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Neuroprotective effects of bone marrow-derived mesenchymal stem cells and conditioned medium in mechanically injured neuroblastoma cells

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Background/aim: Bone marrow-derived mesenchymal stem cells (BMSCs) possess self-renewal characteristics that distinguish them from other cell types. Recent studies have focused on the effects of conditioned medium (CM) that includes the extracellular matrix. Here we examined the neuroprotective effects of BMSCs and CM on damaged neuroblastoma cells.

Materials and methods: The cells were divided into five groups: 1) healthy controls, 2) damaged cells alone, 3) damaged cells treated with BMSCs, 4) damaged cells treated with CM, and 5) damaged cells treated with both BMSCs and CM. Neuroprotective effects were then evaluated based upon the levels of oxidative stress, antitransforming growth factor β1 (anti-TGFβ1) production, and apoptosis.

Results: Significant differences were observed between healthy controls and damaged cells ($P < 0.001$), as well as between damaged cells and those treated with BMSCs alone ($P < 0.05$), CM alone ($P < 0.05$), and both BMSCs and CM in combination ($P < 0.01$). Among the treated groups, the strongest neuroprotective effects were seen in cells treated with both BMSCs and CM.

Conclusion: These results show that both BMSCs and CM exhibit neuroprotective effects in damaged neuroblastoma cells. The strongest benefits were seen following treatment with both BMSCs and CM.

Key words: Bone marrow derived mesenchymal stem cells, conditioned medium, neuroprotective, injured, neuroblastoma cell

1. Introduction

Stem cells exhibit self-renewal characteristics that distinguish them from other cell types. Major subtypes include totipotent cells, which can divide to produce all cells in an organism, pluripotent cells, which are able to differentiate into any of the three germ layers (endoderm, ectoderm, or mesoderm), and multipotent cells, which can produce multiple cell types within a given lineage (1). Mesenchymal stem cells (MSCs) constitute a class of multipotent cells capable of differentiating into many diverse cell types, including neural cells, chondroblasts, adipocytes, bone, skeletal muscle, and connective tissue (2–4). These cells can be isolated from almost any tissue, including bone marrow, Wharton jelly, umbilical cord, placenta, dental pulp, and adipose tissue using a simple procedure (5). Identification of MSCs is primarily performed using specific phenotypic and cell surface markers. MSCs should be able to adhere to a plastic surface and express defined surface antigens such as CD29, CD73, CD90, and CD105; however, they should also be negative for markers CD19, CD34, CD45, CD79a, and HLA class II during in vitro cultivation (6).

Their relative easy isolation and expansion in vitro, along with minimal to no immunoreactivity and graft-versus host reaction, and their ability to differentiation into a wide range of lineages has made these cells an attractive target for clinical applications (7,8). Among the MSCs, bone marrow stromal cells (BMSCs) represent one of the best candidates for treating neural diseases, having been investigated for use in a wide range of clinical applications, including spinal cord injuries, amyotrophic lateral sclerosis, autoimmune encephalomyelitis, and cerebral infarction (4,7,9–11). However, the isolation of stem cells, timing of the transplantation, preparation, dosage, and ideal transplantation methods remain a topic of considerable controversy, with a great deal of research necessary before any of these procedures are ready for widespread use.

In the absence of validated stem cell therapies, conditioned medium (CM) provides several practical

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advantages. CM can be manufactured, freeze-dried, packaged, and transported more easily than stem cells, and because CM does not contain living cells, no need exists to match the donor and the recipient to avoid rejection (12). To date, few studies have examined the effects of CM on neurological diseases such as cerebral injury (13,14), ischemia (15), stroke (16,17), and spinal cord injury (18). The aim of the present study was to determine the potential neuroprotective effects of BMSCs and CM in mechanically injured neuroblastoma cells in vitro via evaluation of oxidative stress, transforming growth factor β1 (TGFβ1) production, and apoptosis.

2. Materials and methods

All relevant study protocols were approved by the ethics committee of Celal Bayar University School of Medicine (No: 20478486-366) prior to initiation of this research.

