Original Article
Comparing the effect of neurotrophic factor induced MSCs (BMSC and DPSC) on the expression of myelin proteins Nogo-A and OMgp in a glaucoma rat model

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Abstract: Glaucoma is a chronic ocular disorder that occurs due to the degeneration of retinal ganglion cells (RGCs). Mesenchymal stem cells (MSCs) have emerged to be potential candidates to regenerate new cells and restore vision. There is an immediate need to find an efficient stem cell based therapy to enhance the secretion of neurotrophic factors (NFs) and promote long term cell survival. There are numerous mechanisms behind the regeneration of cells induced by the secretion of NFs that needs to be validated yet. DPSCs and BMSCs were isolated from the Sprague-Dawley rats and engineered into lentiviral constructs with BDNF and GDNF. The secretion of NFs by the engineered MSCs was quantified by ELISA and then transplanted into glaucoma rat model. The effect of NF secretion by the engineered MSCs on the neurite outgrowth was observed. The expression of myelin Nogo-A and OMgp, was investigated by RT-PCR and western blot analyses. The intraocular pressure in the eye of glaucoma rat model was reduced following the transplantation of the engineered stem cells. DPSCs were more efficient than BMSCs in secretion of neurotrophic factors (BDNF and GDNF) and enhanced the neurite outgrowth. Western blot analyses showed that Nogo-A and OMgp expression was significantly reduced. DPSCs are an alternate source of MSCs that are more efficient than BMSCs. The engineered MSCs secrete significantly higher amount of neurotrophic factors than the normal MSCs. The regeneration of RGCs following the optic nerve injury is associated with the reduced expression of Nogo-A and OMgp.

Keywords: Glaucoma, DPSC, BMSC, neurotrophic factor, myelin proteins

Introduction

The loss of neurons in the central nervous system (CNS) was considered to be irreplaceable with the consequent exacerbation of the CNS disorders. Glaucoma is one such degenerative CNS disorder characterized by the loss of retinal ganglion cells (RGCs) leading to blindness [1]. The death of RGCs is accompanied by a number of pathophysiological mechanisms, for instance, glutamate excitotoxicity and oxidative stress due to excess reactive oxygen species (ROS) that induces DNA damage leading to cellular dysfunction [2-5]. However, these mechanisms mainly result from the elevated intraocular pressure (IOP), one of the most important risk factors for glaucoma [4]. The increased IOP

blocks the retrograde transport leading to the deprivation of neurotrophic factors (NF) and impairs axonal regeneration [6, 7]. The presence of myelin proteins, Nogo-A (Neurite outgrowth inhibitor) and OMgp (Oligodendrocyte-myelin glycoprotein), within a damaged CNS is also reported to impair the axonal regeneration and is expressed in the retina of rats [8-10].

Numerous studies have shown that inducing NFs into the eyes of animals reduces the ocular hypertension [11-14]. One of the best strategies to induce NFs is to engineer NF into the cultured stem cells through viral constructs to provide long term neuroprotection [15]. Mesenchymal stem cells (MSCs) have been recently explored to be highly beneficial in the treatment

of degenerative diseases of CNS [16]. The limited therapeutic strategies to cure glaucoma has largely expanded the use of MSCs to develop regenerative and reparative treatments. MSCs are considered to be potential candidates in stem cell therapy due to their low immune response in host during autologous transplantation and can be isolated from a number of other regions like dental pulp, umbilical cord and vermiform appendix, in addition to the bone marrow [17]. Dental pulp stem cells (DPSCs) are the alternative source of MSCs for cellular therapy in CNS injury [18]. It can be isolated from infant as well as adult mammalian teeth and is easily accessible with less ethical concerns. DPSCs have been reported to be more efficient in producing NFs than the bone marrow derived MSCs (BMSCs) and promotes an enhanced recovery in spinal cord injury [18].

In this study, we compared the efficiency of DPSCs and BMSCs engineered into a lentiviral construct with NFs (BDNF and GDNF). The effect of the engineered MSCs on Nogo-A and OMgp expression was also investigated, where the NF induced MSCs inhibited the expression of myelin proteins (Nogo-A and OMgp). The regeneration of RGCs following the transplantation of MSCs was attributed to the reduced expression of Nogo-A and OMgp.

Materials and methods

Animals

Adult male Sprague-Dawley rats (n=38) that weighed 170 to 200 g were purchased from the SLAC Company (Shanghai China) and the research involving animals was approved by the local ethical committee. The experiments were performed according to the institutional guidelines and National Research Council Guide was followed The rats were housed under optimum conditions (12 hour light/dark cycle, temperature of 22 ± 0.5°C and humidity of 50 ± 10%) and in a pathogen free environment.

