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PAPER

Cryogel microcarriers loaded with glial cell line-derived neurotrophic factor enhance the engraftment of primary dopaminergic neurons in a rat model of Parkinson's disease

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Abstract

Objective. Cryogel microcarriers made of poly(ethylene glycol) diacrylate and 3-sulfopropyl acrylate have the potential to act as delivery vehicles for long-term retention of neurotrophic factors (NTFs) in the brain. In addition, they can potentially enhance stem cell-derived dopaminergic (DAergic) cell replacement strategies for Parkinson's disease (PD), by addressing the limitations of variable survival and poor differentiation of the transplanted precursors due to neurotrophic deprivation post-transplantation in the brain. In this context, to develop a proof-of-concept, the aim of this study was to determine the efficacy of glial cell line-derived NTF (GDNF)-loaded cryogel microcarriers by assessing their impact on the survival of, and reinnervation by, primary DAergic grafts after intra-striatal delivery in Parkinsonian rat brains. *Approach.* Rat embryonic day 14 ventral midbrain cells were transplanted into the 6-hydroxydopamine-lesioned striatum either alone, or with GDNF, or with unloaded cryogel microcarriers, or with GDNF-loaded cryogel microcarriers. *Post-mortem*, GDNF and tyrosine hydroxylase immunostaining were used to identify retention of the delivered GDNF within the implanted cryogel microcarriers, and to identify the transplanted DAergic neuronal cell bodies and fibres in the brains, respectively. *Main results.* We found an intact presence of GDNF-stained cryogel microcarriers in graft sites, indicating their ability for long-term retention of the delivered GDNF up to 4 weeks in the brain. This resulted in an enhanced survival (1.9-fold) of, and striatal reinnervation (density & volume) by, the grafted DAergic neurons, in addition to an enhanced sprouting of fibres within graft sites. *Significance.* This data provides an important proof-of-principle for the beneficial effects of neurotrophin-loaded cryogel microcarriers on engraftment of cells in the context of cell replacement therapy in PD. For clinical translation, further studies will be needed to assess the impact of cryogel microcarriers on the survival and differentiation of stem cell-derived DAergic precursors in Parkinsonian rat brains.

1. Introduction

The progressive and relatively selective dopaminergic (DAergic) neurodegeneration in the nigrostriatal pathway makes Parkinson's disease (PD) an ideal candidate for cell replacement therapy (CRT) (Graybiel *et al* 1990, Schapira and Jenner 2011, Caminiti *et al* 2017). The data from preclinical and clinical studies have shown that grafts of fetal ventral mesencephalic (VM) cells can exhibit survival and functionality in the PD brain, thus serving as an important proof-of-concept, to date, of the potential of such cell replacement strategies in the treatment of PD (Björklund and Stenevi 1979, Björklund *et al* 1981, Dunnett *et al*

1983, Lindvall 1989, Lindvall *et al* 1990, 1992, Li *et al* 2016). While previous research from our laboratory has shown that the engraftment of the fetal VM cells can be significantly enhanced by encapsulating them in a glial cell line-derived neurotrophic factor (GDNF)-enriched collagen hydrogel for delivery to the rat brain (Moriarty *et al* 2017, 2019), the field of CRT for PD has now moved to the use of ethically and logistically advantageous stem cells (embryonic stem cells or induced pluripotent stem cells (iPSCs)) for obtaining transplantable DAergic cells.

Although grafts of the stem cell-derived DAergic precursors (DAPs) have shown to survive and offer functional recovery in preclinical animal models of PD (Doi *et al* 2020, Piao 2021, Kirkeby 2023, Park 2024), their survival is highly variable (<1%–500%, with a median of 51% of the transplanted cells) with the DAergic differentiation being very poor (0%–46%, with a median of 3% of the transplanted cells) (Comini and Dowd 2024). Interestingly, recent research from our laboratory has shown that encapsulating iPSC-DAPs in a neurotrophic factor (NTF)-enriched collagen hydrogel significantly enhances their survival and differentiation in the rat brain (Comini *et al* 2024). However, their DAergic differentiation still remains low, which is in part due to a) the need for long term sustained availability (∼30 d) of DAergic NTFs for efficient terminal differentiation, as suggested by *in vitro* differentiation protocols (Nolbrant *et al* 2017, Drummond *et al* 2020) and b) the inability of the hydrogel to retain the NTFs beyond a week in the brain (Moriarty *et al* 2017), owing to the short half-lives and rapid metabolism of the NTFs *in vivo* (Granholm *et al* 2000, Ejstrup *et al* 2010, Luz *et al* 2016, Wurzelmann *et al* 2017).

Thus, there is a need to identify alternative ways of safe, targeted, local and minimally invasive delivery of NTFs for their sustained availability to the DAP cells transplanted in the brain. In line with this, our recent study using cryogel microcarriers containing poly(ethylene glycol) diacrylate (PEGDA) and 3-sulfopropyl acrylate (SPA) has shown the ability of these biomaterial microcarriers for sustained delivery of NTFs to the rat brain (Hakami *et al* 2024). Following on from this previous study, the aim of the current study was to assess the impact of these cryogel microcarriers on the engraftment of primary DAergic neurons in the context of CRT in PD, to develop a proof-of principle for the beneficial effects of the microcarriers on the outcomes of such treatment strategies. For further information on the structure, synthesis, loading and characterisation of the cryogel microcarriers, as well as their biological impact *in vitro* and *in vivo*, see Hakami *et al* (2024).

2. Methods

2.1. Experimental Design

To study the impact of sustained GDNF availability via cryogel microcarriers, on the survival and reinnervation of primary DAergic neurons post-transplantation in a rat brain, 39 adult female Sprague–Dawley rats received unilateral intra-medial forebrain bundle (MFB) 6-hydroxydopamine (6-OHDA) lesions (15.42 μg in 3 μl 0.03% ascorbate saline per rat). At ∼3.5 weeks post-lesion, the rats were randomly divided into 4 groups to receive intrastriatal transplants of VM cells (400 000 cells in VM transplantation media; per 6 μl transplant) alone ($n = 9$), or with GDNF (1500 ng per 6 μl transplant), or with unloaded SPA cryogel spheres (comprised of 80% SPA and 20% PEGDA) (control/CTL spheres) (0.01 mg per 6 μl transplant) ($n = 10$), or with GDNF-loaded SPA cryogel spheres (GDNF spheres) (0.01 mg per 6 μl transplant) ($n = 10$). At Week 4 post-transplantation, all the animals were sacrificed by transcardial perfusion-fixation for subsequent *post-mortem* histological assessment. A schematic representation of this experimental design is shown in figure 1.