2.1. Isolation of BMSCs

BMSCs were obtained from the tibias of a male Wistar rat weighing 250 g and cultured in alpha-minimum essential medium (α-MEM) containing 15% (v/v) fetal calf serum (FCS), 50 μg/mL gentamycin, 100 UI/mL penicillin, 100 UI/mL streptomycin, and 100 UI/mL amphotericin (All, Biochrom, Berlin, Germany). Briefly, bones were aseptically excised from the hind limbs of the rat following ether euthanasia. The soft tissue was removed, and two tibias were clipped off with sterile scissors, a hole was created in each bone with an 18-gauge needle, and the marrow was flushed from the shaft with control media (19). Stem cells were maintained at 37 °C and 5% CO_2 for 3 days, after which the medium was replaced with fresh medium to remove nonadhesive cells from the flask. Cells were then grown to confluence and passaged until passage 3 (P3) (19). A detailed characterization of BMSCs is shown in Figure 1.

2.2. Preparation of BMSC conditioned medium

Upon reaching passage 3, cells were plated into 25-cm^2 culture flasks containing α-MEM (3000 cells/cm^2) and grown until reaching 70%–80% confluence, with growth medium refreshed every 2 days. Cells were then maintained in α-MEM for 2 days to ensure secretion of sufficient growth factors into the culture medium; media from days 3 or later were not used due to the accumulation of cellular toxic waste. Two-day medium was collected and used as a CM for in vitro application to the injured neuroblastoma cells. Before application, CM was examined under an IX70 inverted microscope (Olympus, Tokyo, Japan) to ensure the absence of bacteria or other contaminants.

2.3. Neuroblastoma cell line culture

NB2a mouse neuroblastoma cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) horse serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 UI/mL streptomycin, and 25 μg/mL gentamicin (All, Biochrom, Berlin, Germany) at 37 °C and 5% CO_2 in a humidified chamber. The medium was changed every 2 days. Cells were subcultured using a 0.25% trypsin/EDTA solution when reaching 70%–80% confluence (20). In the present study, we used an NB2a cell line obtained from mouse. NB2a cell line was obtained commercially from the alum institute as frozen cells and used in culture condition. Mouse was not used as an experimental animal.

2.4. Differentiation and treatments

NB2a cells were seeded into 24-well chamber slide plates (15,000 cells/well) for 24 h, after which the medium was replaced with serum-free medium supplemented with 0.5 mM dibutyryl cyclic AMP (Sigma-Aldrich, St. Louis, MO, USA) to induce differentiation into neurons (21) (Figure 2). Following differentiation, the cells were divided into five groups: 1) healthy controls, 2) damaged cells alone, 3) damaged cells treated with BMSCs, 4) damaged cells treated with CM, and 5) damaged cells treated with both BMSCs and CM. Cell damage was induced as described previously (22). Briefly, a scratch insult was performed on cultured neuroblastoma cells. Cell bodies and processes were cut mechanically with a cataract knife on glass coverslips in 24-well culture plates. Pathological changes associated with injury were then assessed under a light microscope. Following injury, 1×10^6 BMSCs and/or 50% CM were administered to damaged groups; BMSCs were applied to group 3, while CM was applied to group 4; a combination of BMSC and CM was applied to group 5.

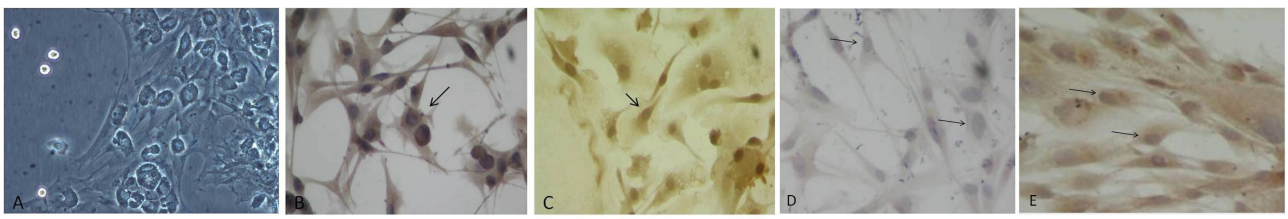

Figure 1. A) In vitro culture of bone marrow-derived mesenchymal stem cells (BMSCs) B, C) Immunocytochemical staining of BMSCs with Stro-1 and c-kit; arrows indicate positive cells. D, E) Immunocytochemical staining of BMSCs with CD45 and CD 105 marker; arrows indicate CD45 negative and CD105 positive cells.