DPSC isolation and culture

DPSCs were isolated from adult male Sprague-Dawley rats (n=3) that weighed 170 to 200 g and were purchased from the SLAC Company (Shanghai China). The upper and lower incisors were extracted followed by the removal of dental pulp in Dulbecco's Modified Eagle's Medium

(DMEM) (Sigma Aldrich) under sterile conditions supplemented with 1% penicillin/streptomycin (P/S). It was then sliced into 1 mm³ small fragments and incubated for 30 minutes at 37°C in 4 mL of 0.25% trypsin EDTA. An equal volume of DMEM containing 1% P/S with 10% fetal bovine serum (FBS) was added to inactivate trypsin. The cell suspension was passed through a 70 µm cell strainer to obtain a single cell population, followed by centrifugation at 150 g for 5 minutes, cell pellets were resuspended in the media (DMEM with 1% P/S and 10% FBS) and seeded into T25 flasks (5 ml). The cultures were maintained under optimum condition (37°C in 5% CO_2) and the medium was changed every 24 hours following the seeding and cells were passaged at 80% confluency. The conditioned media for ELISA was obtained from separate culture of stem cells from each different animal.

Isolation and culture of BMSC

The same animals described above were used for BMSC isolation. The femurs of these animals were used for the isolation of BMSC and their ends were detached under sterile conditions. The bone marrow was washed out with 10 ml DMEM, followed by the centrifugation of cell aspirates at 150 g for 5 minutes, the cell pellet was resuspended in DMEM (with 1% P/S and 10% FBS) and seeded into T25 flasks (volume 5 ml). Same culture conditions were maintained as mentioned above (37°C in 5% CO_2).

Inducing BDNF and GDNF ex-vivo in stem cells using lentiviral vectors

The neurotrophic factors BDNF and GDNF were engineered to the stem cells using lentiviral vectors [19]. DPSCs and BMSCs were plated and allowed to adhere for 12 hours (density of 1000-1200 cells per well). The growth medium (with 2% FBS and 12 µg/mL Sequa-brene (Sigma Aldrich)) was added in each well following the cell adherence. Two lentiviral vector's encoding BDNF (LV-BDNF) and GDNF (LV-GDNF) were added to DPSCs and BMSCs at a multiplicity of infection (MOI) of 15 for each vector. After incubation for 8 hours, media with viral particles were removed and fresh medium was added. At 70-80% confluence, DPSCs and BMSCs were transferred and subcultured. Stem cells engineered with lentiviral vectors were maintained as described above.

Table 1. BDNF and GDNF secretion by engineered (E) and normal (N) MSCs (BMSCs and DPSCs)

NF[1] MSC[2]	BDNF[3] (ng/L)		GDNF[4] (ng/L)		BDNF/GDNF (ng/L)	
	N[5]	E[6]	N	E	N	E
DPSCs[7]	5.28 ± 2.03	23.43 ± 1.56	9.83 ±1.04	40.1 ± 1.47	5.4 ±1.23/9.24 ± 1.40	25.6 ± 0.21/40.4 ± 0.57
BMSCs[8]	3.95 ± 0.76	15.21 ± 0.26	6.45 ± 2.42	19.78 ± 1.45	4.2 ± 0.73/6.2 ± 1.89	15.3 ± 0.29/20.2 ± 0.83

The amount of BDNF and GDNF secreted by BMSC and DPSC was quantified using ELISA. The DPSCs produced a greater amount of NFs when compared to BMSCs that shows its efficiency as an alternate source of MSC therapy. The BDNF/GDNF combination was secreted approximately in the equal amounts when expressed alone in the MSCs. Values are represented as mean ± SD and significant at P<0.05. [1]Neurotrophic Factor; [2]Mesenchymal Stem cell; [3]Brain Derived Neurotrophic Factor; [4]Glial cell line derived Neurotrophic Factor; [5]Normal cells; [6]Engineered cells; [7]Dental Mesenchymal Stem cells; [8]Bone Marrow Mesenchymal Stem cells.
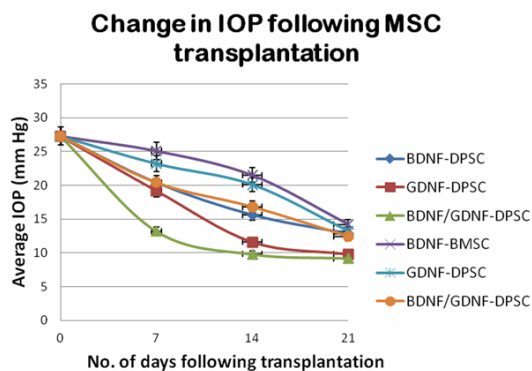