2.2. Animals

All animal procedures were approved by the Animal Care and Research Ethics Committee at University of Galway and carried out under the licence and authorisation issued by the Irish Health Products Regulatory Authority, in compliance with the European Union Directive 2010/63/EU and S.I No. 543 of 2012. 39 female Sprague–Dawley rats (weighing 200–225 g on arrival) and 9 time-mated female Sprague–Dawley rats (for derivation of primary VM cell suspensions) were sourced from Janvier Labs, France. Animals were housed in groups of three per cage, on a 12:12 h light:dark cycle, at 20 °C–24 °C, with relative humidity levels maintained between 40%–70%. For the duration of the experiment, animals had access to food and water *ad libitum*. All treatment groups were assigned randomly and the *ex vivo* analyses were carried out by researchers blinded to the treatments.

2.3. Preparation of SPA cryogel spheres and GDNF loading into the spheres

The preparation and purification of the cryogel spheres, followed by loading of GDNF into the spheres, were performed as previously described (Hakami *et al* 2024). Briefly, a water-in-oil-emulsion technique was used, where the water phase consisted of a mixture of PEGDA (Mn 700, 455008, Merck, UK) and SPA (31098-20-1, Merck, UK) at

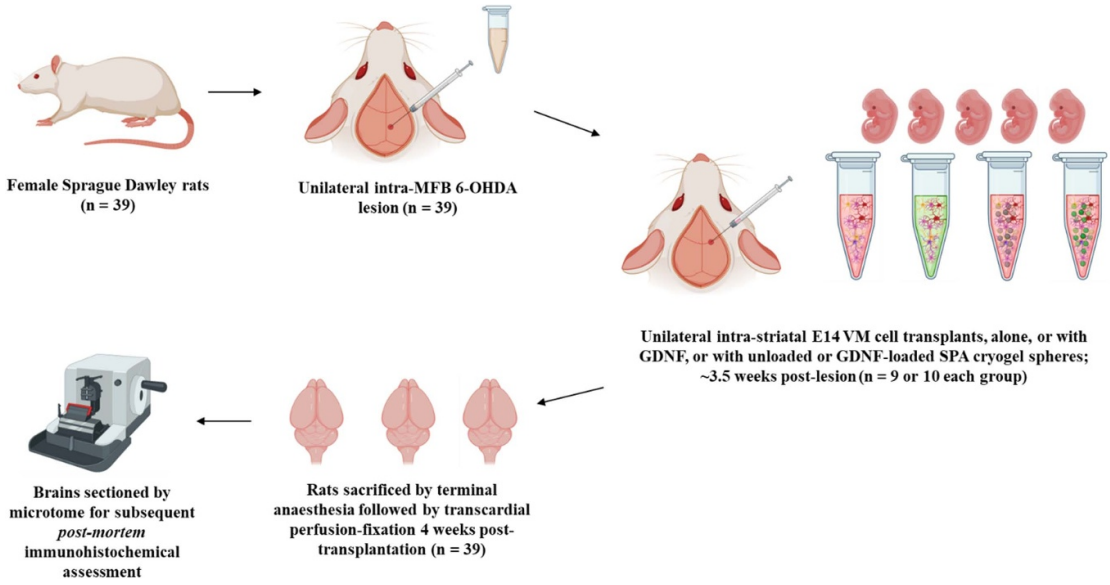

Figure 1. Experimental design. E14 VM cells (400 000 cells) were delivered along with GDNF-loaded cryogel spheres (0.01 mg) and kept on ice prior to unilateral transplantation into the striatum of 6-OHDA-lesioned rat brains. At Week 4 post-transplantation, the brains were assessed for cell survival, striatal reinnervation, and GDNF retention.

a molar ratio of 1:4 (to form spheres with 80% SPA) with a photoinitiator (PEGylated Irgacure 2959, 2 mg ml^{-1}) and a fluorescent probe (iFluor^{TM} 647 maleimide, 0.05% *v*/*v* of a 10 mg ml^{-1} solution) (1131944, Stratech, UK) in deionized water. The oil phase contained perfluoropolyether–polyethylene glycol (PFPE-PEG-PFPE) as a surfactant (2% *w*/*v*) in Novec^{TM} 7500 hydrofluoroether engineered fluid (HFE-7500) (F051243, 3M^{TM}, Fluorochem Ltd, UK). Droplets were generated using a microfluidic device (Fluidic 947) (10001336, Microfluidic ChipShop, Germany) with a *T* junction size of 20 μm. The water phase and oil phase were filled, using needles, into separate 3 ml syringes, which were then separately connected by polyethylene tubes and a soft silicone sleeve, via male mini luer fluid connectors to their specific inlets on the microfluidic device. Syringe pumps (Landgraf Laborsysteme LA-30) were used to pump the oil and water phases at a specific flow rate of 400 $\mu l\ h^{-1}$ and 150 $\mu l\ h^{-1}$, respectively. The resulting emulsion was collected and immersed into an ethanol cooling bath at −60 °C overnight. The following day, the frozen emulsion droplets in the cooling bath were irradiated with UV light (Omnicure S1500, Excelitas, 23 W cm^{-2}, 365 nm) for 3 min to allow for crosslinking in the droplets, thus forming cryogel microcarriers/microspheres. This was followed by purification of the cryogels.

To load GDNF into the cryogel spheres, protein LoBind micro tubes (72.706.600, Sarstedt, Germany) were initially blocked with a blocking solution (PBS + 1% BSA + 0.04% ProClin^{TM} 300, Sigma, 48914-U) for a minimum of 2 h at room temperature (RT). Then, the blocking solution was discarded and the tubes were filled with 1 ml of loading solution (PBS + 0.1% BSA) containing 2000 ng GDNF (Human GDNF, Cell Guidance Systems, GFH2-100) and 0.01 mg SPA cryogels. The tubes were left overnight on a steady bench top at RT. The next day, the tubes were centrifuged at 1300 rpm for 2 min using a microcentrifuge until the pellet of spheres could be seen at the bottom of the tubes. Then, all the supernatant was discarded, and the sphere pellet was washed 3 times with the solution of PBS + 0.1% BSA to wash off any unbound NTFs, and ultimately resuspended in VM transplantation media (DMEM/F12 having 0.6% D-glucose, 1% penicillin–streptomycin, 1% L-glutamine, 1% FBS, and 2% B27 supplement) for transplantation.

