G-CSF augments the neuroprotective effect of conditioned medium of dental pulp stem cells against hypoxic neural injury in SH-SY5Y cells

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OBJECTIVES: Dental pulp stem cells (DPSCs) can differentiate into functional neurons and have the potential for cell therapy in neurological diseases. Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein family shown neuroprotective effect in models of nerve damage.

Dental pulp stem cells (DPSCs) can differentiate into functional neurons and have the potential for cell therapy in neurological diseases. Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein family shown neuroprotective effect in models of nerve damage. We evaluated the protective effects of G-CSF, conditioned media from DPSCs (DPSCs-CM) and conditioned media from transfected DPSCs with plasmid encoding G-CSF (DPSC-CMT) on SH-SY5Y exposed to CoCl2, as a model of hypoxia-induced neural damage.

MATERIALS AND METHODS: SH-SY5Y exposed to CoCl2 were treated with DPSCs-CM, G-CSF, simultaneous combination of DPSCs-CM and G-CSF and finally DPSC-CMT. Cell viability and apoptosis were determined by resazurin (or lactate dehydrogenase (LDH) assay alternatively) and propidium iodide (PI) staining. Western blot analysis was performed to detect changes in apoptotic protein levels. The interleukin-6 and interleukin-10/IL6/IL10 levels were measured with Enzyme-Linked Immunosorbent Assay (ELISA).

RESULTS: DPSCs-CM and G-CSF were able to significantly protect SH-SY5Y against neural cell damage caused by CoCl2, according to resazurin and LDH analysis. Also, the percentage of apoptotic cells decreased when SH-SY5Y were treated with DPSCs-CM and G-CSF simultaneously. After transfection of DPSCs with G-CSF plasmid, DPSC-CMT could significantly improve the protection. The amount of β-catenin, cleaved PARP and caspase-3 were significantly decreased and the expression of survivin decreased when SH-SY5Y were treated with DPSCs-CM and G-CSF according to Western blot. Decreased level of IL-6/IL10, which exposed to CoCl2 after treatment with DPSCs-CM indicated the suppression of inflammatory mediators.

CONCLUSION: Combination therapy of G-CSF and DPSCs-CM improved the protective activity.

Introduction

Migration of MSCs to injured sites and differentiation to mature cells with functional activity is a process of tissue repair. Modulating the immune function is an important effect of MSCs besides the ability to promote cell growth, stimulate proliferation, inhibit cell death and development of pro-angiogenic factors. All modalities via paracrine influence or direct cell to cell contact made them as an appropriate tool to recuperate the injuries to various tissues (1).

HDPSCs (Human dental pulp stem cells) are ectomesenchymal derived stem cells, originating from migrating neural crest cells (2). Furthermore, DPSCs express nestin and βIII-tubulin as neuronal lineage markers that support the notion that these cells may be capable to differentiate into neural cells (3). DPSCs also express immunomodulatory factors that stimulate the formation of blood vessels and augment the regeneration and repair of injured nerves. Hence DPSCs are a suitable candidate as a source of replacement cells for injured neuronal cells (4).

The mechanism of action after DPSCs transplantation is not fully understood but it is likely to be a paracrine-mediated mechanism, with the secretion of neurotrophic factors coordinated for neuronal survival and axonal regeneration (2).

Treatment with neurogenic factors such as epidermal growth factor (EGF), retinoic acid and fibroblast growth factor (FGF) are effective in the differentiation of DPSCs into neural cells (5).

DPSCs show a higher proliferative rate, higher number of stem/progenitor cells in the population and a greater clonogenic potential compared to BM-MSCs (Bone marrow-mesenchymal stem cells) (6). In addition, they exhibit superior neuroprotective effects in neurological injuries and pathologies as compared with BM-MSCs and ADSCs (Adipose stem cells). This superiority might be related to higher expression of neurotrophic factors (7).
factors including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell-line derived neurotrophic factor (GDNF), nerve growth factor (NGF), platelet-derived neurotrophic factor (PDGF) and vascular endothelial growth factor (VEGF) (2).

DPSCs secretum has been widely used in researches as a cell-free therapeutic tool that decreases the risks of immune reactions and the development of ectopic tissue which are associated with the engraftment of stem cells (7). The DPSCs secretum has high concentrations of fms-related tyrosine kinase 3 (FLT-3), monocyte chemoattractant protein 1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Compare to BM-MSCs and ADSCs, the DPSCs secretum show higher angiogenic and neurogenic potentials in ectopic transplantation models and exhibit the highest migration capacity. DPSCs secretum also mediate stronger anti-apoptotic effects in a microenvironment challenged by oxidative and serum deprivation (8).

Due to these properties, DPSCs have been considered as a potential source for cell-based therapy for neural diseases such as Parkinson’s disease (2), Alzheimer’s disease (9), amyotrophic lateral sclerosis (10) and stroke (8).

Granulocyte colony-stimulating factor (G-CSF) is a 19.6-kDa glycoprotein that belongs to the cytokine family of growth factors (11). It is clinically applied for treating neutropenia in humans (12). Several studies have been demonstrated the neuroprotective effect of G-CSF in a variety of experimental brain injury models (13, 14). For example, administration of G-CSF in rats model of focal cerebral ischemia improved the survival rate and neurological behavior, also lowered the infarction volume compared to the vehicle group (15).

