4: mBM Soup 24 h together with 1 mM morphine, T5: mBM Soup 48 h and T6: mBM Soup 48 h together with 1 mM morphine. All data represented by mean ± standard. * p < 0.05 as evaluated by paired ANOVA.**

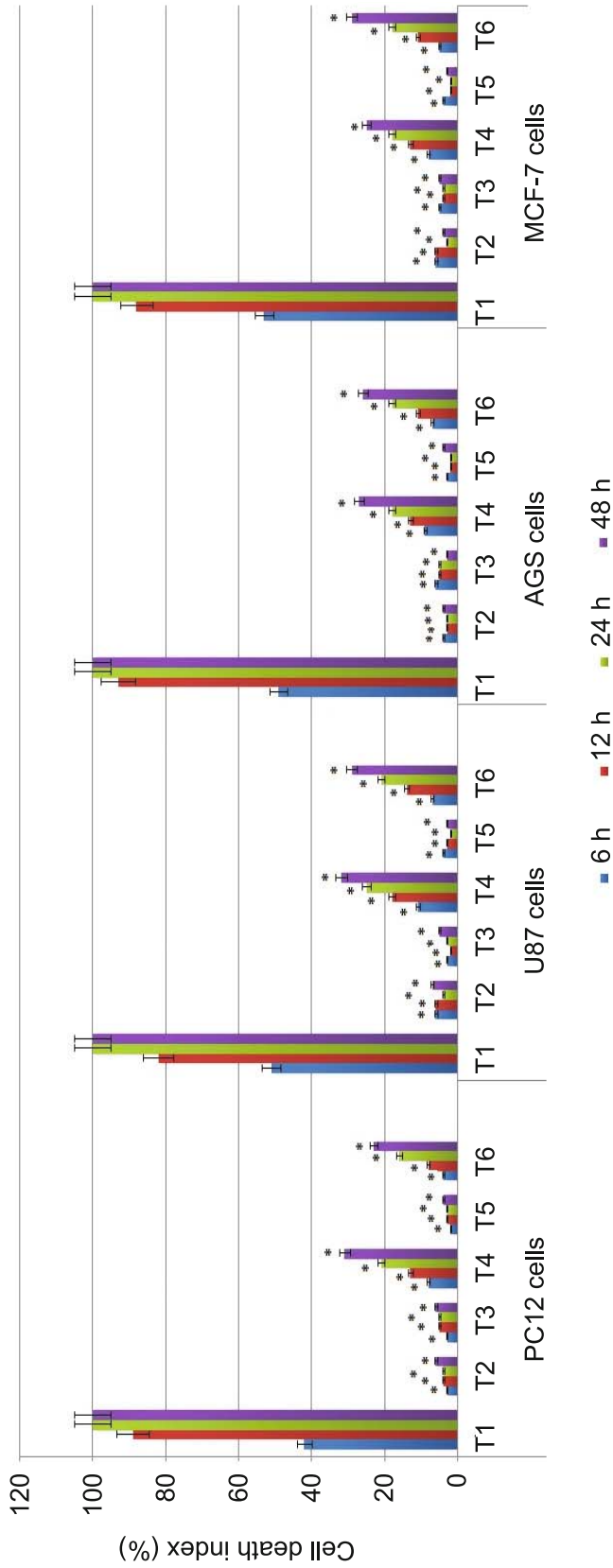


**Fig. 4. The effects of different treatment media on cell death in cell lines. Quantitative analysis of apoptotic cells by fluorescence microscopy in various treatments. T1: 1 mM morphine, T2: control cells, T3: mBM Soup 24 h, T4: mBM Soup 24 h together with 1 mM morphine, T5: mBM Soup 48 h and T6: mBM Soup 48 h together with 1 mM morphine. All data represented by mean ± standard. * p < 0.05 as evaluated by paired ANOVA.**