2.4. Preparation of E14 VM cell suspension

Using laparotomy, E14 embryos were obtained from time-mated female Sprague–Dawley rats by anaesthesia under isoflurane anaesthesia (5% in 0.5 l O_2) followed by quick decapitation using a guillotine. The VM tissue was micro-dissected from each embryo as previously described (Dunnett and Björklund 1997). The tube containing dissected VM tissue in Hibernate-E medium was centrifuged at 1100 rpm for 5 mins at RT. The supernatant was then discarded, and the tissue pellet was incubated in 40% trypsin–Hank's balanced salt solution at 37 °C with 5% CO_2 for 4 mins. Fetal bovine serum (FBS) was then added to the tube, to neutralise the trypsin, and centrifuged at 1100 rpm for 5 mins at RT. The resultant cell pellet was resuspended in 1 ml VM transplantation media (DMEM/F12 having 0.6% D-glucose, 1% penicillin–streptomycin, 1% L-glutamine, 1% FBS, and 2% B27

supplement), using a P1000 pipette (set at 500 μl), followed by a P200 pipette (set at 100 μl), and finally with a 25-gauge needle and 1 ml syringe. Cell density was then estimated using a haemocytometer.

2.5. Surgeries

All surgeries were performed following aseptic technique, under isoflurane anaesthesia (5% with O_2 for induction and 2% with O_2 for maintenance) using a stereotaxic frame with the nose bar set at −4.5 for intra-MFB lesion surgeries and −2.3 for intra-striatal implant/transplant surgeries. To infuse 6-OHDA (3 μl; at a rate of 1 μl min^{-1}) in the MFB, co-ordinates Antero–posterior (AP) −4.0, medio-lateral (ML) −1.3 from bregma and dorso-ventral (DV) −7.0 below the level of dura were used. To transplant/implant the cells/microcarriers (6 μl; at a rate of 1 μl min^{-1}) in the striatum, co-ordinates AP 0.0, ML ±3.7 from bregma and DV −5.0, −4.5, and −4.0 (2 mins per level) below the level of dura were used. The infusion period was followed by a 2 min diffusion period.

2.6. Immunohistochemistry (IHC)

Animals were sacrificed by terminal anaesthesia (140 mg kg^{-1} pentobarbital intraperitoneal (i.p)) followed by transcardial perfusion of heparinised saline (5000 units per litre) followed by fixation using 4% paraformaldehyde (PFA). The extracted brains were then placed at 4 °C in 4% PFA overnight, before storing in a 25% sucrose solution containing 0.1% sodium azide for cryoprotection until they sink to the bottom of the storage container. Then, serial coronal sections (30 μm) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, UK) and the tissue sections were collected in a series of 12, into wells of 24 well plates containing TBS azide at pH 7.4. Free floating IHC for tyrosine hydroxylase (TH) and GDNF was performed as previously described (Hoban *et al* 2013, Moriarty *et al* 2017, Cabré *et al* 2021). In brief, the endogenous peroxidase activity was first quenched with a solution of 3% hydrogen peroxide (H_2O_2) and 10% methanol in distilled water. Then, sections were incubated with a solution of 3% serum (normal horse serum) in TXTBS was used to block the non-specific binding. Then, a solution of primary antibody (Mouse anti-TH, 1:1000; Merck, Mouse anti-GDNF, 1:200; R & D systems) and 1% serum in TXTBS was added to the sections and left overnight at RT. On the following day, sections were incubated in a solution having biotinylated secondary antibody (Horse anti–mouse, 1:200, Fisher Scientific) and 1% serum in TBS. *A* streptavidin–biotin–horseradish peroxidase solution (0.5% reagent *A* + 0.5% reagent *B* and 1% serum in TBS) was then added to the sections and incubated for 2 h. Then, the sections were incubated in a solution of TNS having 0.5 mg ml^{-1} of diaminobenzidine tetrahydrochloride and 0.3 μl ml^{-1} of a 30% H_2O_2 solution, to develop staining. The stained sections were then mounted onto gelatin-coated glass slides and left to dry. The dried slides were dehydrated in a series of ascending alcohols, cleared in xylene, and coverslipped using DPX mountant (Sigma).

2.7. Image analysis

Immunostained sections were imaged using an Olympus VS120 Virtual Slide Microscope. The graft volume, reinnervation density, reinnervation volume, graft density, and GDNF density were assessed using ImageJ software (U.S. National Institute of Health), as previously described (Hoban *et al* 2013, Moriarty *et al* 2017). For measuring graft volume and reinnervation volume, the graft areas and reinnervation areas were measured in a 1-in-6 series through the striatum and converted to volumes using Cavalieri's principle. For cell counts, TH^+ cell bodies were counted in a 1-in-6 series through the striatum, and these were corrected using Abercrombie's principle. The GDNF density was measured in a 1-in-12 series through the striatum, while the TH^+ reinnervation density and graft density were measured in a 1-in-6 series through the striatum.

2.8. Statistical analysis

All data are shown as graphs of individual data points with mean ± standard error of mean (SEM) and were analysed using 1-way analysis of variance (ANOVA), with Tukey's *post-hoc* test, when needed. Throughout the results text, the main effects from the initial ANOVA are cited in the body of the results, while the results of the *post-hoc* analyses are shown in the corresponding figure and explained in the figure legend.

3. Results

3.1. Cryogel spheres retain the delivered GDNF up to 4 weeks in the brain

PEGDA-SPA cryogel spheres have previously shown to be capable of delivering NTFs for up to 2 weeks in the implant site in the brain (Hakami *et al* 2024). So, to assess the ability of the cryogel spheres for longer retention times of the NTFs (i.e. up to 4 weeks) in the brain, in the current study, assessment of GDNF immunostained brain sections revealed the intact presence of the cryogel spheres within the graft sites and also the retention of GDNF within the implanted cryogel spheres up to 4 weeks in the brain (figure 2; Group, $F_{(3,\,34)} = 5.45$, $P < 0.01$). In addition, GDNF immunostaining revealed no GDNF in the brains when administered as a bolus injection (figure 2).