Neuroprotective effect of G-CSF in a model of Parkinson’s disease in male mice demonstrated by reduction of striatal dopamine depletion after MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine) application (16).

It was found that the conditioned medium (CM) of DPSCs contains a wide range of bioactive secreted factors (17) so, in this study, we evaluated the neuroprotective effects of simultaneous application of DPSCs-CM and G-CSF on SH-SY5Y cells exposed to CoCl$_2$ as an appropriate model of hypoxic neural injury.

Furthermore, G-CSF gene transfection to DPSCs was used to improve the problem of short half-life of G-CSF, thereby the cellular carrier which provided longer access to G-CSF was designed and prepared and finally the protective effect of conditioned medium derived from transfected cells (DPSC-CM$_2$) was investigated. To the best of our knowledge, so far, no combination therapy using DPSC-CM$_2$ and G-CSF has been done in any study.

Materials and Methods

Materials

Collagenase type I, dispase type II, fetal bovine serum (FBS), penicillin/streptomycin, trypsin and L-glutamine were purchased from Gibco, USA. Plasmid pAdTrack-CMV was prepared as a gift from Dr. Bert Vogelstein, National Cancer Institute, Maryland, USA. Escherichia coli XL1-Blue was transformed with the plasmid and amplified in Escherichia coli strain XL1-Blue and then extracted by a Plasmid Mega Kit (Qiagen, Germany). Ascorbic acid 2-phosphate, dexamethasone, glycerol 2-phosphate, resazurin and alpha modification of Eagle’s medium (α-MEM) were obtained from Sigma-Aldrich, Oakville, ON, Canada. Oil Red O and Alizarin Red S were purchased from Merck KGaA, Germany. Monoclonal antibodies were taken from Cell Signaling, USA. IL-6 (CN: KPG-HI6P) and IL-10 (CN: KPG-HI10P) ELISA kits were purchased from Karmania Pars Gene. Lipofectamine 3000 reagent was obtained from ThermoFisher USA Invitrogen.

Cell culture and treatments

Isolation and expansion of dental pulp stem cells

Pulp of third molar teeth were obtained from 18-29 years adults due to orthodontic surgery at the Dental Institute of the Institute of Dental and Craniofacial Regeneration of Mashhad University of Medical Sciences for experimental and clinical purposes. Isolation and expansion of dental pulp stem cells was carried out using the protocol previously described (18). Briefly, dental pulps were cut into 2-3 mm fragments and enzymatically digested using 3 mg/ml of collagenase type I and 4 mg/ml of dispase type II (Gibco, USA) solution for 45 minutes at 37°C. Following centrifugation at 500 × g for 5 min, the cell pellet was suspended and seeded in a culture flask with α-modification of Eagle’s medium (α-MEM; Sigma, Germany) supplemented with 20% fetal bovine serum (FBS; Gibco, USA) and the final concentration of 1% penicillin/streptomycin (Gibco, USA) and 0.01% amphotericin B (Simintak, Iran). The culture flask was cultivated at 37°C in 5% CO$_2$. The medium was changed every three days until stem cells reached 70% confluence (19). No specific blinding or randomization was used in the current study.

Hypoxia induction in SH-SY5Y cells by treating with CoCl$_2$

SH-SY5Y cells were cultivated in α-MEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin with the density of 1×10$^4$ cells/well at 37°C and 5% CO$_2$ humidified atmosphere. Hypoxia was induced in SH-SY5Y cells by treating with CoCl$_2$ at the concentration of 0.6 and 1.2 mM and different times of exposure (6, 12, 24 and 48 hr) to determine the optimum treating situation for subsequent experiments (20). The cell viability was measured by resazurin (7-Hydroxy-3H-phenoaxazin-3-one 10-oxide) assay. In brief, 20 µl of resazurin (0.15 mg/ml) (Sigma, Germany) was added to each well and incubated at 37°C for 4 h. The absorbance was measured at 600 nm via Synergy H4 Multi-Mode microplate reader (BioTek, Winooski, USA).

Flow cytometry analysis for surface markers

DPSCs at passage 3 were used to determine the human surface markers associated with mesenchymal and hematopoietic lineages using the following monoclonal antibodies (mAbs); mouse anti human CD45 FITC, mouse anti human CD34 FITC, mouse anti human CD29 FITC and mouse anti human CD44 FITC. Flow cytometry was performed using BD Calibur flow cytometer (Germany) and data were analyzed by Flowjo version 7.6 software (Flowjo LLC, USA).

Preparation of DPSCs conditioned medium

To prepare the conditioned medium, DPSCs at 3×10$^4$ passages with the density of 1×10$^4$ cells/plate were incubated in serum-free α-MEM for 48 h in 37°C and 5%
Neuroprotective Effect of DPSC and G-CSF in SH-SY5Y Cells

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Protective effects of DPSCs-CM and G-CSF on SH-SY5Y cells in hypoxic condition

SH-SY5Y cells at the density of 1×10^4 cells/well were incubated with CoCl_2 (0.6 and 1.2 mM). DPSCs-CM and DPSC-CM plus G-CSF at concentration of 10, 100 and 500 ng/ml were added to SH-SY5Y cells and incubated at 37°C for 6, 12, 24 and 48 h. Assessment of cell viability was performed by resazurin (Sigma, Germany) analysis.