Figure 1. Change in IOP following transplantion of engineered DPSC. There was a consistent reduction of IOP following the transplantation of NF induced DPSC. GDNF-DPSC showed a greater improvement compared to DPSC-GDNF. BDNF-GDNF-DPSC had an additive effect where IOP was reduced from 7th day. Engineered BMSCs showed a gradual but significant decrease in IOP at 21 days following transplantation. Values are represented as mean ± SD and significant at P<0.05.

Quantification of BDNF and GDNF production using ELISA

The neurotrophins produced by stem cells (DPSCs and BMSCs) before and after the ex-vivo induction was quantitated using ELISA. The difference between the neurotrophins produced by BMSC and DPSC was noticed. The assay was performed using EMAX Immunoassay kits (Promega) for BDNF and GDNF in rats according to manufacturer's instructions. From the neurotrophin standards, a standard curve was constructed to quantify the BDNF and GDNF production.

Glaucoma rat model

The rats (n=35) were anesthetized by the intra-peritoneal injection of ketamine (50 mg/kg, Sigma Aldrich), and xylazine (2.2 mg/kg; Sigma Aldrich) to induce ocular hypertension. A few drops of the local anesthetic proparacaine (Sigma Pharmaceuticals) was applied to the rat's left eye. The half of anterior chamber of the left eye was selectively damaged by directing laser light at 90 spots of diameter 50 μm. The IOP was measured using iCare tonometer (iCare USA) once in a week.

Transplantation of NF induced stem cells in the glaucoma rat model

Intraocular injections were performed by collecting neurotrophin induced BMSCs and DPSCs that were detached (using 0.05% trypsin and 0.1% EDTA) and pelleted at 500 rpm for 5 minutes. The pellet was resuspended (50,000 cells per μl) in Earle's Balanced Salt Solution (Invitrogen). The neurotrophin induced BMSC and DPSC cell suspension (2 μl) was injected intravitreally using a beveled glass microelectrode that was attached to a syringe through polyvinyl chloride tubing. The left eye served as the experimental group and the right eye served as the control. The different group of injections included: (1) BDNF-DPSC (2) GDNF-DPSC (3) BDNF/GDNF-DPSC (4) BDNF-BMSC (5) GDNF-BMSC (6) BDNF/GDNF-BMSC (7) EBSS as control. Each group consisted of n=5 rats and the IOP was measured at day 0, day 7, day 14 and day 21 following the transplantation. After 21st day of transplantation of MSCs, the rats were euthanized and RGCs were then isolated as previously decribed [37].