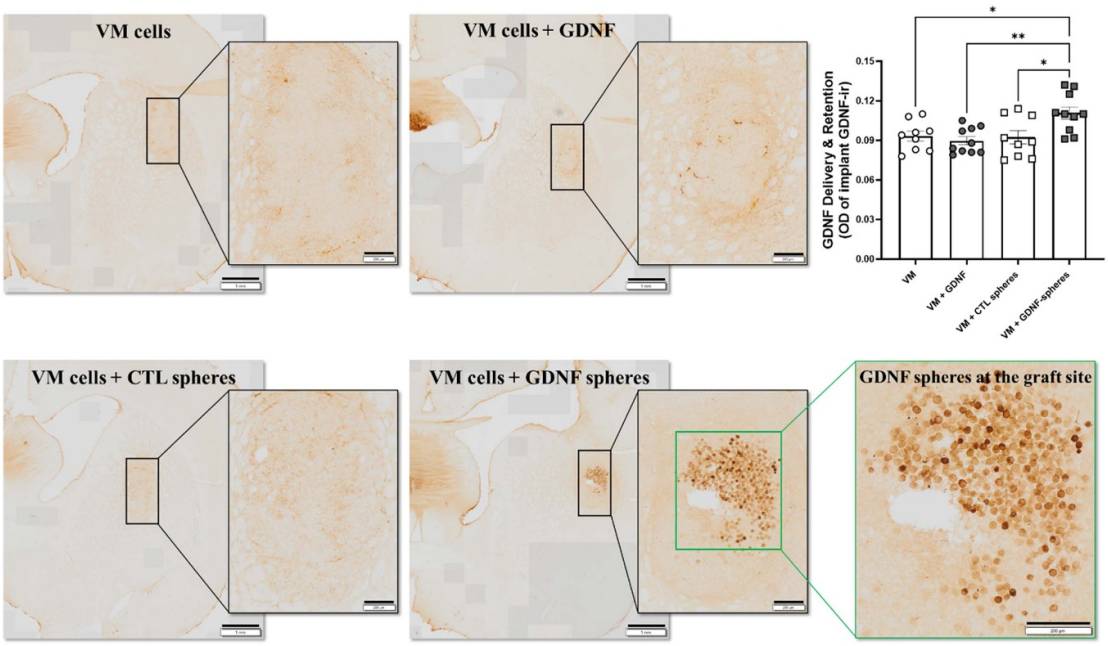

Figure 2. GDNF retention within the implanted cryogel spheres at 4 weeks in the brain. Quantification of the density of GDNF immunostaining revealed a significantly higher presence of GDNF in the implant sites of grafts containing GDNF-loaded cryogel spheres. Visual inspection of the immunostained sections revealed the presence of GDNF within the cryogel spheres at the graft sites, indicating their ability for long-term retention of the delivered NTFs. Data represented as photomicrographs and mean ± SEM of $n = 9$ or 10 rats. Data analysed by one way ANOVA (*A*, *B*). $^{*}P < 0.05$, $^{**}P < 0.01$ by one way ANOVA with Tukey's *post-hoc*. Scale bar represents 1 mm, 200 μm and 200 μm (inset).

3.2. GDNF-loaded cryogel microcarriers improve the survival of primary dopaminergic neurons, in addition to enhancing the sprouting of fibres within, and out from the graft, leading to an enhanced striatal reinnervation

As this was the first time that rat primary dopaminergic neurons were transplanted along with the cryogel spheres, we first wanted to confirm the *in vivo* compatibility of the cryogel spheres with the primary dopaminergic grafts. TH^+ staining confirmed the survival of dopaminergic grafts and successful transplantation of the dopaminergic neurons in all the groups (figure 3). No differences were seen in the graft volumes between the groups (figure 3(A); Group, $F_{(3, 34)} = 1.01$, $P > 0.05$).

The graft survival was also assessed by counting the number of TH^+ cell bodies in the striatal graft site. While statistically no significant differences were seen between the groups, in the number of TH^+ cell bodies present in the grafts (figure 3(B); Group, $F_{(3, 34)} = 2.45$, $P > 0.05$), there was a strong trend for higher cell survival when cells were delivered along with GDNF-loaded cryogel spheres than when delivered alone (1.9-fold), or with GDNF (1.6-fold), or with CTL spheres (1.7-fold). Additionally, 0/9 rats in the 'VM cells alone' group versus 7/9 rats in the 'VM cells + GDNF spheres' group had >6,000 surviving TH^+ cells in the grafts (figure 3).

We then assessed the re-innervation of the surrounding striatal tissue by the graft-derived neuronal outgrowths. TH immunostained brain sections were analysed to determine the density of the TH^+ fibres extending out from the graft into the immediate peri-graft region, and the volume of the striatum into which these fibres were growing out. TH^+ fibres were seen growing out from the graft and extending into the surrounding striatum in all the groups (figure 4). However, an enhanced density of TH^+ fibres were seen to be growing out from the grafts that contained cells along with GDNF-loaded cryogel spheres (figure 4(A); Group, $F_{(3, 30)} = 4.66$, $P < 0.01$). Furthermore, although no statistically significant differences were seen in the volume of striatal reinnervation by the graft-derived fibres (figure 4(B); Group, $F_{(3, 30)} = 2.18$, $P > 0.05$), a strong trend for higher volumes of striatal reinnervation by the TH^+ fibres was seen from the grafts that contained cells along with GDNF-loaded cryogel spheres, when compared to grafts of cells alone (1.95-fold) (figure 4(B)).

In addition to an enhanced sprouting of TH^+ fibres out from the grafts containing GDNF-loaded cryogel spheres, we wanted to assess if the neuronal sprouting within the graft site itself was also enhanced in the presence of GDNF-loaded cryogel spheres. In grafts containing cells along with GDNF-loaded cryogel spheres, regions of clearly enhanced