IL-6 and IL-10 release assessment

IL-6 and IL-10 protein levels in culture supernatants were measured using commercial ELISA kits (Karmania Pars Gene). Standard solutions were prepared according to manufacturer protocol and 50 µL of each standard and supernatants from DPSCs were added to wells coated with IL-6 or IL-10 separately. Plates were incubated with 200 µL of blocking solution for 2 h at 37°C. After that, 50 µL of detection antibody were added to each well and incubated at 200 rpm for 1 h at 37°C. Plates were washed 3 times and incubated with Avidin-HRP at 200 rpm for 1 h at 37°C, followed by the addition of 25 µL of stop solution. The absorbance was measured at 450 nm via Synergy H4 Multi-Mode microplate reader (BioTek, Winooski, USA).

Flow cytometry analysis for the cell apoptosis

1×10^4 SH-SY5Y cells/well were treated with DPSC-CM, DPSC-CM plus G-CSF (10, 500 ng/ml) and exposed to 0.6 mM of CoCl_2 for 24 h. Then cells were harvested and washed twice with phosphate-buffered saline (PBS) and centrifuged at 500 g for 5 min. 300 µL of the PI solution was added to each tube and incubated for 30 min at 37°C and finally analysed with a flow cytometer.

Statistical analysis

The data was reported as the mean ± SD of three independent experiments each in triplicate. The data were analyzed using Graph Pad Prism software, version 6.0.

Cell viability was analyzed using two-way analysis of variance (ANOVA) followed by Tukey as post-test. One-way ANOVA and Tukey’s multiple comparisons test was used to assess the significant differences between the experimental groups in other tests. P value <0.05 was considered to indicate a statistically significant difference.

Ethics approval

The protocol for this work (No. 970850 and 960882) and reference number (IR.MUMS.sp.REC.1396.202_October14,2020) have been approved by Mashhad University of Medical Sciences.

Results

Hypoxia induction and investigation of the protective effect of DPSCs-CM and DPSCs-CM plus G-CSF

CoCl_2 at the concentration of 0.6 and 1.2 mM significantly (P<0.001) reduced the cell viability of SH-SY5Y after 6, 12, 24 and 48 h exposure [F (3, 48) = 1422] (Figure 1). A significant enhancement in the viability of cells exposed to hypoxia was seen when cells were treated with DPSCs-CM (P<0.001) (Figure 1).

In the next step, the results of the viability assay confirmed the neuroprotective effect of DPSCs-CM and DPSCs-CM plus G-CSF on SH-SY5Y cells against the
In hypoxic condition, DPSCs-CM plus G-CSF (10, 100 and 500 ng/ml) significantly reduced the cellular damage in SH-SY5Y cells exposed to CoCl$_2$ [F (4, 10) = 118.7, P < 0.001] (0.6 mM at 24 hr) determined by viability assay (P = 0.0244, 0.0030 and 0.0002 respectively) (Figure 2). DPSCs-CM plus G-CSF had superior protective activity in comparison with G-CSF or DPSCs-CM alone [F (4, 10) = 88.07, P < 0.001] (P values for G-CSF10, 100 and 500 vs. CM:G-CSF10, 100 and 500 were 0.0083; 0.0024 and <0.001, respectively).

Assessment of IL-6 and IL-10 release

Levels of Interleukin 6 and 10 were evaluated as important factors involved in neural protection. The results of ELISA test showed that the levels of IL-6 [F (3, 8) = 1182, P < 0.001] and IL-10 [F (3, 8) = 637.8, P < 0.001] in cells exposed to 0.6 mM of CoCl$_2$ for 24 hr were increased compared to control and significantly decreased when cells were treated with DPSCs-CM compared to the hypoxia group (P < 0.001) (Figure 3).

Analysis of survivin, cleaved PARP, β-catenin and cleaved caspase-3 proteins by western blot

Western blotting was performed to detect changes in apoptotic protein levels such as β-catenin, cleaved PARP, cleaved caspase-3 and survivin. As shown in Figure 4, the expression of survivin significantly increased in cells treated with DPSCs-CM, G-CSF 500 and G-CSF 500 plus DPSCs-CM [F (4, 10) = 99.13, P < 0.001] (P < 0.001) compared to cells treated with 0.6 mM of CoCl$_2$. Whereas the amount of β-catenin [F (4, 10) = 64.82, P < 0.001], cleaved caspase-3 [F (4, 10) = 97.36, P < 0.001], and cleaved PARP [F (4, 10) = 79.30, P < 0.001] were significantly decreased in cells treated with DPSCs-CM, G-CSF 500 and DPSCs-CM plus G-CSF 500 (P < 0.001) compared to cells treated with 0.6 mM of CoCl$_2$. DPSCs-CM plus G-CSF 500 had superior protective activity in comparison with G-CSF 500 alone for β-catenin (P < 0.001) and caspase 3 (P < 0.001).