Neurite outgrowth quantification

Following the MSC transplantation, the isolated RGCs were subjected to neurite outgrowth assay. The protocol was followed as illustrated in the manufacturer's catalog (Chemicon Catalog No. NS200). The assay was performed on membrane inserts and the neurites were stained for visualization and quantification. The neurite outgrowth was quantified by measuring the absorbance at 562 nm.
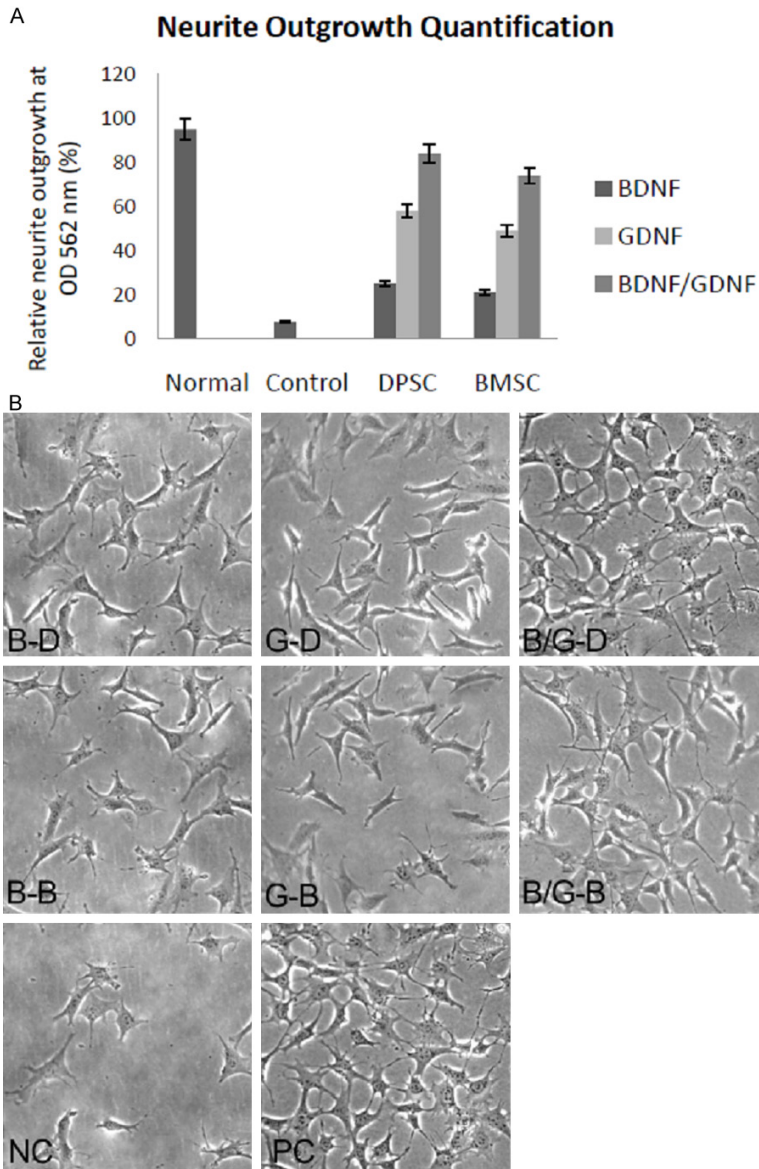
Figure 2. Neurite outgrowth quantification. (A) The neurite outgrowth following the transplantation of engineered MSCs was quantified by measuring the absorbance at 562 nm. The relative neurite outgrowth is expressed in terms of percentage (B) Phase contrast photomicrographs of RGCs cultured following the MSC transplantation. B-D represents BDNF-DPSC, G-D represents GDNF-DPSC, B/G-D is BDNF/GDNF-DPSC, B-B is BDNF-BMSC, G-B is GDNF-BMSC and B/G-B is BDNF/GDNF-BMSC treated samples. The negative control (NC) is the sample treated with EBSS and positive control (PC) is the normal RGCs isolated from the right eye of the rat model.

Nogo-A and OMgp expression analysis using RT-PCR

Total RNA was extracted using Trizol reagent and converted to cDNA. qPCR (Applied Bio-system (Carlsbad, CA, USA) was performed to assess the relative mRNA expression levels of Nogo-A and OMgp following the transplantation of engineered MSCs. Primer probes for Nogo-A and OMgp were acquired commercially from Applied Biosystems. Nogo-A forward primer: CAGG-TGATGTACGCTCTGGA, Nogo-A reverse primer: TGAGGGAAG-TAGGATGTGC; OMgp forward primer: ACCTCAAGCTTATTTA-CTATGAAG, OMgp reverse primer: AGTGTTTCCATTTGCA-GTTGGTT; Actin forward primer: GAAAATCTGGCACCACAC-CT, Actin reverse primer: GG-CCGGACTCGTCATACTC. Beta-Actin was used as an internal control to normalize the mean Ct values.