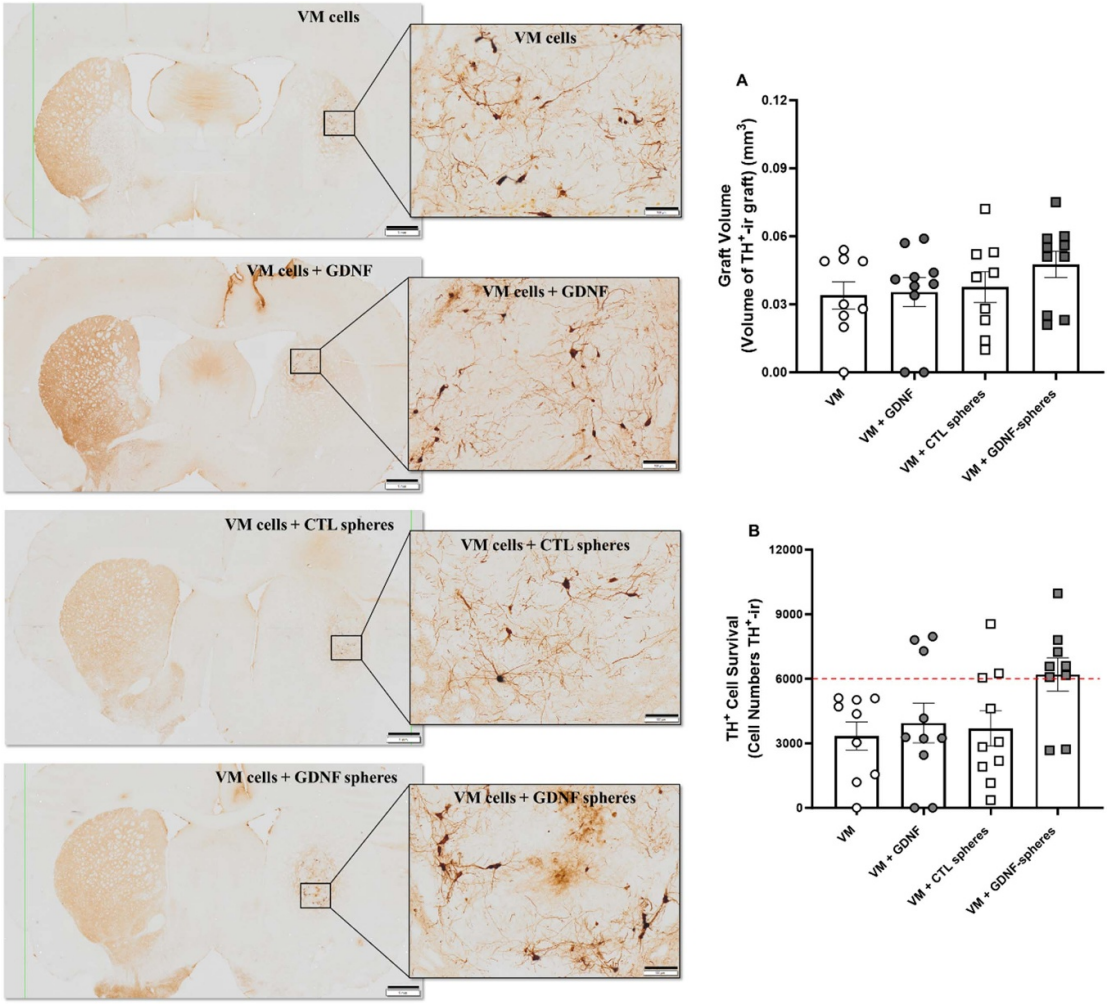

Figure 3. *In vivo* compatibility of SPA cryogel spheres with, and their impact on the survival of, rat primary dopaminergic neurons, after 4 weeks of co-transplantation in the 6-OHDA-lesioned rat brain. Measurements of the graft volume (A) and the number of surviving cells (B) in the TH-immunostained grafts revealed the *in vivo* compatibility of the cryogel spheres with the co-transplanted primary dopaminergic neurons. A trend for enhanced TH^+ cell survival was seen in the grafts that contained cells along with GDNF-loaded cryogel spheres. Data represented as photomicrographs and mean ± SEM of $n = 9$ or 10 rats. Data analysed by one way ANOVA (*A*) and (*B*). Scale bar represents 1 mm and 100 μm (inset).

sprouting of TH^+ fibres were seen within the graft site (figure 5). Quantification of the density of TH^+ immunostaining revealed significantly higher TH^+ fibre sprouting within the graft sites containing cells along with GDNF-loaded cryogel spheres, than in the grafts that contained cells alone, or with GDNF, or with CTL spheres (figure 5; Group, $F_{(3,34)} = 6.26$, $P < 0.01$).

3.3. Correlations between E14 VM graft survival and striatal reinnervation, and graft density and striatal reinnervation

After observing that delivering primary dopaminergic neurons along with GDNF-loaded cryogel spheres results in (a) a trend for higher graft survival, (b) higher striatal reinnervation, and (c) higher neuronal sprouting within the graft, we aimed to determine if an increase in the TH^+ cell survival or TH^+ density within the graft correlated with an improvement in the striatal reinnervation by the graft.

A strong correlation was seen between the number of surviving dopaminergic neurons in the graft and (a) the density of neuronal outgrowths/fibres growing out from the graft (figure 6(A); $r = 0.83$, $P < 0.0001$), and (b) the volume of the striatum into which the fibres were extending out (figure 6(B); $r = 0.90$, $P < 0.0001$). A strong correlation was also seen between the density of TH^+ immunostaining within the graft and (a) the density of neuronal outgrowths/fibres growing out from the graft (figure 6(C); $r = 0.87$, $P < 0.0001$), and (b) the volume of the striatum into which the fibres were extending out (figure 6(D); $r = 0.81$, $P < 0.0001$).

This indicates that delivering primary dopaminergic neurons along with GDNF-loaded cryogel spheres results in an enhanced graft survival which in