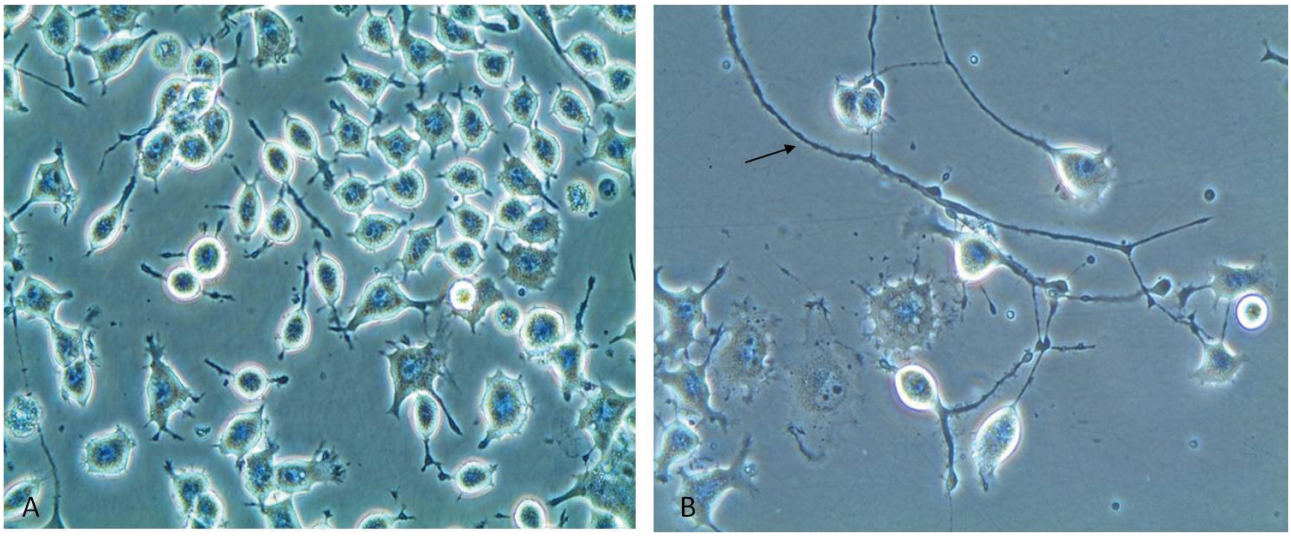

Figure 2. In vitro culture of Nb2a cells. A) Cells were grown in a monolayer from individual cells. B) After administration of d-CAMP, cells developed long neurite-like projections, indicated by arrows.

Treatments were applied only once to damaged cells; groups 1 and 2 were not treated.

2.5. Immunocytochemistry

BMSCs were examined using immunocytochemistry to evaluate the levels of oxidative stress (eNOS, iNOS) and healing (TGFβ1). After application, cells were fixed with 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) for 30 min and washed three times with phosphate-buffered saline solution (PBS; Invitrogen, Carlsbad, CA, USA) for 5 min. Next, permeabilization was performed using 0.1% Triton X-100 (AppliChem GmbH, Darmstadt, Germany) in PBS at 4 °C for 15 min and then washed with PBS. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide for 5 min at room temperature. Cells were incubated for 1 h with blocking serum, followed by the addition of the primary antibodies monoclonal anti-endothelial nitric oxide synthase (e-NOS; Neomarkers, Fremont, CA, USA), anti-inducible nitric oxide synthase (i-NOS; Neomarkers), and anti-TGFβ1 (Santa Cruz Biotechnology, Heidelberg, Germany) at 4 °C overnight to assess healing and oxidative stress (Figures 3–5); additional antibodies against stro-1 (Santa Cruz