Transfection experiment and evaluation of LDH release from SH-SY5Y cells

To design a cellular carrier which could express G-CSF in addition to conditioned media, we transferred the plasmid encoding G-CSF-GFP to DPSCs. The expression of this plasmid was investigated using fluorescent microscopy. As indicated in Figure 5a, 48 hr after transfection, gene expression increased in DPSCs cells. Afterwards, results of the LDH assay from SH-SY5Y cells exposed to CoCl$_2$ confirmed the neuroprotective effect of DPSCs-CM, DPSCs-CM and DPSCs-CM plus G-CSF on SH-SY5Y cells against the hypoxic condition. DPSCs-CM, DPSCs-CM and DPSCs-CM plus G-CSF significantly reduced the cellular damage in SH-SY5Y cells exposed to CoCl$_2$ (0.6 mM at 24 hr) determined by LDH assay [F (5, 12) = 755.5, P < 0.001] (Figure 5b). DPSCs-CM had superior protective activity in comparison with DPSCs-

Figure 1. Effect of CoCl$_2$ on SH-SY5Y cells viability and DPSCs-CM on SH-SY5Y exposed to CoCl$_2$. 1×10$^4$ cells/well of SH-SY5Y cells were exposed to 0.6 and 1.2 mM concentration of CoCl$_2$ for 6, 12, 24 and 48 hr. The cell viability was assessed by resazurin assay. The value presented as mean ± SD (n=9). ***P<0.001

DPSCs-CM: Dental pulp stem cells- conditioned medium

Figure 2. Protective effect of G-CSF and DPSCs-CM on SH-SY5Y exposed to CoCl$_2$. 1×10$^4$ cells/well were exposed to hypoxic condition by 0.6 mM of CoCl$_2$ for 24 hr. Cells were treated with DPSCs-CM and different concentration of G-CSF (10,100,500 ng/ml) for 24 hr. Value presented as mean±SD (n=9), ***P<0.001 in comparison with CoCl$_2$.

G-CSF: Granulocyte macrophage-colony stimulating factor; DPSCs-CM: Dental pulp stem cells- conditioned medium

Figure 3. Effect of CoCl$_2$ on secretion of IL-6 and IL-10 in SH-SY5Y cells. The value presented as mean ± SD (n=9), ***P<0.001 in comparison with CoCl$_2$.
Anti-apoptotic effect of DPSCs-CM and G-CSF on SH-SY5Y cells exposed to CoCl$_2$ by Flow cytometry method

To investigate the protective effect against apoptosis caused by CoCl$_2$, propidium iodide was used. Flow cytometry analysis of SH-SY5Y cells stained with PI revealed that exposure to CoCl$_2$ (0.6 mM) for 24 hr increased apoptotic cells as compared to control (Figure 5c, Figure 6). DPSCs-CM, DPSCs-CM$_T$ and DPSCs-CM plus G-CSF (500 ng/ml) significantly decreased the apoptotic cells in SH-SY5Y cells exposed to CoCl$_2$ ($P<0.001$) compared to hypoxic condition. DPSCs-CM$_T$ had superior protective activity in comparison with DPSCs-CM or DPSCs-CM plus G-CSF.

**Discussion**

In the present study, we showed that combination therapy of G-CSF and DPSCs-CM could significantly improve the protective activity against neural cell damage caused by CoCl$_2$. After that, the cellular carrier was designed with transfection of plasmid encoding G-CSF to provide conditioned media containing G-CSF. The results showed that DPSC-CM$_T$ caused considerable protection against hypoxia.

Cell therapy using MSCs has emerged as a novel and promising neuroprotective strategy in neural diseases such as ischemic stroke, Alzheimer and Parkinson’s diseases. Accumulating data have suggested DPSCs as an important candidate for cell therapy. DPSCs may enhance neuronal survival and neurite outgrowth via a paracrine mechanism. In addition, evidence indicated
that DPSCs play an important role in neurogenesis, neural maintenance and repair due to the secretion of several neurotrophic factors (23). Studies have been shown that DPSCs can promote neuroprotection after nerve injury (2, 24).

In a study, the secretum obtained from DPSCs was able to reduce the cytotoxicity and apoptosis caused by the amyloid-beta peptide in Alzheimer’s disease (9). Administration of DPSCs during acute ischemic stroke provided neuroprotective effects by modulating inflammation and blood-brain barrier permeability in post-ischemia/reperfusion brain injury in rats (25). Intracerebral transplantation of DPSCs into ischemic regions of the brain significantly improves the forelimb sensory-motor function in a rodent model after 4 weeks post-treatment (26).

SH-SY5Y is a thrice cloned subline of SK-N-SH cells which were originally obtained from a bone marrow biopsy of a neuroblastoma patient in the early 1970s (27). In this study, the cytotoxicity of CoCl₂ on SH-SY5Y cells was demonstrated at the concentration of 0.6 and 1.2 mM at 6, 12, 24 and 48 hr (20). The culture of SH-SY5Y cells with DPSCs-CM indicated the neuroprotective effect of DPSCs-CM by increasing the cell viability. Similar to our study, the therapeutic potency of MSCs-CM has been explored in an experimental model of spinal cord injury (28), brain injury (29), bone defects (30), and ischemic heart disease (31).

Several studies have been reported that hematopoietic cytokines like granulocyte macrophage-colony stimulating factor (GM-CSF), G-CSF, or erythropoietin had neuroprotective effects and has an important role in recovering the neurologic functions after central nervous system injury (32).

G-CSF is a hematopoietic growth factor that plays important role in hematopoiesis (33). Number of different cell types such as endothelium, macrophages and some immune cells produce G-CSF and stimulate the bone marrow to release stem cells and granulocytes. The receptor of G-CSF is expressed on precursor cells in the bone marrow which when activated, initiates proliferation and differentiation into mature granulocytes. It has also been shown that neurons in the brain and spinal cord express G-CSF receptor and their activation by G-CSF induce neurogenesis, increases neuroplasticity and reduces apoptosis (34). Moreover, activation of G-CSF receptor resulted in stimulating survival, proliferation and development of neuronal stem cells. Usage of G-CSF as a therapy for ischemic stroke was conducted in a phase I/II clinical trial. Patients who received G-CSF showed greater improvement in neurologic functioning between baseline and 12-month follow-up than in the control group (35).