*Cell viability and cell proliferation*

Comparison of the mean PC12, U87, MCF-7 and AGS cell viabilities 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 to the treatment 2 (97 %) ($p<0.05$). After 6, 12, 24 and 48 hours, the percentages of cell viability were not different in treatments 3 and 5 compared to treatment 2. The percentage of cell viability was increased in treatment 3 compared to the treatment 4 ($p<0.001$) (Fig. 1). The percentage of cell viability was increased in treatments 4 and 6 compared to the treatment 1 ($p<0.05$). Comparison of the mean cell proliferation analyzed using the MTT assay after 24 and 48 hours revealed a significant increase between the treatments compared to the treatment 1, respectively ($p<0.05$). Cell proliferation was again significantly decreased by increasing the time in treatment 1 compared to the treatment 2 ($p<0.05$). The cell proliferation was increased in treatments 4 and 6 compared to the treatment 1 ($p<0.05$). The percentage of cell proliferation was lowest in the treatment 1 and was highest in the treatments 3 and 5 ($p<0.05$) (Fig. 2).

*NO levels*

NO concentration was evaluated using the Griess method. The effect of different concentrations of staurospurine on PC12, U87, MCF-7 and AGS cells after 24 and 48 hours indicated a time-dependent decrease in NO secretion compared to the control treatment, respectively ($p<0.05$).

Figure 4 shows NO concentration amounts (μM) in the culture medium of PC12, U87, MCF-7 and AGS cells that contained staurospurine (1 μM) plus different mBM soup for 24 and 48 incubation times. After 24 and 48 h incubation, the NO concentration in treatment 1 was decreased in the medium compared to the control sample (treatment 2), respectively ($p<0.05$). After the 24 and 48 h incubation, NO concentration significantly increased, when cells were treated with treatments 2–6 compared to the treatment 1 ($p<0.05$). Data showed that the highest NO concentration was in the treatment 6 and the lowest concentration was in the treatment 1 in culture medium for 24 and 48 h, respectively ($p<0.05$) (Fig. 3).

*Cell death indexes*

The cell death index of PC12, U87, MCF-7 and AGS cells treated with different treatments showed an increase from the treatment 1 to other treatments in order of the time, respectively ($p<0.05$). After 6, 12, 24 and 48 h, the cell death index was highest in the treatment 1 and lowest in the treatments 3 and 5,

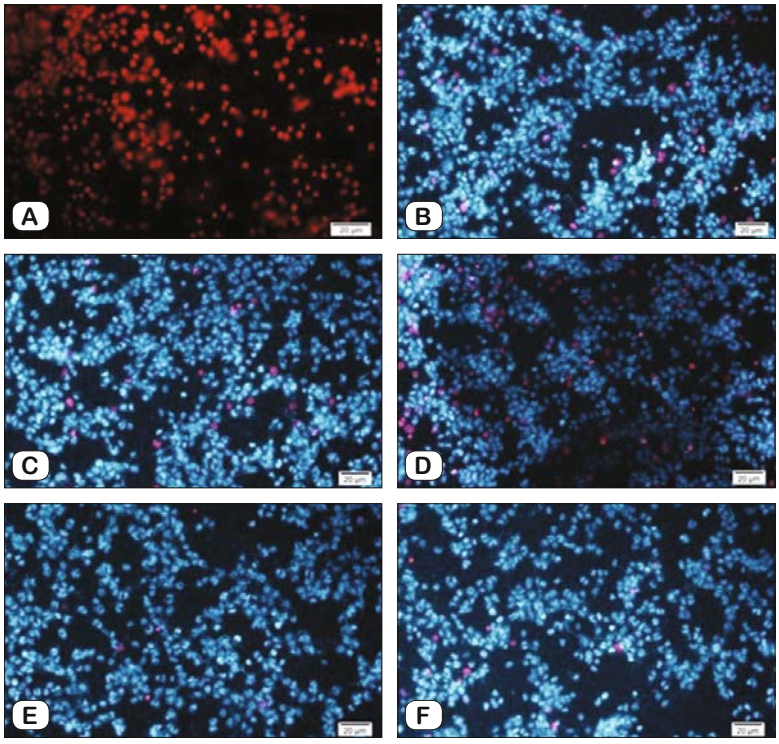


**Fig. 5. Morphology of PC12 cells as examined by florescence microscopy. A: 1 mM morphine, B: control cells, C: mBM Soup 24 h, D: mBM Soup 24 h together with 1 mM morphine, E: mBM Soup 48 h and F: mBM Soup 48 h together with 1 mM morphine.**