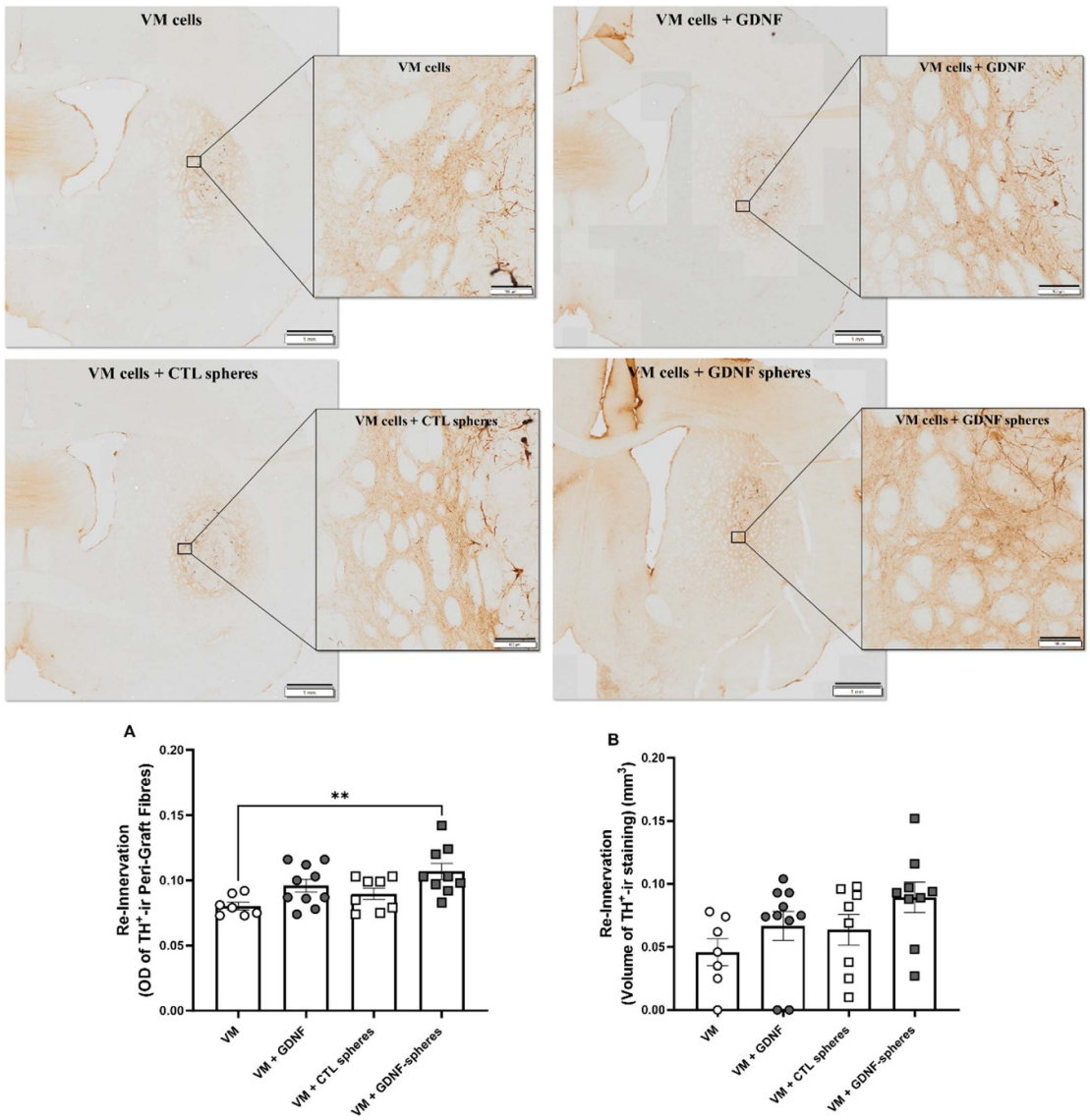

Figure 4. Impact of GDNF-loaded cryogel spheres on the striatal reinnervation by primary dopaminergic grafts at Week 4 post-transplantation in the 6-OHDA-lesioned rat brain. Quantification of the density (A) and volume (B) of striatal reinnervation by the graft-derived TH^+ fibres in the TH-immunostained brain sections revealed an enhanced density of fibres extending out into the peri-graft region from the grafts containing cells along with GDNF-loaded cryogel spheres (A). Additionally, a strong trend for enhanced volume of the striatum into which these graft-derived fibres were extending was seen in the presence of GDNF-loaded cryogel spheres (B). Data represented as photomicrographs and mean ± SEM of $n = 9$ or 10 rats. Data analysed by one way ANOVA (*A*) and (*B*). $^{**}P < 0.01$ by one way ANOVA with Tukey's *post-hoc*. Scale bar represents 1 mm and 100 μm (inset).

turn triggers an enhanced sprouting of TH^+ fibres, both within the graft itself, and out from the graft into the surrounding striatum.

4. Discussion

In the TRANSEURO clinical trial for the safety and efficacy assessment of human fetal VM cell transplants (NCT01898390), the patients at an early stage of PD, who show a significant improvement in their motor scores in response to dopamine therapy, in addition to several other criteria were included, with the hypothesis that the subjects who satisfy these inclusion criteria would benefit the most from the dopaminergic grafts in CRT. Barker and consortium (2019) also recommended to use a similar approach of patient selection criteria for the future trials of stem cell-derived DAPs. The stem cell-derived DAP transplants for CRT have several advantages over fetal-derived DAP cells, including (1) a more ethically-acceptable source, (2) large availability of transplantable cells, and (3) opportunity for several patients to receive the dopaminergic grafts.

Although stem cell-derived dopaminergic CRT has reached testing in multiple clinical trials (NCT04802733, NCT05635409, NCT05887466, UMIN000033564, UMIN000033565), the grafts may not show any effects on the dopamine–resistant