Multiple studies have indicated the neuroprotective effect of G-CSF in a variety of in vivo brain injury models (36). It was reported that treatment with G-CSF promotes somatic growth, prevented brain atrophy and improved long-term neurological outcome in the neonatal hypoxic-ischemia model (37). G-CSF administration after middle cerebral artery occlusion revealed a significant reduction in the amount of edematous tissue, brain water content and cortical lesion volume (38).

Co-administration of G-CSF with BM-MSCs produced synergistic beneficial effects by promoting cell proliferation and differentiation of bone marrow stem cells led to early neuronal development, reduction the cerebral infarct size and improved the brain regeneration and functional recovery in an experimental mouse model of cerebral ischemia (39).

The effect of combinational therapy of G-CSF and BM-MSCs was evaluated in Sprague-Dawley rats after stroke (40). Although, the combination therapy produced remarkable neurogenesis in the formerly infarct area and beyond in the islet of regeneration, it wasn’t more efficient in reducing the mortality rate and improving the post-stroke recovery than G-CSF treatment alone (40). Adjunct treatment of G-CSF with hUCB-MSC (human umbilical cord blood-derived mesenchymal stem cells) in traumatic brain injury (TBI) in adult rats indicated synergetic effect in functional improvement in TBI rats than that exerted by monotherapy with hUCB or G-CSF (41).

In this study, we transfected DPSCs with plasmid encoding G-CSF-GFP. It was shown that 48 h after transfection, the expression of G-CSF increased in DPSCs. Moreover, DPSCs-CM improved the protective effect which caused a decrease in apoptotic cells and cellular damage induced by CoCl₂. Western blotting was used to detect changes in apoptotic protein levels such as β-catenin, cleaved PARP, cleaved caspase-3 and survivin.

The Wnt signaling pathway has a role in regulating diverse cellular processes, including cell proliferation and cell death. Following the activation of Wnt/β-catenin pathway, cytoplasmic β-catenin becomes stabilized, enters the nucleus and interacts with transcription factors, notably TCF/LEF to regulate the transcription of target genes (42). Wnt/β-catenin pathway activation contributes to functional recovery and induces neuroprotective processes and neurogenesis after focal cerebral ischemia (43). Also, it was shown that the inactivation of β-catenin by small interfering RNA increased the ischemia-induced infarct volume in rats (44).

Hypoxia may induce the activation of the Wnt signaling pathway in the adult brain. Dysfunction of the Wnt/β-catenin signaling pathway has been linked to neurodegenerative disorders such as schizophrenia, autism and Alzheimer’s disease (45, 46). Overexpression of β-catenin in NIH-3T3 cells and human H1299 cells mimics the induction by apoptotic stimuli of transcriptionally active p53 (47). Destabilization of β-catenin through missense mutation of presenilin-1, which is the most commonly mutated gene in familial Alzheimer patients resulted in inducing neuronal apoptosis (48).

The wnt signaling pathway regulates apoptosis through a variety of mechanisms including those through SFRP2 (secreted Frizzled-related protein-2) gene expression, wnt-BMP signaling, GSK 3-β-NF-κBeta, β-catenin, c-Jun N-terminal kinase signaling, or gene expression of Dickkopf-1, nemo, sox 10 and tau (49).

PARP-1 (poly (ADP-ribose) polymerase-1) is a nuclear mediator that performs central roles in the repair of damaged DNA. In addition, PARP-1 plays important roles in vasocostriction, transcription, cardiac remodeling, regulation of astrocyte and microglial function, aging and long term memory (50). Activation of caspase-mediated
cell death through the cleavage and activating effector which drive the process of apoptosis. Cleavage of PARP-1 by caspases is considered to be a hallmark of apoptosis and has been implicated in several neurological diseases like Alzheimer’s disease, cerebral ischemia, multiple sclerosis, traumatic brain injury, Parkinson’s disease, NMDA-mediated cytotoxicity and brain tumors (51).

Survivin is a unique member of the inhibitor of the apoptosis gene family. The consequence of the interaction of caspases-3 with survivin is the inhibition of apoptosis (52).

A decrease in the amount of β-catenin also inhibiting the cleavage of caspase-3 and PARP established the neuroprotective effect of DPSCs-CM and G-CSF on SH-SY5Y cells exposed to the hypoxic condition. On the other side, increased levels of survivin, imply the positive effect of DPSCs-CM and G-CSF on neuroprotection and inhibition of apoptosis.

IL-6 is a pleiotropic and multifunctional cytokine that plays an important role in cell proliferation, differentiation, survival and apoptosis (53). IL-6 maintains the homeostasis in the brain by directing neurogenesis, astrogliosis, microgliosis and controlling blood-brain barrier integrity (54). The role of IL-6 in the injured brain is controversial. In one study, it was reported that an increased level of IL-6 may lead to exacerbation of cerebral ischemic damage by increasing harmful mediators and mediating inflammatory cascades (55). On the other hand, Swartz et al., reported that IL-6 promotes post-traumatic healing in the nervous system by increasing angiogenesis (56).

The neuroprotective effect of MSCs in hypoxic-ischemic brain damage (HIBD) rats was demonstrated to be mediated by endogenous IL-6. It performed its anti-apoptotic role via the IL-6/STAT3 signaling pathway (56).

