

Exploring the potential application of dental pulp stem cells in neuroregenerative medicine

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The trigeminal nerve and its peripheral branches are susceptible to injury in the dental practice due to surgical removal of impacted third molars and placement of dental implants. Although peripheral trigeminal nerve injuries can undergo spontaneous regeneration, some injuries may be permanent with varying degrees of continued sensory impairment and neuropathic pain ranging from mild numbness to complete anaesthesia (Tay and Zuniga, 2007). Adult neurons require continued neurotrophic support from surrounding cells to sustain neural viability, inhibit death-inducing pathways activating a variety of cell survival pathways (Zheng and Quirion, 2004).

Stem cell therapies for neural repair and regeneration are rapidly advancing as they can function as an important source for trophic factors. Dental pulp stem cells (DPSC), which are isolated from the central pulpal core of teeth, have shown promise for neuroregenerative properties in various *in vitro* and *in vivo* experimental studies (Mead et al., 2017; Mohan and Ramalingam, 2020). DPSC can relatively easily be harvested from extracted teeth have the distinction, that they are derived from neural crest cells and are considered closer to neuronal lineages compared to other sources of mesenchymal stem/stromal cells (MSC) such as bone marrow-derived MSC (Sakai et al., 2012). DPSC have been reported to differentiate along neural lineages when treated with neurotrophic growth factors such as nerve growth factor (NGF), epidermal growth factors, retinoic acid, fibroblastic growth factor (Ovindasamy et al., 2010). With this inherent capacity, DPSC are also able to readily form neurospheres. The therapeutic effect of DPSC, however, is mostly considered through a paracrine mode of action, which entails various trophic and anti-inflammatory factors. Because of the aforementioned neurogenic and neurotrophic properties, DPSC have a tremendous impact on neuroregenerative therapy (Ovindasamy et al., 2010; Mead et al., 2017; Mohan and Ramalingam, 2020).

Previous studies examining co-cultures

of molar pulpal explants with trigeminal ganglia (TG) from neonatal rat pups, demonstrated distinct neurite outgrowth suggesting that tooth pulp tissue secreted neurotrophic neurite-promoting factors to attract axons from local nerve trunks in TG/pulpal co-cultures (Lillesaar et al., 1999).

Mechanism of action of DPSC in the repair of neural elements: Studies from different laboratories have highlighted that paracrine mechanism is responsible for the improved neuronal function rather than neuronal differentiation from the stem cells (Mead et al., 2013, 2017; Mohan and Ramalingam, 2020). Mead et al. compared the neurotrophic activities of human DPSC, human bone marrow-derived MSC and human adipose-derived stem cells on axotomised adult rat retinal ganglion cells *in vitro* and concluded that human DPSC promoted significant multi-factorial paracrine-mediated retinal ganglion cells survival and neurite outgrowth and may be considered a potent and advantageous cell therapy for retinal nerve repair (Mead et al., 2014).

We have previously explored the application of DPSC-derived trophic factors to mediate axonal guidance and neuronal survival in TG neurons which was due to the presence of neurotrophic factors (NTFs) in the collected media from DPSC cultures (DPSC-CM/conditioned media). CM was collected from sub-confluent DPSC that were cultured in serum free media for 72 hours. Specific enzyme-linked immune sorbent assays were used to detect the secreted NTFs including NGF, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3). Quantitative polymerase chain reaction qPCR confirmed upregulation of neuronal markers in TG neuronal cultures underscoring that DPSC-derived NTFs upregulated genes responsible for neuronal survival and neurite outgrowth extension (Sultan et al., 2020).

To explore the role of DPSC-secreted NTFs on neuronal protection and neurite

outgrowth extension further, specific neutralizing antibodies against GDNF and NT-3 were used to block the biological activity of the corresponding NTFs in DPSC-CM (**Figure 1**). The neutralizing experiments were conducted to determine the exact role of NTFs detected in DPSC-CM on neuronal survival and neurite outgrowth by adding neutralizing antibodies against; GDNF and NT-3 to the DPSC-CM before adding it to the cell culture. To further validate that these NTFs were totally knocked out, enzyme-linked immune sorbent assays analysis was assessed on the CM after adding the neutralizing antibodies. It was found that adding 5 $\mu\text{L}/\text{mL}$ GDNF antibody and 10 $\mu\text{L}/\text{mL}$ NT-3 antibody totally blocked the corresponding NTFs level in the DPSC-CM.

Morphometric analysis of immunocytochemically stained images using ImageJ software (National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>, 1997-2018) revealed that neutralization of GDNF in DPSC-CM treated primary TGNC cultures resulted in only a slight reduction of the number of surviving neurons as assessed by NeuN immune marker, but significantly attenuated the neurite outgrowth as assessed by MAP-2 immune marker (**Figure 1**). This result suggested that GDNF was particularly important for TG neurite outgrowth promoted by DPSC-derived factors. Cellular processes influenced by GDNF such as proliferation, differentiation, maturation and neurite outgrowth of GDNF have been recognized but seem to have been less studied than its neurosurvival promoting effect. Our finding that inhibiting GDNF resulted in effects on both TG neuronal survival and neurite outgrowth underlined a critical dual function of this NTF.