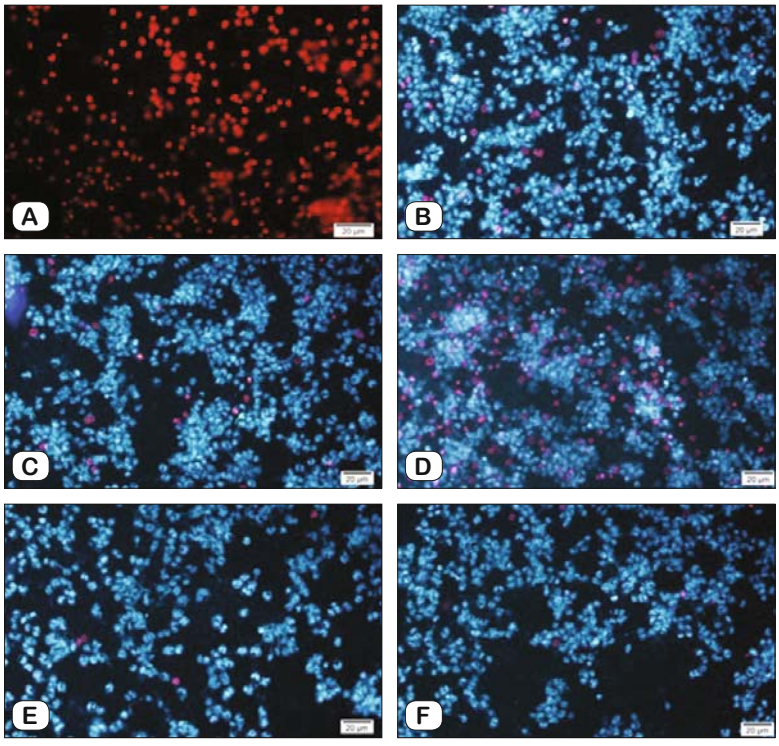


**Fig. 6. Morphology of U87 cells as examined by florescence microscopy. A: 1 mM morphine, B: control cells, C: mBM Soup 24 h, D: mBM Soup 24 h together with 1 mM morphine, E: mBM Soup 48 h and F: mBM Soup 48 h together with 1 mM morphine.**

respectively ($p<0.05$). The cell death index was increased in the treatment 1 compared to the treatment 2, respectively ($p<0.05$). The cell death index of PC12, U87, MCF-7 and AGS cells in treatment 4 was increased compared to the treatment 3 ($p<0.05$). The cell death index of cells in the treatment 6 was increased compared to the treatment 5 ($p<0.05$). The cell death index of PC12, U87, MCF-7 and AGS cells were decreased in the treatments 2, 3 and 5 compared to the treatment 1 after 6–48 h incubation, respectively ($p<0.05$) (Fig. 4). Morphology of PC12 (Fig. 5), U87 (Fig. 6), MCF-7 (Fig. 7) and AGS (Fig. 8) were examined by a florescence microscopy.

## Discussion

Morphine as an opioid drug exerts a variety of biological effects, which may be mediated through mechanisms other than the classic opioidergic receptors. The exact mechanism whereby morphine modulates cell survival and cell death is not clearly understood (34).

Cell viability was measured by MTT assay. On the other hand, apoptosis was detected by Hoechst/Propodium Iodide staining test (specific test for apoptosis) and NO secretion.