The neuroprotective effect of MSCs in hypoxic-ischemic brain damage (HIBD) rats was demonstrated to be mediated by endogenous IL-6. It performed its anti-apoptotic role via the IL-6/STAT3 signaling pathway (56).

Figure 6. Schematic representation of protective mechanism of DPSCs-CM and G-CSF in SH-SY5Y cells exposed to hypoxic condition. DPSCs-CM: Dental pulp stem cells-conditioned medium; G-CSF: Granulocyte macrophage-colony stimulating factor.
studies may elucidate the preference of engineered-cell therapy in neural injuries.

Conclusion

Overall, we can conclude that combination therapy of G-CSF and DPSCs-CM can improve the neuroprotective activity of DPSCs on neural cells exposed to hypoxic conditions. These results were confirmed by evaluating the apoptotic cells which indicated the anti-apoptotic effect of DPSCs-CM, DPSCs-CM<sub>2</sub>, and DPSCs-CM plus G-CSF with the superior effect of DPSCs-CM on SH-SYSY cells exposed to CoCl<sub>2</sub>. It seems that DPSCs-CM and G-CSF were able to regulate the apoptosis pathways which resulted in to decrease in the β-catenin and cleaved form of caspase-3 and PARP-1 also increase in the level of survivin. Furthermore, the results of the LDH assay emphasize the neuroprotective effect of DPSCs-CM, DPSCs-CM<sub>2</sub>, and G-CSF.

It is shown that DPSCs-CM<sub>2</sub> and DPSCs-CM plus G-CSF had a superior protective effect in comparison with DPSCs-CM or G-CSF alone to fight against the hypoxic condition. With transfection of plasmid encoding G-CSF, we designed a cellular carrier which has two advantages: 1-These engineered cells can express G-CSF for a longer time and may overcome the short half-life of G-CSF. 2-This cellular carrier, probably, play the role of combination therapy itself by secretion of G-CSF in addition to conditioned media. Further clinical studies may elucidate the preference of engineered-cell therapy in neural injuries.

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Authors' Contributions

FA performed the experiments, computations, analyzed the data, and wrote the manuscript. ZS conceived, designed, and supervised the project and approved the final manuscript. MM designed and supervised some parts of the project. AE performed the experiments, computations. ZT conceived, designed, and supervised the project, wrote the manuscript, provided financial support and approved the final draft of the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest, financial or otherwise.

References

1. Volkman R, Olen D. Concise review: Mesenchymal stem cells in neurodegenerative diseases. Stem Cells 2017; 35:1867-1880.
2. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA. Concise review: Dental pulp stem cells: a novel cell therapy for retinal and central nervous system repair. Stem Cells 2017; 35:61-67.
3. Yamada Y, Nakamura-Yamada S, Kusano K, Baba S. Clinical potential and current progress of dental pulp stem cells for various systemic diseases in regenerative medicine: A concise review. Int J Mol Sci 2019; 20:1132.
4. Luo L, He Y, Wang X, Key B, Lee BH, Li H, et al. Potential roles of dental pulp stem cells in neural regeneration and repair. Stem Cells Int 2018; 7:2018.
5. Mohan SP, Ramalingam M. Dental pulp stem cells therapy in neuroregeneration. J Pharm Bioallied Sci 2020; 12:560-566.
6. Nuri N, Corallo C, Chan B, Ferrari M, Gerami-Naini B. Multipotent differentiation of human dental pulp stem cells: A literature review. Stem Cell Rev Rep 2016; 12:511-523.
7. Hayashi Y, Murakami M, Kawamura I, Ishizaka R, Fukuta O, Nakashima M. CXCL14 and MCP1 are potent trophic factors associated with cell migration and angiogenesis leading to higher regenerative potential of dental pulp side population cells. Stem Cell Res Ther 2015; 6:111.
8. Lan X, Sun Z, Chu C, Bolzle J, Li S. Dental pulp stem cells: An attractive alternative for cell therapy in ischemic stroke. Front Neurol 2019; 10:824.
9. Ahmed NE-MB, Murakami M, Hirose Y, Nakashima M. Therapeutic potential of dental pulp stem cell secretome for Alzheimer’s disease treatment: An in vitro study. Stem Cells Int 2016; 2016:8102478.
10. Wang J, Zuzzio K, Walker CL. Systemic dental pulp stem cell secretome therapy in a mouse model of amyotrophic lateral sclerosis. Brain Sci 2019; 9:165.
11. Frank T, Schlachetzki JC, Göricone B, Meuer K, Rohde G, Dietz GP, et al. Both systemic and local application of granulocyte-colony stimulating factor (G-CSF) is neuroprotective after retinal ganglion cell axotomy. BMC Neurosci 2009; 10:49.
12. Frampton JE, Lee CR, Faulds D. Filgrastim. Drugs 1994; 48:731-760.
13. Tsai ST, Chu S-C, Liu S-H, Pang C-Y, Hou T-W, Lin S-Z, et al. Neuroprotection of granulocyte colony-stimulating factor for early stage Parkinson’s disease. Cell Transplant 2017; 26:409-416.
14. Liu P-K, Wen Y-T, Lin W, Kapupara K, Tai M, Tsai R-K. Neuroprotective effects of low-dose G-CSF plus meloxicam in a rat model of anterior ischemic optic neuropathy. Sci Rep 2020; 10:1-10.
15. Yanqing Z, Yu-Min L, Jian Q, Bao-Guo X, Chuan-Zhen L. Fibronectin and neuroprotective effect of granulocyte colony-stimulating factor in focal cerebral ischemia. Brain Res 2006; 1098:161-169.
16. Meuer K, Pitzer C, Teismann P, Krüger C, Göricone B, Laage R, et al. Granulocyte-colony stimulating factor is neuroprotective in a model of Parkinson’s disease. J Neurochem 2006; 97:675-686.
17. Kichenbrand C, Velot E, Menu P, Moby V. Dental pulp stem cell-derived conditioned medium: An attractive alternative for regenerative therapy. Tissue Eng Part B Rev 2019; 25:78-88.
18. Pourgonabadi S, Ghorbani A, Tayarani-Najaran Z, Mousavi SH. In vitro assessment of alendronate toxic and apoptotic effects on human dental pulp stem cells. Iran J Basic Med Sci 2018;21:905-910.
19. Pilhauerova N, Soukup T, Kleplova TS, Suchanek J. Enzymatic isolation, amplification and characterization of dental pulp stem cells. Folia Biologica 2019; 65:124-133.
20. Feng J, Wang W. Hypoxia pretreatment and EPO-modification enhance the protective effects of MSC on neuron-like PC12 cells in a similar way. Biochem Biophys Res Commun 2017; 482:232-238.
21. Johnson TV, DeKorver NW, Levasseur VA, Osborne A,
Neuroprotective Effect of DPSC and G-CSF in SH-SY5Y Cells Ahmadi et al.