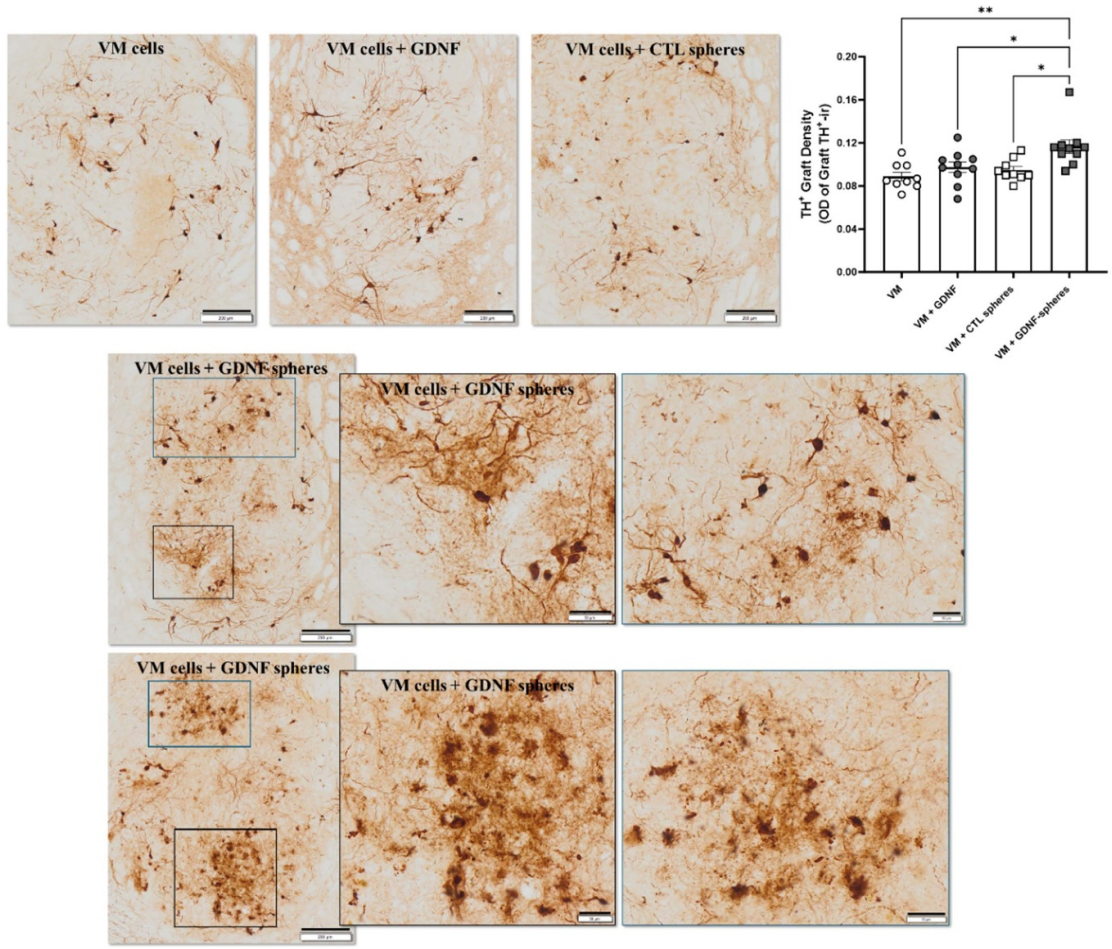

Figure 5. Impact of GDNF-loaded cryogel spheres on the sprouting of TH^+ fibres from the cells within the graft site itself, at Week 4 post-transplantation in the 6-OHDA-lesioned rat brain. Quantification of the TH immunostaining within the striatal graft sites revealed an enhanced neuronal sprouting in the graft sites containing cells along with GDNF-loaded cryogel spheres. Data represented as photomicrographs and mean ± SEM of $n = 9$ or 10 rats. Data analysed by one way ANOVA (*A*) and (*B*). $^{*}P < 0.05$, $^{**}P < 0.01$ by one way ANOVA with Tukey's *post-hoc*. Scale bar represents 200 μm and 50 μm (inset). See figure 3 for lower power magnification of graft site for striatal context.

motor features and the non-motor symptoms associated with the disease, and may thus only be clinically beneficial in patients with a predominant motor symptomatology (Barker and consortium, 2019). So, for the transplants to induce a significant motor improvement, an optimal and efficient survival and dopaminergic differentiation would be essential. However, data from preclinical studies of stem cell-derived DAPs indicate variable graft survival and poor dopaminergic differentiation (Comini and Dowd 2024), suggesting that such transplants might have poor efficacy in clinical trials. With no clinical trial yet showing the long-term efficacy of stem cell-derived grafts, and given that just ∼1% of the transplanted DAP cells differentiate into TH^+ dopaminergic neurons in the brains of non-human primates (Comini and Dowd 2024), the stem cell-derived transplants may not be efficacious enough, when grafted on their own. So, it is imperative to continue preclinical research to enhance the outcomes and fully realise the therapeutic & reparative potential of such strategies.

The variable survival and poor dopaminergic differentiation of the stem cell-derived DAP grafts is in part because the cells are lifted at an immature/-progenitor stage from an NTF-rich culture (Nolbrant *et al* 2017, Drummond *et al* 2020) and transplanted into the neurotrophin-impoverished adult diseased brain. Thus, there is a need to identify strategies to replicate the sustained *in vitro* availability of NTFs, like GDNF, for long time periods (∼30 d) for efficient dopaminergic differentiation post-transplantation in the brain (Drummond *et al* 2020). In this context, when iPSC-derived DAPs were delivered to the striata of 6-OHDA-lesioned rat brains by encapsulation in an NTF-enriched collagen hydrogel, although their survival and differentiation were significantly enhanced, the dopaminergic differentiation still remained quite low (Comini *et al* 2024). This could potentially be due to the inability of the hydrogel to retain the delivered NTFs for longer than a week in the brain (Moriarty *et al* 2017).

As previously reported, NTFs, when administered as a single dose bolus injection, is not retained beyond

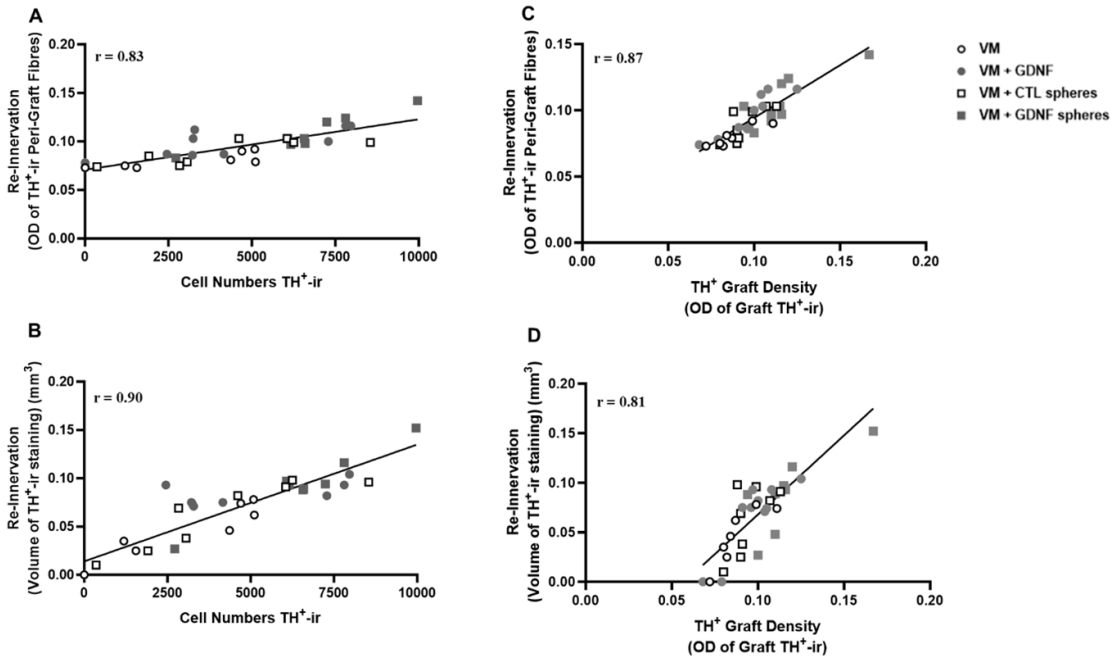

Figure 6. Correlation between graft survival and striatal reinnervation (*A*) and (*B*) and graft density and striatal reinnervation (C), (D). A strong positive correlation (Pearson) was seen between the dopaminergic neuronal survival and the density of TH^+ fibres extending out from the graft (A) and the striatal volume into which the fibres were extending (B). A strong correlation was also observed between the density of TH^+ fibres sprouting within the graft and the density of the TH^+ fibres sprouting out from the graft (C) and the striatal volume into which the fibres were extending (D).

a week in the rat brain due to their relatively short half-lives (i.e. 3–4 d in the brain) (Granholm *et al* 2000, Ejstrup *et al* 2010, Luz *et al* 2016, Wurzelmann *et al* 2017), whereas cryogel spheres made of PEGDA and SPA have the ability for long-term sustained delivery of NTFs to the brain (Hakami *et al* 2024), and can potentially enhance the outcomes (survival and differentiation) of the cells transplanted for CRT in PD. *In vitro* studies reported in Hakami *et al* (2024) have previously demonstrated the cytocompatibility of these cryogel spheres, both unloaded and NTF-loaded, with neuronal and microglial cell lines, in addition to a functional enhancement of the metabolic activities of rat primary neural cultures in the presence of NTF-loaded cryogel spheres. The *in vivo* biocompatibility of implants of these cryogel spheres in the brain have also been studied, confirmed, and reported in Hakami *et al* (2024). Immunohistochemical studies for assessing the host neuroimmune response towards the cryogel implants were conducted using immunostaining for host microglia and astrocytic cells, and revealed no additional neuroimmune response when compared to implants of vehicle alone (Hakami *et al* 2024).