Interestingly, blocking of NGF by the neutralizing anti-NGF antibody did not significantly affect both neuronal survival and neurite outgrowth in TG cultures (data not shown). The cultured neurons were still able to extend processes even after the addition of neutralizing antibody against NGF. These results were in accordance with Lillesaar et al. (1999) who demonstrated that NGF antibody failed to inhibit TG neurite growth promoted by DPSC suggesting that unidentified factor(s) induce neurite outgrowth *in vitro*. While we previously confirmed that neutralization of NGF in DPSC-CM was critical for PC-12 neurite outgrowth (Sultan et al., 2021).

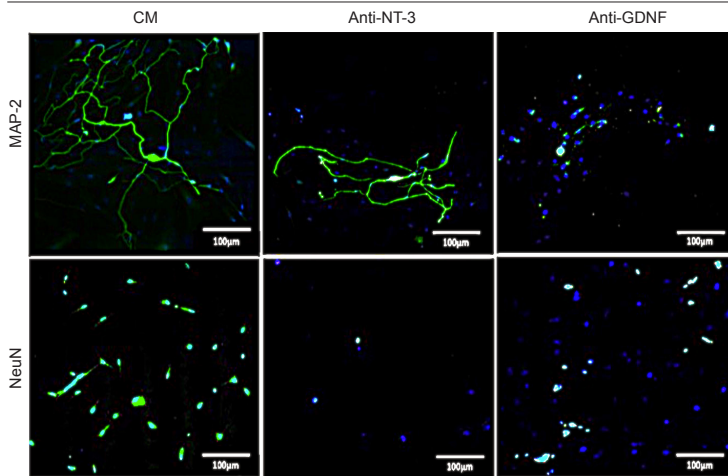


Figure 1 | Laser confocal fluorescence microscopic images.

The impact of DPSC-CM on trigeminal ganglion survival and neurite length after 4 days of culture in the presence of specific neutralising antibodies. Antibodies against GDNF (1 μg/mL; R&D systems, Oxon, UK; AF-212-NA) and NT-3 (2 μg/mL; R&D systems; Cat# AF1404) were used to test the impact of their absence on TG neuronal survival using NeuN (neuronal nucleus immune stain marker; Cat# ab128886 at a 1:1000 dilution; Abcam, Cambridge, MA, USA) and neurite outgrowth extension using MAP-2 (mature neuronal immune stain marker; Cat# ab11267 at a 1:500 dilution; Abcam). Immunolocalization of neuronal markers NeuN in neuronal cell body and MAP-2 in neuronal cell body and processes showing the difference in the number of surviving neurons and the neurite length, respectively. DAPI (blue) was used as a nuclear staining. Anti-GDNF antibody completely blocks CM mediated neurite outgrowth effect while anti-NT-3 markedly reduce the number of surviving neurons. Scale bars: 100 μm. Unpublished data. CM: Conditioned medium; DAPI: 4',6-diamidino-2-phenylindole; GDNF: glial cell line-derived neurotrophic factor; MAP-2: microtubule-associated protein 2; NeuN: neuronal nuclear protein; NT-3: neurotrophin-3.

At the same time, neutralization of NT-3 did not significantly affect TG neurite length; however, it did significantly reduce the number of viable TG neurons as assessed by immunocytochemical mature neuronal marker NeuN (Figure 1). Buchman and Davis (1993) suggested that NT-3 is a target factor for TG neuronal support as they observed abundant expression of NT-3 mRNA in TG culture. They also found that TG cell survival depends on different NTFs at different developmental stages with NT-3 mainly impacting neuronal cell survival. Noteworthy, our previously reported data showed that blocking of NT-3 did not result in any observed influence on DPSC-CM stimulated neural cell line (PC-12) survival or neurite outgrowth which means the possibility of having two different actions of the same neutralizing antibody on two different cell lines (Sultan et al., 2021).

In conclusion, the soluble secreted factors from DPSC promote neuronal cell survival and axonal regeneration and may be considered as cell-free therapy for nerve repair. The secreted NTFs can be easily harvested, purified and stored, thus avoiding complications associated with cell therapy, such as unwanted proliferation/differentiation. Here we have provided further evidence of a critical role of GDNF and NT-3 in TG neuronal cell survival and regeneration. Further studies are warranted to identify the

key DPSC-associated neural bioactive factors, to investigate the therapeutic efficiency of the DPSC secretome to support development of novel DPSC-based neuronal therapies.

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