Western blotting analysis

The cells were subjected to lysis buffer consisting of 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA. The lysis buffer was also supplemented with protease (10%) and phosphatase (1%) inhibitor (Invitrogen). The cell debris was removed and the supernatant was separated by centrifugation at 13,000 rpm for 30 minutes. From each sample, an aliquot of protein (40-80 μg) was subjected to SDS-PAGE and then transferred onto a nitrocellulose membrane. The blot was blocked using 5% blocking solution (non-fat dry milk in PBS) for 1 hour at room temperature and incubated with primary antibody to Nogo-A (goat anti Nog-A) (1:100; Santa Cruz) and OMgp for 16 hours at 4°C and antimouse β-actin antibody (1:500; Santa Cruz, Cat. No. sc-4778) as control. The blots were washed thrice with PBS/0.1% Tween and incubated in the secondary antibody which was peroxidase-conjugated donkey anti-goat IgG (1:5000; Santa Cruz Cat No. sc-2032) for Nogo-A and goat antimouse IgG for β-actin. The blots were again washed and the proteins were detected using a chemilumi-
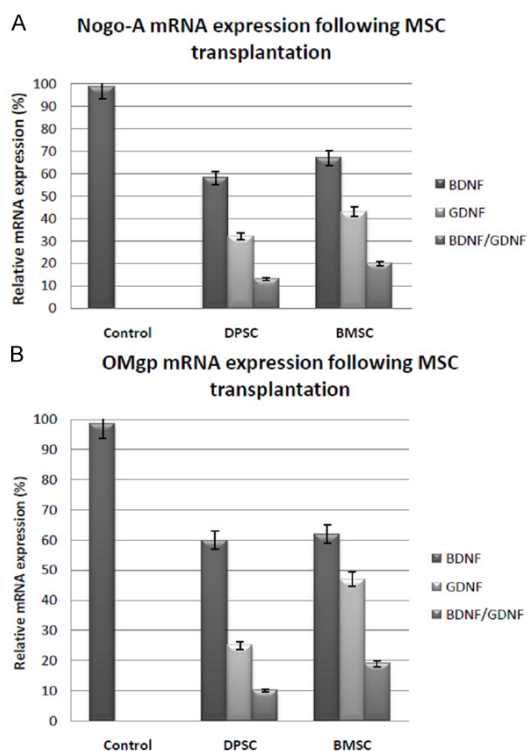
Figure 3. Effect of engineered MSCs on the mRNA expression of Nogo-A and OMgp. The graphs represent relative mRNA expression levels of Nogo-A and OMgp in the glaucoma rat samples transplanted with NF induced DPSCs and BMSCs. A. Nogo-A mRNA expression level is lowest for the BDNF/GDNF-DPSC transplanted sample. Samples treated with NF induced BMSCs exhibit a greater Nogo-A expression. B. OMgp mRNA expression rate is similar to Nogo-A and is significantly reduced in the treated samples with a greater suppression in DPSC treated samples.

nescence system. The protein bands were visualized using Image J software and quantified.

Statistics

One way analysis of variance (ANOVA) was used for the comparison of the variables and the statistical significance was set at P<0.05 using SPSS 15.0 software.

Results

Increased secretion of neurotrophic factors by the engineered DPSCs

The engineered DPSCs secreted significantly increased amount of BDNF and GDNF when compared to engineered BMSCs. There was

a significant difference in the neurotrophic factor (BDNF and GDNF) secretion levels between the engineered MSCs and the untreated MSCs. The secretion levels were analysed using ELISA and it was found that DPSCs secreted greater amounts of BDNF and GDNF than the BMSCs. BDNF secretion was increased from 5.28 ± 2.03 ng/L in untreated DPSCs to 23.43 ± 1.56 ng/L in the engineered DPSCs but there was no detectable levels of GDNF. There was also a significant increase in the GDNF levels from 9.83 ± 1.04 ng/L in untreated DPSCs to 40.1 ± 1.47 ng/L in engineered DPSCs in the engineered DPSCs with no detectable levels of BDNF. Similarly, the NF secretion was increased in engineered BMSCs (from 3.95 ± 0.76 to 15.21 ± 0.26 ng/L for BDNF and from 6.45 ± 2.42 ng/L in untreated BMSCs to 19.78 ± 1.45 ng/L in engineered BMSCs for GDNF). The combination of BDNF/GDNF-MSCs secreted comparable amounts of both the NFs with the higher amount being secreted by DPSCs. The ELISA results are clearly demonstrated in the (**Table 1**). Efficiency of NF induction on MSCs after transfection with lentivirus is shown in the (Supplementary Figure 1).

Changes in IOP level following transplantation

The measurement of IOP in the eyes is one of the methods to determine the improvement in glaucoma condition. The IOP of the glaucomatous rat model following MSC transplantation was measured on 0, 7, 14 and 21th day. At 0th day, the IOP was measured to be 27.3 ± 0.21 mm Hg in all the rats (left eye) and normal IOP level in the right eye was measured to be 9.1 ± 0.36 mmHg. Consistent with the ELISA results, engineered DPSCs rendered significantly greater protection than the engineered MSCs (**Figure 1**).