Tassoni A, Lorber B, et al. Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain 2014; 137:503-519.

22. Salmasi Z, Hashemi M, Mahdipour E, Nourani H, Abnous K, Ramezani M. Mesenchymal stem cells engineered by modified polyethyleneimine polymer for targeted cancer gene therapy, in vitro and in vivo. Biotechnol Prog 2020; 36:e3025.

23. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA. Intraventricularly transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 2013; 54:7544-7556.

24. Le Teunis E, Martínez E, Menozza-Núñez VM, Santiago-Osorio E. Mesenchymal stem cells derived from dental pulp: a review. Stem Cells Int 2016; 2016:64709572.

25. Sowa K, Nito C, Nakajima M, Suda S, Nishiyama Y, Sakamoto Y, et al. Impact of dental pulp stem cells overexpressing the hematopoietic factor G-CSF is a neuronal promoter. CMAJ 2006; 174:927-933.

26. Fathali N, Lekic T, Zhang JH, Tang J. Long-term evaluation of granulocyte colony-stimulating factor on hypoxic-ischemic brain damage in infant rats. Intensive Care Med 2010; 36:1602-1608.

27. Gibson CL, Jones NC, Prior MJ, Bath PM, Murphy SP. G-CSF suppresses edema formation and reduces interleukin-1β expression after cerebral ischemia in mice. J Neuropathol Exp Neurol 2005; 64:763-769.

28. Zhang X-M, Du E, Yang D, Wang R, Yu C-J, Huang X-N, et al. Granulocyte colony-stimulating factor increases the therapeutic efficacy of bone marrow mononuclear cell transplantation in cerebral ischemia in mice. BMC Neurosci 2011; 12:61.

29. Balseanu AT, Buga A-M, Catalin B, Wagner D-C, Boluze J, Zagrean A-M, et al. Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. Front Aging Neurosci 2014; 6:130.

30. Acosta SA, Tajiri N, Shinozuka K, Ishikawa H, Sanberg PR, Sanchez-Ramos J, et al. Combination therapy of human umbilical cord blood cells and granulocyte colony stimulating factor reduces histopathological and motor impairments in an experimental model of chronic traumatic brain injury. PLoS One 2014; 9:e90953.

31. Sun F-L, Wang W, Zuo W, Xue J-L, Xu J-d, Ai H-X, et al. Promoting neurogenesis via Wnt/β-catenin signaling pathway accounts for the neurorestorative effects of morroniside against cerebral ischemia injury. Eur J Pharmacol 2014; 738:214-221.

32. Albadawi H, Crawford RS, Atkins MD, Watkins MT. Role of β-catenin signaling pathway in disrupted hippocampal neurogenesis of temporal lobe epilepsy: A potential therapeutic target? Neurochem Res 2015; 40:1319-1332.

33. Lovestone S, Killick R, Di Forti M, Murray R. Schizophrenia as a GSK-3 dysregulation disorder. Trends Neurosci 2007; 30:142-149.

34. Inestrosa N, Tapia-Rojas C, Griffith T, Carvajal F, Benito M, Rivera-Dictter A, et al. Tetrahydroperforin prevents cognitive deficit, Aβ deposition, tau phosphorylation and synaptotoxicity in the APPswe/PSEN1ΔE9 model of Alzheimer's disease: a possible effect on APP processing. Transl Psychiatry 2011; 1:e20.

35. Zalvide J, DeCaprio JA. Role of pRb-related proteins in simian virus 40 large-T-antigen-mediated transformation. Mol Cell Biol 1995; 15:5800-5810.

36. Pećina-Šlaus N. Wnt signal transduction pathway and tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res 1973; 33:2643-2652.

37. Schneider A, Krüger C, Steigleder T, Weber D, Pitzer C, Noël A, et al. Conditioned medium from mesenchymal stem cells enhances early bone regeneration after maxillary sinus floor elevation in rabbits. Implant Dent 2015; 24:657-663.