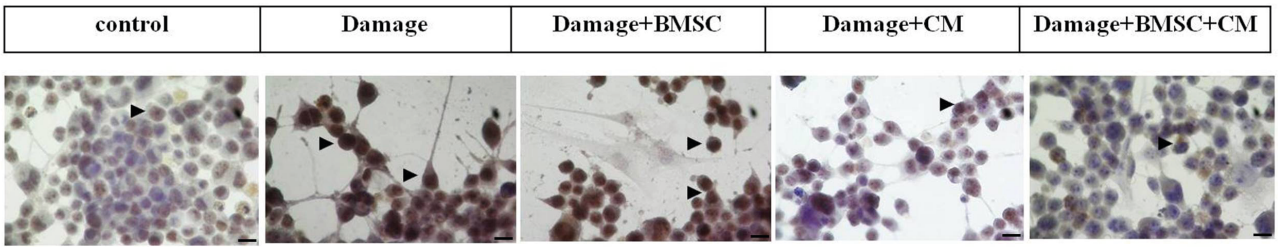

Figure 3. Immunocytochemical staining of anti-endothelial nitric oxide synthase (e-NOS) in Nb2a cells. e-NOS expression was highest in group 2 and lowest in group 5 in damaged groups. Arrows indicate positive cells (bar = 20 μm).

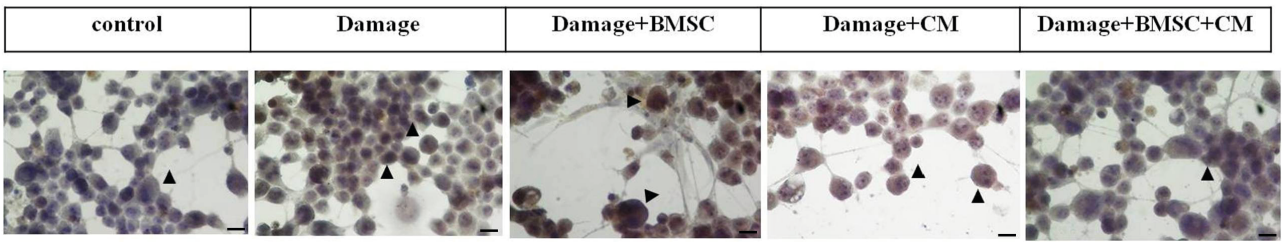

Figure 4. Immunocytochemical staining of anti-inducible nitric oxide synthase (i-NOS) in Nb2a cells. i-NOS expression was highest in group 2, and lowest in group 5. Arrows indicate positive cells (bar = 20 μm).

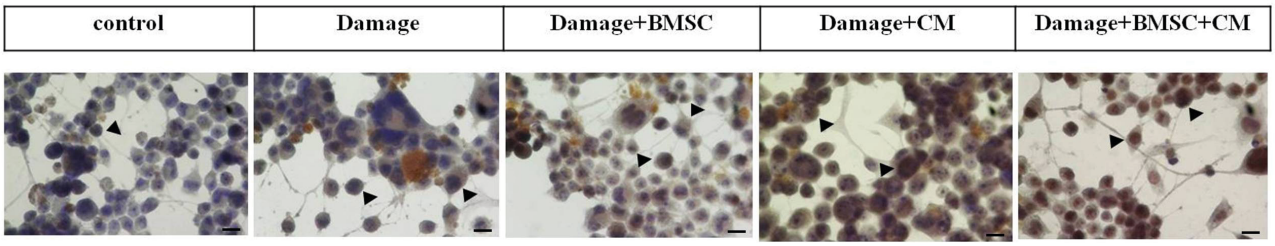

Figure 5. Immunocytochemical staining of TGFβ1 in Nb2a cells. The strongest expression of TGFβ1 was seen in group 5. Arrows indicate positive cells (bar = 20 μm).

Biotechnology) and c-kit (Santa Cruz Biotechnology) were also used. Following incubation, cells were washed in PBS and treated with an anti-mouse biotin–streptavidin horseradish peroxidase secondary antibody (Zymed, San Francisco, CA, USA). Cells were then incubated with diaminobenzidine (DAB; Invitrogen) for 5 min at room temperature and counterstained with Mayer's hematoxylin (ScyTek Laboratories, Logan, UT, USA). After washing in distilled water, cells were covered with mounting medium and staining was evaluated under an Olympus BX40 light microscope by an observer blinded to the treatment conditions.