In the first step of the present study, PC12, U87, AGS and MCF-7 cancer cell lines were exposed to 1mM concentration of morphine as an apoptotic dose. The proapoptotic and necrotic effects of morphine were shown in multiple cell lines (35–39). In agreement with the other investigations, the results of Trypan blue, MTT assay and Hoechst/Propodium Iodide staining test showed that morphine decreased the cell viability and increased the number of apoptotic cells, respectively (37, 38). In the second part of this investigation, the protective effect of mBM Soup was investigated. There are not any common grounds for the interaction between mBM soup and morphine. Here, for the first time, we showed that administrations of mBM Soup were effective for a suppression of morphine-induced cell death in neuronal (PC12), glioblastoma (U87), gastric cancer (AGS) and Breast cancer (MCF-7) cells. Our data showed that application of mouse adult bone marrow stromal cells (BMSC) Soup treatment is mediated by enhanced trophic support of the cancer cells. Another study demonstrated that MAPK activation provided cells with type-specific signals important for cellular differentiation, proliferation, and survival. MAPK activation is an important survival signal in the neurons and may mediate the pro-survival effects for cAMP in neuronal and neuroglial

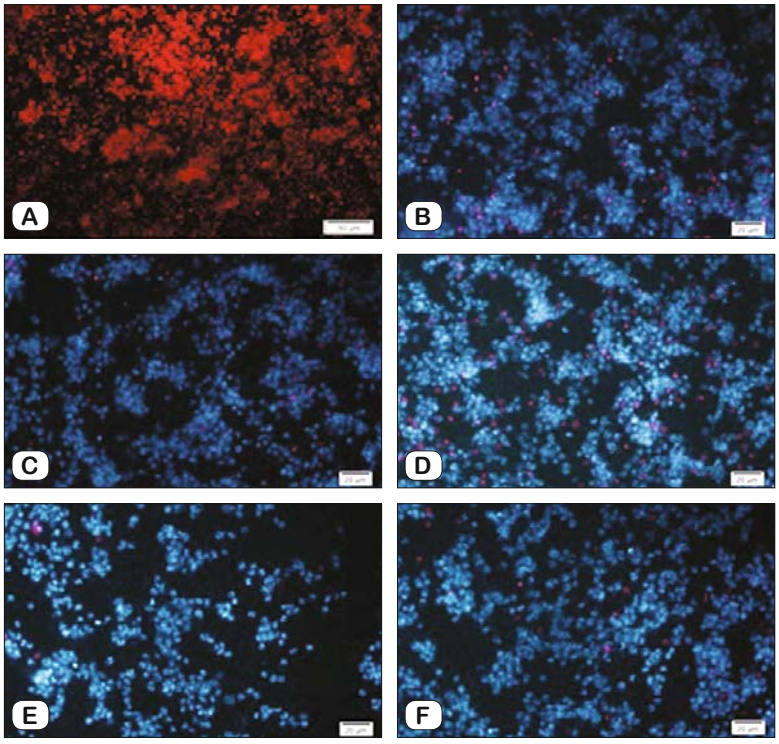


**Fig. 7. Morphology of MCF-7 cells as examined by florescence microscopy. A: 1 mM morphine, B: control cells, C: mBM Soup 24 h, D: mBM Soup 24 h together with 1 mM morphine, E: mBM Soup 48 h and F: mBM Soup 48 h together with 1 mM morphine.**