Increased neurite outgrowth following the transplantation of engineered MSCs

The neurite outgrowth in the isolated RGCs from glaucoma rat model was quantified following MSC transplantation. BDNF/GDNF-DPSCs rendered the most enhanced regeneration whereas BMSCs showed less neurite outgrowth. The combination of NFs (GDNF and BDNF) had an additive effect on the activity of MSCs. The neurite outgrowth results are depicted in the (**Figure 2**).
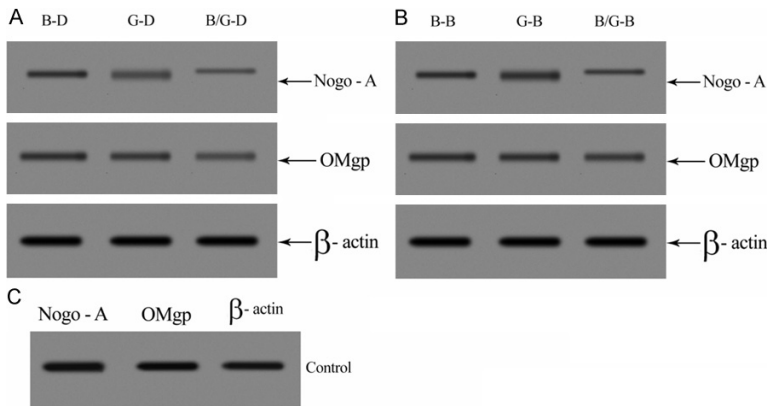
Figure 4. Effect of engineered MSCs on the expression of myelin proteins Nogo-A and OMgp. The figure shows the western blot analysis of myelin proteins, Nogo-A and OMgp, in the engineered MSCs transplanted glaucoma rat samples. B-D represents BDNF-DPSC, G-D represents GDNF-DPSC, B/G-D is BDNF/GDNF-DPSC, B-B is BDNF-BMSC, G-B is GDNF-BMSC and B/G-B is BDNF/GDNF-BMSC treated samples. β-actin acts as the internal control. A. The expression of Nogo-A and OMgp was significantly reduced in the samples treated with engineered DPSCs. BDNF/GDNF-DPSC was most effective in reducing the expression of these myelin proteins. B. In consistent with the ELISA results, engineered BMSCs could reduce the expression of Nogo-A and OMgp to an extent but not as efficient as engineered DPSCs. C. Control samples treated with EBSS.

Figure 5. Quantification of the western blot. A. Relative expression of OMgp following different treatment conditions as mentioned in the **Figure 4**. B. Relative expression of NOGO-A following the treatment. Values were significant at P<0.05 with respect to the control.

Effect of engineered MSCs on the expression of Nogo-A and OMgp

mRNA expression level of Nogo-A and OMgp was quantified to elucidate the action of transplanted NF induced MSCs in the glaucoma rat samples (**Figure 3**). It was found that Nogo-A and OMgp expression was significantly reduced and the effect was greater in the DPSC treated samples. The combined NF induced DPSCs showed to have an additive effect on reducing the expression of Nogo-A and OMgp. The expression was quantified and the relative expression levels are shown in (**Figure 3**). It was inferred that there was a significant reduction in the myelin proteins following transplantation which was otherwise found to be highly expressed following glaucoma.

Decreased expression of Nogo-A and OMgp in NF-MSC transplanted glaucomatous rats

The expression of Nogo-A and OMgp in NF-MSC treated samples was investigated by western blotting. The **Figure 3** shows the expression of Nogo-A and OMgp in DPSC-BDNF, DPSC-GDNF, DPSC-BDNF/GDNF BMSC-BDNF, BMSC-GDNF treated samples. NF induced DPSC treated samples reduced the myelin protein (Nogo-A and OMgp) expression more than the NF induced BMSCs (**Figures 4**, **5**).

Discussion

Glaucoma is mainly associated with the degeneration of RGCs that lead to complete loss of vision [1]. Elevated IOP is one of the most important risk factors for occurrence of glaucoma resulting in obstruction of axonal transport in the optic nerve head [2]. Consequently, the retrograde transport of BDNF is blocked leading to apoptosis of RGCs due to inadequate neurotrophic support [6, 7]. BDNF belongs to NGF family, that binds to tropomyosin receptor kinase (Trk) and activates a number of signaling pathways, for instance,
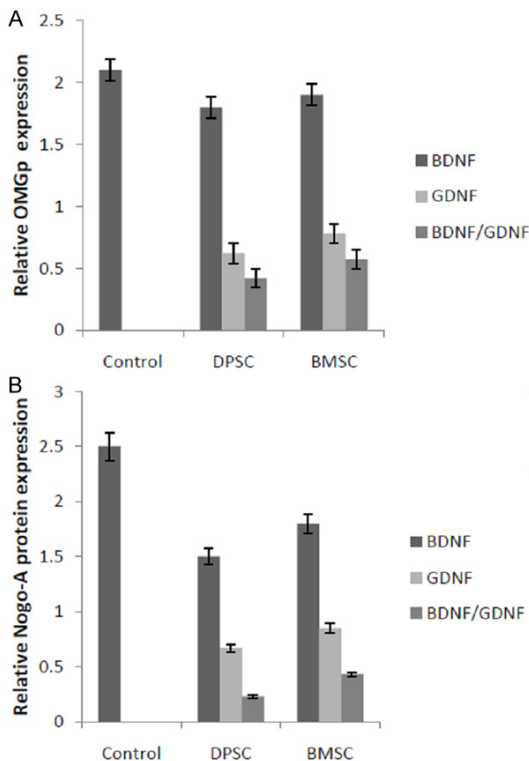
PI3K/Akt/mTOR, Ras/Raf/MEK/ERK and MAPKinase pathways thereby promoting cell survival and growth [20-22]. BDNF also has a low affinity to p75NT receptor that results in apoptosis through the activation of Jun N-terminal kinase (JNK) [20]. Previous studies have shown that BDNF is expressed in mature mouse retina and the exogenous introduction of BDNF increases the survival of RGCs and also promotes the regeneration of RGC axons [23-25]. GDNF also plays a significant role in cell survival and is expressed during retinal development as well as in response to a nerve injury [26-28].