2.6. TUNEL assay

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-biotin nick end-labeling (TUNEL) using the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI, USA) according to the manufacturer's instructions (Figure 6). After application, cells were fixed in 4% paraformaldehyde for 30 min and rinsed three times in PBS for 5 min. Then cells were then incubated with 20 μg/mL Proteinase K for 10 min and washed three times in PBS for 5 min. To inhibit endogenous activity, cells were treated with 3% hydrogen peroxide and rinsed in PBS. Next, cells were treated with equilibration buffer for 5 min and then incubated with Tdt-enzyme for 60 min at 37 °C. Following Tdt-enzyme treatment, cells were treated with 2× SCC solution for 15 min and then washed three times in PBS for 5 min. Streptavidin–peroxidase treatment was performed for 45 min, after which cells were rinsed in PBS and incubated with DAB; Mayer's hematoxylin was used for counterstaining. Cells were rinsed in distilled water and mounted using mounting medium. TUNEL-positive staining was assessed by a blinded observer under an Olympus BX40 light microscope.

2.7. Statistical analysis

During the evaluation of results, the percentage and intensity of the immunostaining were obtained with H-scoring and determined as the ratio of positive labeled cells to all cells in the chosen fields. Moreover, apoptotic index was determined as the ratio of positive labeled cells to all cells in chosen fields. However, in the graph, grading was used to demonstrate the relationship of oxidative stress and apoptosis for both immunochemical intensity and TUNEL, which was done previously in the literature (23). Immunohistochemical intensity was scored by a blinded observer as 0: no staining, 1: weak staining, 2: moderate staining, 3: moderate–strong staining, 4: strong staining, and 5: very strong staining. For TUNEL staining, each section was counted for 100 TUNEL-positive cells from randomly chosen fields. The percentage of apoptotic cells was calculated by a blinded observer as 0: no apoptosis, 1: 1%–10% apoptosis, 2: 11%–25% apoptosis, 3: 26%–50% apoptosis, 4: 51%–75% apoptosis, and 5: more than 75% apoptosis. The results were calculated on GraphPad (GraphPad Software, San Diego, CA, USA) using one-way ANOVA and presented as the mean ± SD. Statistical significance was defined as $P \leq 0.05$ (23).

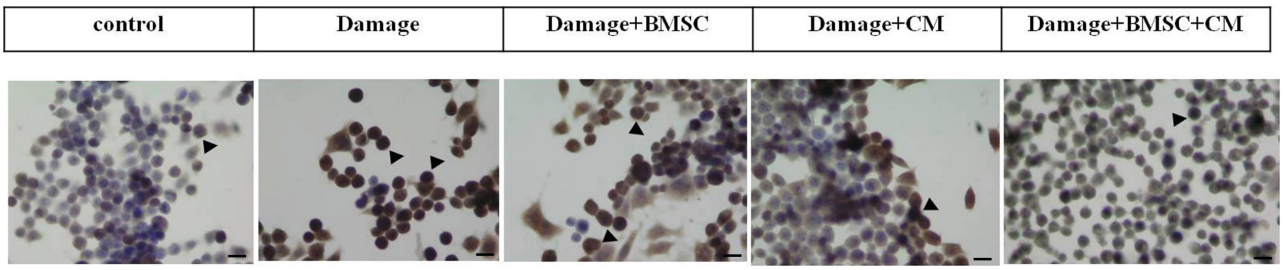

Figure 6. Terminal deoxynucleotidyl transferase-biotin nick end-labeling (TUNEL) staining. Apoptotic cells were most abundant in group 2; the fewest apoptotic cells were seen in group 5. Arrows indicate apoptotic cells (bar = 20 μm).

3. Results

In vitro expression of i-NOS and e-NOS, along with the induction of apoptosis, was used to assess the neuroprotective effects of BMSCs and CM. Following differentiation, cells were divided into five groups: 1) healthy controls, 2) damaged cells alone, 3) damaged cells treated with BMSCs, 4) damaged cells treated with CM, and 5) damaged cells treated with both BMSCs and CM. Both BMSCs and CM were shown to positively affect healing, with significant differences observed between healthy controls and damaged cells ($P < 0.001$), as well as between damaged cells and those treated with BMSCs alone ($P < 0.05$), CM alone ($P < 0.05$), and both BMSCs and CM in combination ($P < 0.01$). Among the treated groups, the strongest neuroprotective effects were seen among cells treated with both BMSCs and CM; however, no significant differences were observed between healthy controls and treated cells. Together, these data indicate that the combined application of BMSCs and CM exerts a stronger neuroprotective effect than either BMSCs or CM alone (Figure 7).