38. Lin MT. Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury. Clin Sci 2013; 124:165-176.

39. Katagiri W, Osugi M, Kinoshita K, Hibi H. Conditioned medium from mesenchymal stem cells enhances early bone regeneration after maxillary sinus floor elevation in rabbits. Implant Dent 2015; 24:657-663.

40. Tsimmis I, Stavrianou D, Bracchi S, Rossi L, Wanet T, Noël A, et al. Conditioned medium from bone marrow-derived mesenchymal stem cells improves recovery after spinal cord injury in rats: An original strategy to avoid cell transplantation. PLoS One 2013; 8:e95515.

41. Chang C-P, Chio C-C, Cheong C-U, Chao C-M, Cheng B-C, Lin M-T. Conditioned medium from mesenchymal stem cells after optic nerve injury. Invest Ophthalmol Vis Sci 2013; 54:1608.

42. Damalas A, Ben-Ze'ev A, Simcha I, Shutman M, Leal JFM, Rivera-Dictter A, et al. Tetrahydroperforin prevents cognitive deficit, Aβ deposition, tau phosphorylation and synaptotoxicity in the APPswe/PSEN1ΔE9 model of Alzheimer’s disease: a possible effect on APP processing. Transl Psychiatry 2011; 1:e20.

43. Balseanu AT, Buga A-M, Catalin B, Wagner D-C, Boluze J, Zagrean A-M, et al. Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. Front Aging Neurosci 2014; 6:130.

44. Acosta SA, Tajiri N, Shinozuka K, Ishikawa H, Sanberg PR, Sanchez-Ramos J, et al. Combination therapy of human umbilical cord blood cells and granulocyte colony stimulating factor reduces histopathological and motor impairments in an experimental model of chronic traumatic brain injury. PLoS One 2014; 9:e90953.

45. Huang C, Fu X-H, Zhou D, Li J-M. The role of Wnt/β-catenin signaling pathway in disrupted hippocampal neurogenesis of temporal lobe epilepsy: A potential therapeutic target? Neurochem Res 2015; 40:1319-1332.

46. Sun F-L, Wang W, Zuo W, Xue J-L, Xu J-d, Ai H-X, et al. Promoting neurogenesis via Wnt/β-catenin signaling pathway accounts for the neurorestorative effects of morroniside against cerebral ischemia injury. Eur J Pharmacol 2014; 738:214-221.

47. Albadawi H, Crawford RS, Atkins MD, Watkins MT. Role of β-catenin signaling pathway in disrupted hippocampal neurogenesis of temporal lobe epilepsy: A potential therapeutic target? Neurochem Res 2015; 40:1319-1332.

48. Lovestone S, Killick R, Di Forti M, Murray R. Schizophrenia as a GSK-3 dysregulation disorder. Trends Neurosci 2007; 30:142-149.

49. Inestrosa N, Tapia-Rojas C, Griffith T, Carvajal F, Benito M, Rivera-Dictter A, et al. Tetrahydroperforin prevents cognitive deficit, Aβ deposition, tau phosphorylation and synaptotoxicity in the APPswe/PSEN1ΔE9 model of Alzheimer’s disease: a possible effect on APP processing. Transl Psychiatry 2011; 1:e20.

50. Damalas A, Ben-Ze’ev A, Simcha I, Shutman M, Leal JFM, Rivera-Dictter A, et al. Excess β-catenin promotes accumulation of transcriptionally active p53. EMBO J 1999; 18:3054-3063.

51. Albadawi H, Crawford RS, Atkins MD, Watkins MT. Role of poly(ADP-ribose) polymerase during vascular reconstruction. Vascular 2006; 14:362-365.

52. Chaitanya GV, Alexander JS, Babu PP. PARP-1 cleavage fragments: Signatures of cell-death proteases in neurodegeneration. Cell Biol Int 2010; 34:1-15.

53. Albadawi H, Crawford RS, Atkins MD, Watkins MT. Role of poly(ADP-ribose) polymerase during vascular reconstruction. Vascular 2006; 14:362-365.
cytokine in the central nervous system. Int J Biol Sci 2012; 8:1254-1266.
55. Huang J, Upadhyay UM, Tamargo RJ. Inflammation in stroke and focal cerebral ischemia. Surg Neurol 2006; 66:232-245.
56. Swartz KR, Liu F, Sewell D, Schochet T, Campbell I, Sandor M, et al. Interleukin-6 promotes post-traumatic healing in the central nervous system. Brain Res 2001; 896:86-95.
57. Gu Y, He M, Zhou X, Liu J, Hou N, Bin T, et al. Endogenous IL-6 of mesenchymal stem cell improves behavioral outcome of hypoxic-ischemic brain damage neonatal rats by suppressing apoptosis in astrocyte. Sci Rep 2016; 6:1-16.
58. Mosser DM, Zhang X. Interleukin-10: New perspectives on an old cytokine. Immunol Rev 2008; 226:205-218.
59. Garcia JM, Stillings SA, Leclerc JL, Phillips H, Edwards NJ, Robicsek SA, et al. Role of interleukin-10 in acute brain injuries. Front Neurol 2017; 8:244.
60. Porro C, Cianciulli A, Panaro MA. The regulatory role of il-10 in neurodegenerative diseases. Biomolecules 2020; 10:1017.