The efficient delivery of NFs has been constantly experimented to protect the degenerating retinas. Stem cells have emerged to be promising source of NF secretion by acting as a cellular vehicle for the delivery of NFs [29]. Injection of BMSCs into the vitreous cavity has been reported to exert neuroprotection on the RGC axons [29]. Another study demonstrated the structural and functional protection of degenerating retina by means of MSCs engineered to secrete BDNF [15]. The combined action of NF has also enhanced the RGC survival like the injection of BDNF and neurturin or BDNF and GDNF which proves the additive effect of NFs [30-32].

Mesenchymal stem cells (MSCs) have been largely used as a potential source for regenerative and reparative therapy in various degenerative diseases [33]. Bone marrow-derived MSCs (BMSCs) were the first powerful source for cell based therapy for ocular diseases. BMSCs greatly increased the survival of RGCs and promoted the regeneration of axons following a traumatic optic injury [18]. However, the neuroprotection by BMSCs was considered to be due to the paracrine mediated effect cause by the activation of retinal glia or by the signaling between the injured RGCs and transplanted stem cells [18]. Therefore, dental pulp stem cells were being explored as the alternative source of MSCs for the ocular disorders.

The present study deals with the comparison of neurotrophic secretion by BMSCs and DPSCs, where these MSCs were specially engineered into lentiviral constructs and induced with BDNF and GDNF. The lentiviruses are efficient in delivering genes and provide a stable long term expression [34]. The engineered MSCs were transplanted into a glaucoma rat model and the secretion of BDNF and GDNF was quantified. Our results consistently demonstrated that engineered DPSCs have more potential in producing neurotrophic factors than the engineered BMSCs (Table 1). The combined effect of BDNF and GDNF administered via BMSCs and DPSCs was also demonstrated in this study, that renders an additive effect on the survival of RGCs (Figure 2). This proved that BDNF and GDNF function independently to promote RGC survival.

The effect of engineered MSCs on the glaucomatous retina was also investigated by measuring the IOP levels in the experimental glaucoma rat model (Figure 1). The decreasing IOP following the transplantation showed that engineered MSCs were efficient as a therapeutic strategy for the glaucoma. The impact of engineered DPSCs on the IOP further proved that DPSCs can serve to be alternate source for MSCs rather than BMSCs. NF-DPSCs showed a significant reduction in IOP from day 7 following transplantation. Easy accessibility and ability to secrete increased amount of multiple source of NFs makes DPSCs one of potential sources for the regenerative and reparative cell based therapies.

We also studied the effect of these engineered MSCs on the mRNA and protein expression of Nogo-A and OMgp, in a glaucoma rat model (Figures 3, 4). Early studies indicate that myelin proteins are confined to oligodendrocytes, however recent evidences state the prominent expression of myelin proteins in CNS. Nogo-A inhibits neurite outgrowth and the genetic deletion of Nogo-A has demonstrated significant survival of neural cells in mice with amyotrophic lateral sclerosis [35]. Though the expression of OMgp in glaucoma is not clearly defined, OMgp is reported to be the inhibitor of neurite outgrowth that binds to Nogo receptor to form a receptor complex [36]. Our study identified that the engineered MSCs were able to reduce the expression of Nogo-A and OMgp. Therefore, the results demonstrate that MSCs enhance the survival and regeneration of RGCs through the suppression of Nogo-A and OMgp that accumulates in CNS following an injury.