To better understand the mechanisms underlying this apparent neuroprotective effect, cells were stained to determine the production of e-NOS, i-NOS, and TGFβ1. For e-NOS, significant differences were seen between damaged cells and those treated with BMSCs alone ($P < 0.05$), CM alone ($P < 0.05$), or BMSCs and CM in combination ($P < 0.01$), with the lowest overall staining seen in the group treated with both BMSCs and CM. e-NOS staining was higher in cells treated with CM alone relative to those treated with BMSCs alone (Figure 7). No significant differences were seen among any of the treatment groups.

For i-NOS, we observed significant differences between damaged cells and those treated with BMSCs alone ($P < 0.01$), CM alone ($P < 0.05$), or BMSCs and CM in combination ($P < 0.001$). As with e-NOS staining, i-NOS was lowest in the group treated with both BMSCs and CM, with cells treated with CM alone exhibiting higher staining relative to those treated with BMSCs alone (Figure 7). No significant differences were seen among any of the treatment groups.

For TGFβ1 staining, significant differences were seen between damaged cells and those treated with BMSCs alone ($P < 0.01$), and BMSCs and CM in combination ($P < 0.01$); the difference between CM alone and BMSCs in combination with CM was also significant ($P < 0.01$), supporting the notion that BMSCs secrete TGFβ in response to injury (Figure 7).

Finally, we used TUNEL to evaluate apoptosis to clarify the relationship between oxidative stress and programmed cell death. Treatment with both BMSCs and CM, either alone or in combination, decreased the rate of apoptosis relative to untreated controls.

Significant differences were seen between damaged cells and those treated with BMSCs alone ($P < 0.01$), CM alone ($P < 0.05$), or BMSCs and CM in combination ($P < 0.001$). TUNEL staining was lowest among cells treated with both BMSCs and CM (Figure 7). No significant differences were seen between any of the treatment groups.

Taken together, the data presented here demonstrated clear neuroprotective effects for both BMSCs and CM in injured neuroblastoma cells. While BMSCs were more effective than CM alone, the strongest effects were seen in cells treated with a combination of BMSCs and CM, indicating an additive effect between treatments.

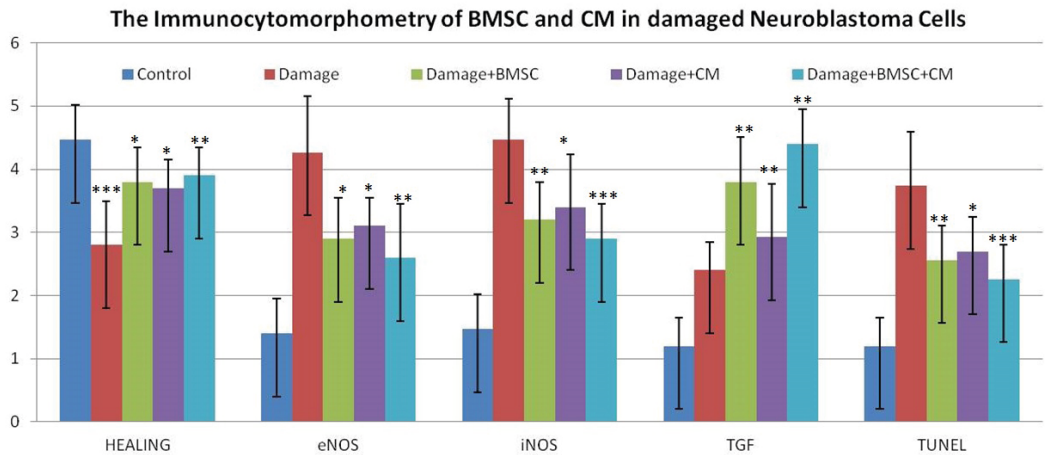

Figure 7. Immunocytomorphometry of bone marrow-derived mesenchymal stem cells (BMSCs) and conditioned medium (CM) in damaged neuroblastoma cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4. Discussion