Overall, the work in this study showed that delivering rat E14 VM cells along with GDNF-loaded PEGDA-SPA cryogel spheres resulted in a 1.9-fold increase in the primary dopaminergic cell survival, a 1.95-fold increase in the striatal reinnervation by the graft, and enhanced sprouting of TH^+ fibres within the graft site, due to the ability of the cryogel spheres to retain the delivered GDNF up to 4 weeks in the brain. There was no GDNF or TH^+ (cell body) staining on the intact side of the rat brain, or on the lesioned side of the brain outside of the graft site, indicating that all GDNF and TH staining on the lesioned side was graft/implant–derived. These findings highlight the potential of such biomaterial microcarrier-aided NTF delivery strategies to enhance the outcomes of the cells transplanted in the context of CRT in PD.

These PEGDA-SPA cryogel spheres have previously shown their ability to retain the delivered NTFs up to 2 weeks in the brain, while no NTFs were retained by Day 7 post-bolus administration in the brains (Hakami *et al* 2024). Following on from this, in the current study, we aimed to assess the retention of the delivered GDNF up to 4 weeks in the brain. At Week 4 post-transplantation, while no GDNF was present in the brains when administered as a bolus injection, the cryogel spheres were present intact in the graft sites and showed their ability to retain the delivered GDNF within the graft site at this time point. Histological assessment of the effect of long-term retention of GDNF in the graft site, on the grafted cells, revealed no differences in the graft volumes between any transplant groups (i.e. cells alone, or with GDNF, or with CTL spheres, or with GDNF spheres), but 0/9 rats in the 'cells alone' group, and 7/9 rats in the 'cells + GDNF spheres' group showed presence of >6,000 surviving TH^+ cells in the graft. The survival of TH^+ cells when transplanted with GDNF

spheres was 1.7-fold higher than when delivered with CTL spheres, suggesting that the GDNF delivered by the cryogel spheres was functional and beneficial. This is an encouraging finding because, in previous studies, no benefit on the survival of the grafted primary dopaminergic cells was seen when GDNF was delivered by loading into poly(lactic-co-glycolic acid) microspheres (Clavreul *et al* 2006) or polymer rods (Törnqvist *et al* 2000), due to a burst release of GDNF, from the former, on the 1st day and negligible release in the following days, and an insufficient GDNF release (only ∼10 ng d^{-1}), from the latter, in the initial days post-transplantation when the majority of the transplanted cells die (Barker *et al* 1996, Emgård *et al* 1999, Sortwell *et al* 2000). However, due to optimal release kinetics of GDNF from the cryogel spheres (Hakami *et al* 2024), the beneficial effects on the grafted cell survival were manifested in this current study. Also, while previous studies have either used GDNF at higher doses (∼3–67 times higher than the dose used in the current study) or by repeated administration (i.e. every 3rd day for 3 weeks, or daily for 10 d) to show an enhanced survival of the grafted primary dopaminergic neurons (Rosenblad *et al* 1996, Sinclair *et al* 1996, Sullivan *et al* 1998), it is quite promising to see such beneficial effects in this study by simply using a single administration of GDNF-loaded cryogel spheres.

Grafts containing cells along with GDNF-loaded cryogel spheres showed a significantly enhanced density of TH^+ fibres extending out from the graft into the immediate peri-graft region and a 1.95-fold increase in the volume of striatum into which these fibres were extending out. Previous studies that have reported an increase in the TH^+ fibre outgrowth from primary dopaminergic grafts have used either repeated GDNF administration via invasive cannulae/ports/pumps (Rosenblad *et al* 1996, Sinclair *et al* 1996, Yurek 1998) or have used genetically modified cells that overexpress GDNF by *ex vivo* gene therapy (Sautter *et al* 1998, Wilby *et al* 1999, Ahn *et al* 2005, Perez-Bouza *et al* 2017). It is therefore encouraging to see that a relatively safe and minimally invasive strategy of using biomaterial-based cryogel spheres can offer sustained GDNF availability, at amounts needed for an enhanced TH^+ fibre outgrowth from the grafted cells.

An enhanced density of TH^+ fibres within the graft site itself, has been reported in previous studies, upon sustained availability of GDNF (Rosenblad *et al* 1996). In line with this, in the current study, a significant enhancement in the sprouting of TH^+ fibres was seen in the graft sites that contained cells along with GDNF-loaded cryogel spheres, suggesting a positive impact of the long-term GDNF availability to the grafted cells. Additionally, a strong positive correlation was seen both (a) between the graft survival and the reinnervation of the host (i.e. both, the density of TH^+ fibres reinnervating the host, and the volume of reinnervation by the graft) and (b) between the sprouting of TH^+ fibres within the graft and the sprouting of TH^+ fibres out from the graft, indicating that when cells are delivered along with GDNF-loaded cryogel spheres, it triggers an enhanced TH^+ cell survival resulting in an enhanced sprouting of TH^+ fibres from the cells within the graft site, and also an enhanced sprouting of TH^+ fibres growing out from the graft site into the surrounding striatal tissue.

In summary, the cryogel spheres were able to retain the delivered GDNF up to 4 weeks post-transplantation, demonstrating their efficacy for long-term retention of neurotrophins in the brain. This resulted in positive graft-based outcomes like, (a) enhanced cell survival, (b) enhanced reinnervation of the host by the graft, and (c) enhanced sprouting of TH^+ fibres within the graft site.

5. Conclusion

The present study shows, for the first time, that GDNF-loaded cryogel spheres enhance the survival of, and reinnervation by, the primary dopaminergic neurons by providing a sustained availability of GDNF to the transplanted graft. Therefore, this study shows the significant potential of biomaterial microcarrier-based NTF delivery strategies for enhancing the outcomes of cells grafted in the context of CRT in PD. Moving forward, preclinical studies will be needed to assess the impact of such NTF-loaded cryogel spheres on the survival and differentiation of stem cell-derived DAPs in the context of stem cell-derived CRT in PD.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Conflict of interest

The authors declare no competing financial or non-financial interests.

Author contributions

E D led the design and implementation of the project and wrote the manuscript with K N; K N completed the embryonic tissue harvesting and VM microdissection and the *in vivo* aspects of the study with assistance from G C and T P; K N completed the *post-mortem* analyses; A H and B N generated the cryogel microcarriers.

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