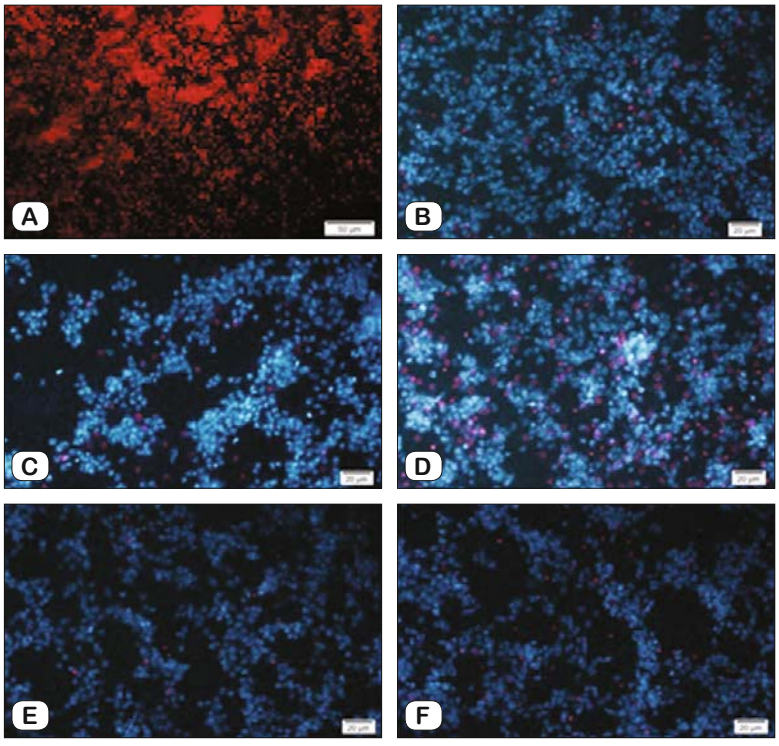


**Fig. 8. Morphology of AGS cells as examined by florescence microscopy. A: 1 mM morphine, B: control cells, C: mBM Soup 24 h, D: mBM Soup 24 h together with 1 mM morphine, E: mBM Soup 48 h and F: mBM Soup 48 h together with 1 mM morphine.**

cells (40). The previous study showed that MSC could express a number of glial cell markers such as: S100 and GFAP and that these cells promoted neurite outgrowth (41). It has been shown that MSC significantly enhanced neurite outgrowth of DRG neurons. Data showed that mRNA transcripts for NGF, GDNF, NT3, BDNF, TGFβ and VEGF were expressed in undifferentiated MSC (42). This result is consistent with another study in rat MSC (43). It showed that BDNF levels correlated with the enhancement of SH-SY5Y (44) and DRG (45) neurite outgrowth in response to MSC. Meanwhile, Tyrosine kinase receptor signaling by MSC soup can induce MAPK activation and increase cell viability and cell proliferation and decrease cell death in the cells.

Meanwhile, Tyrosine kinase receptor signaling activation like TGF-β or NGF by BM-MSC soup can induce MAPK activation and increase cell viability and cell proliferation and decrease cell death in the cells. It is confirmed by a recent study. It was shown that Condition Medium from MSCs, particularly from genetically modified MSCs overexpressing Akt-1 (Akt-MSCs), exerted cardiomyocyte protection (46). It has been showed that injected MSCs acted *via* a paracrine mechanism and secreted trophic factors, which enhance angiogenesis, synaptogenesis, and neurogenesis (47). Meanwhile, MSC secretes trophic factors that inhibit scar formation (mainly caused by astrocytosis) as well as stimulate neural progenitor cells (NPCs) proliferation, migration and differentiation (48, 49). Hung, et al (2007) showed that conditioned medium of BM-MSCs can activate the PI3K/Akt pathway in hypoxic endothelial cells resulting in an inhibition of apoptosis, and increased cell survival, and stimulation of angiogenesis. Data showed that BM-MSC Soup had a higher content of anti-apoptotic and angiogenic factors, such as: IL-6, VEGF, and monocyte chemoattractant protein (MCP)-1 (50).

However, opposing results were documented. For example, in PC3 cells (an animal model of prostate cancer) MSCs promote fibroblast growth factor 2 (FGF2) secretions. It has been shown that MSCs promoted tumor angiogenesis by enhancing the expression of endothelin-1 in colon cancer cell lines (50, 51). It has been shown that co-culturing gastric cancer cell lines with MSCs increased the proportion of CD133-positive gastric cancer cells (52, 53). It has been showed that TGF-β expression in MSCs promoted tumor growth of prostate cancer cell lines in mice. This tumor growth-promoting effect of MSCs can be blocked by siRNAs against TGF-β (54). 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 prevented cell apoptosis, while a dominant-negative Akt induced cell apoptosis (55). In the other side, recent studies showed that inhibitors of the PI3K/Akt pathway could sensitize cells to apoptotic stimuli (56, 57). For example, Osaki et al, showed that inhibition of PI3K caused an 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 (58).

The present study might suggest new strategies for the treatment and enhancement of cell proliferation and suppression of opioids induced cell death, using mBM soup as anti-apoptosis or necrosis inducer.

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