Transplantation of MSCs offers a novel and exciting prospect for the repair of injured tissues, although the exact mechanism of MSC activity remains unclear. Potential mechanisms include neuroprotection, the replacement of damaged cells, or the creation of an environment that enables the regeneration of endogenous cells (9). MSCs have been shown to differentiate into neural cells and astrocytes both in vitro and in vivo, exhibiting the ability to differentiate into astrocyte-like cells when transplanted into the normal or ischemic brain (24). However, despite these apparent abilities, the prevailing belief regarding the neuroprotective effects of MSCs is that of paracrine factors, which are secreted by these cells in response to damage (4,5,7). MSCs are known to secrete several growth factors, including brain-derived neurotrophic factor, vascular endothelial growth factor, neural growth factor, glia cell line-derived neurotrophic factor, and insulin-like growth factor, which play major roles in neuroprotection (5,25). One of these factors, TGFβ-1, is produced by BMSCs in the bone matrix niche, exerting significant effects on cell proliferation, survival, and apoptosis, depending on the cellular context (26). Here, we examined culture conditions in vitro and demonstrated that BMSCs exert neuroprotective effects in damaged neuroblastoma cells, consistent with the existing data. In addition, TGFβ1 staining was more prominent in cells treated with BMSCs than in those treated with CM alone, indicative of robust TGFβ production by BMSCs.

One of the questions yet to be answered is the extent to which CM affects tissue regeneration. Previous studies have shown that CM includes extracellular matrix (ECM) compounds providing an architectural framework that affects MSC differentiation and lineage specification. The ECM contains a variety of compounds including proteoglycans, heparan sulfate, fibroblast growth factors, Wnts, and fibronectin, all of which play important roles in the differentiation of MSCs into neural lineages. In addition, the ECM plays a role in mechanical signaling, cell differentiation, and intracellular signaling (1,27). We determined that TGFβ1 staining was higher in the CM alone treated group than in healthy and untreated damaged groups, which indicates that CM includes TGFβ1. Although we did not determine all content of our CM, this neuroprotective effect may be due to the combined action of several factors such as vascular endothelial growth factor, osteopontin, matrix metalloproteinase-13 (MMP-13), fibroblast growth factor-binding protein (FGF-BP), and TGFβ1, determined in CM in previous studies (17,18)

Endogenous MSCs are able to migrate to injured tissue and participate in healing. The concentrated CM derived from MSCs can modulate wound repair in the absence of MSCs, indicative of mechanisms that are likely independent, but not mutually exclusive (28). Cantinieaux et al. reported that BMSC-CM administered after spinal cord contusion improves motor recovery in rats (18). Moreover, Liang et al. showed that neural stem cell conditioned medium promoted locomotor recovery in adult rats after spinal cord injury (29). In addition, Torrente et al. reported that paracrine factors of human mesenchymal stem cells are a useful neuroprotective approach for brain recovery following injury (30). Similar to the literature, our study demonstrated that CM has been shown to make a significant contribution to the improvement in the neuroblastoma trauma model in the absence of BMSCs.

In vitro, BMSC-derived CM protects neurons from apoptosis, activates macrophages, and promotes angiogenesis (18). Rats treated with CM isolated from both normoxic- and hypoxic-preconditioned MSCs performed significantly better than controls in terms of both motor and cognitive function in a rat model of traumatic brain injury (13). CM derived from human adipose stem cells also induced significant functional and structural recovery after stroke as a consequence of enhanced neovascularization and reduced neural cell apoptosis (17). Similar activities were also seen with CM derived from other cell types, with effects on neural cell death and damage occurring in a dose-dependent manner (31). We determined that oxidative stress and apoptosis were lower in all treatment groups relative to controls, indicating that BMSCs and CM minimize apoptosis by reducing oxidative stress. Overall, oxidative stress and apoptosis were lowest in the BMSC–CM co-treatment group, with BMSCs alone exhibiting greater protective effects than CM alone. Together, these results suggest an additive, or potentially synergistic, effect between CM and BMSCs on cell repair.

4.1. Importance of this study

Stem cells are a major topic of research for the treatment of spinal cord and traumatic brain injuries. Recent studies have suggested positive effects of CM on neural cell injury in vitro, but the scope of the data remains limited. Here, we showed that a combination of BMSCs and CM was the most effective method for repairing injured neuroblastoma cells, suggesting potential therapeutic uses for BMSCs and CM in cases of neurological damage.

4.2. Conclusion

BMSCs and CM exert significant neuroprotective effects through oxidative stress and apoptosis in damaged neuroblastoma cells in vitro; co-treatment with BMSCs and CM increases this effect. This combination treatment may represent a novel therapeutic option for the treatment of traumatic neurological diseases; however, further studies will be needed to identify conditions under which this combination therapy would be most effective in clinical applications.

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