Conclusions

To conclude, NF-induced DPSCs were more efficient than NF-induced BMSCs and might prove

to be an important alternative source for the stem cell based therapy. The reparative property of MSCs is attributed to the reduced expression of myelin proteins, Nogo-A and OMgp, following MSC transplantation in a glaucomatous retina. The increased activity of DPSCs might be associated with different molecular mechanisms that requires an elaborate study.

Disclosure of conflict of interest

None.

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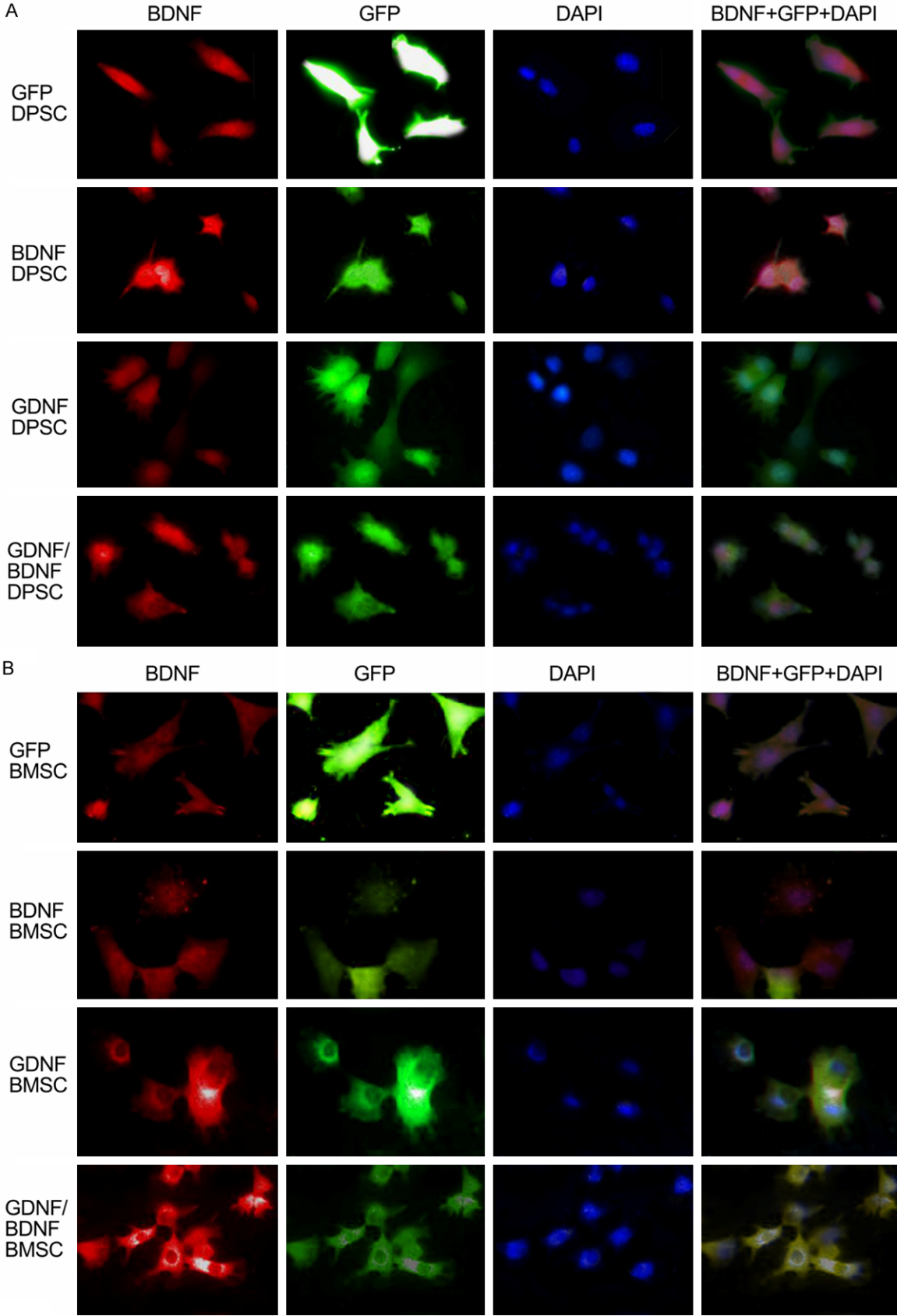
Supplementary Figure 1. Expression of BDNF and GDNF on BMSCs and DPSCs after transfection with lentivirus.