Developing A New Strategy for Delivery of Neural Transplant Populations using Precursor Cell Sprays and Specialised Cell Media

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Aerosolization; neural repair; stem cell spray, cell delivery; traumatic injury

Abstract
Neural precursor/stem cell transplantation therapies promote regeneration in neurological injuries but current cell delivery methods have drawbacks. These include risks with surgical microinjection (e.g. haemorrhage, embolism), and high cell loss with systemic delivery/passage through fine gauge needles. Aerosolized cell delivery offers significant benefits including rapid and minimally invasive cell delivery, and ease of delivery to end users. To develop this approach, it is necessary to prove that (a) aerosolization does not have detrimental effects on transplant cells and (b) suitable media can be identified to support cell delivery. To achieve these aims, cells were sprayed using a commercial spray device or stored in Hibernate-A™, a CO2 independent nutrient solution. Histological assessments consisted of cell viability analysis, immunocytochemistry and EdU labelling. We show that a major neural precursor transplant population – oligodendrocyte precursor cells (OPCs) – survive following aerosolized delivery and retain their capacity for proliferation and differentiation (key to their repair function). Hibernate-A™ could support OPCs survival without specialised maintenance conditions, with no detrimental impact on cell fate. We consider this data supports the concept of a novel class of advanced medical spray devices to facilitate transport and delivery of transplant populations in neural cell therapy.

Introduction

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Neural cell transplantation therapies are being utilised for several neurological injuries and diseases, with proven benefit.[1–5] The potential mechanisms of pro-regenerative actions include replacement of lost cells, immunomodulatory effects and expression of therapeutic proteins such as growth factors or enzymes for glial scar breakdown, which result in an environment more conducive to regeneration.[6–8]

Neural cell therapies (for clinical use or preclinical testing) are usually delivered through fine bore stereotactic cannulae directly into the brain/spinal cord parenchyma. Such invasive delivery methods have significant drawbacks from a patient safety and cell therapy efficacy perspective. Needle or instrument insertion causes mechanical trauma and carries haemorrhage risk,[2,9–12] of particular concern for traumatic injuries where injection into injured tissue increases risks of clinical complication. Transplant cells can be damaged due to the mechanical pressure required to inject cells through a fine bore cannula into densely packed neural tissue;[9,13] studies suggest that less than ca 5% of cells survive in-vivo.[14–16] Transplant solutions for in vivo applications contain high cell densities. For example, 20 million neural stem cells in 400 µL of HypoThermosol (5 x 10^7 cells/mL) were injected into the putamen of stroke patients in one recent clinical trial.[17] Cell death and clumping in these suspensions means that injecting cells into such injuries does not achieve homogenous cell delivery. Insertion of multiple injection cannulae into the neural parenchyma carries a risk of introducing pathogens when instruments are inserted from the external environment through neural tissues, which in clinically vulnerable patients, could lead to adverse outcomes.

Alternative methods for cell delivery include vascular administration such as intravenous or intra-arterial delivery. However, few cells reach target tissues due to systemic clearance by organs such as the lungs and spleen and the risk of embolism is significant.[18–23] Given these barriers to clinical translation, there is a need to develop efficacious transplant delivery strategies to support administration of neural cell therapy in complex and serious injuries.

Aerosolized delivery of neural transplant cells could potentially provide a novel and realistic solution to this translational challenge. It can be envisaged that transplant populations could be transported to the end user in a sterile format that allows for cell spray delivery. This approach to provision of cells is rapid, does not require specialised stereotactic equipment and is compatible with early surgical procedures exposing neural tissue. The minimally invasive nature of spraying from a short distance onto neural tissue has no predictable clinical complications. For traumatic neural injuries, cells could be delivered with homogenous distribution over areas of extensive pathology/damage. We predict spraying will reduce the risk of pathogen introduction to the patient.

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as no instrumentation would come into direct contact with the patient during spray delivery unlike current other methods of injection. The potential advantages of a neural cell spray are summarised in Figure 1.

Cell spray technology has been used in a limited number of non-neurological clinical therapies. ReCell® technology is an alternative to skin grafts for partial thickness burn wounds where a patient skin biopsy is used to generate a mixture of keratinocytes, melanocytes and fibroblasts which is spray delivered onto the burn site.\[^{24}\] An airbrush spray has also been adapted to deliver chondrocytes leading to cartilage repair for osteoarthritic knees.\[^{25}\] A single study assessed an intra-nasal mesenchymal stem cell delivery approach as an inexpensive and non-invasive cell delivery technique to circumvent the blood brain barrier. When tested in a rodent model of Parkinson’s disease, an improvement in motor function was reported.\[^{26}\] To the best of our knowledge, however, a spray delivery strategy has never been tested for the direct delivery of neural transplant cells to sites of traumatic injuries.

Critical to the success of such a delivery approach, including to end users, would be the identification of suitable media that can support the safe transport of cells. Ideally, this would be

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*Figure 1. Advantages of a neural cell spray for transplantation of neural cell therapies. Image created using Biorender.com.*
achieved without the use of specialised maintenance or transport conditions, such as cold chain delivery, which can add substantially to logistical complexity and cost.

To address these issues, this study has used the major neural transplant population of oligodendrocyte progenitor cells (OPCs) to establish whether: (i) OPCs can survive aerosolization without detriment to key regenerative properties of the cells; and (ii) a widely used tissue transport medium (Hibernate-A™) can support OPC maintenance/survival and subsequent cell recovery without the requirement for specialised growth conditions. OPCs are a highly promising transplant population for neuro-regeneration having been utilised in clinical trials for acute spinal cord injury (SCI) and multiple sclerosis.[27,28] We present data to support the concept that transport and spray delivery of neural transplant cells for neurological applications is feasible.

Results
OPCs retained high cell viability post-spraying
Immediately following spraying, light microscopy examination revealed even coverage of plate surfaces by OPCs. Viable OPCs were observed 48 hours post-spraying, with large numbers of viable cells observed in control and experimental conditions and displaying similar morphological profiles between conditions (Figure 2a-b). Quantification revealed OPC viability at 48 hours following spray delivery was reduced (58.6 ± 11.7%) compared to controls (84.5 ± 2.2%; Figure 2c). In contrast, following differentiation after spraying, the viability of the spray delivered cells (70.8 ± 15.1%) did not significantly differ from controls (76.9 ± 19.6%; Figure 2d-f).

Figure 2. OPCs show high viability following spray delivery. Live cells were stained with calcein (green arrow), dead cells with ethidium homodimer (EH; red arrow) and cell nuclei stained blue with Hoechst dye. (a-b) Representative, (c) Cell viability, 48h. (d-e) Cell viability post-differentiation.
Spray delivered cells retained cell specific marker expression and proliferative capabilities

At 48 hours, NG2 staining revealed OPCs with typical bipolar and multipolar morphology in both control and spray conditions, with no obvious differences in cell morphologies between conditions (Figure 3a-b). The proportions of cells expressing NG2 at 48 hours (Fig. 3a-c) did not differ significantly between spray delivered cells (78.2 ± 13.2%) and controls (89.4 ± 8.3%). OPCs also continued to proliferate following spray delivery and the proportions of proliferating OPCs (labelled with EdU over a 24 h period) post-spray (37.6 ± 18.4%) did not statistically differ from controls (41.6 ± 11.6%; Figure 3a-b, d).

Figure 3. Sprayed OPCs express NG2 and retain their ability to proliferate. (a-b) Representative triple-merged fluorescence images of OPCs showing NG2 (green) and EdU staining (red) and cell nuclei stained with Hoechst dye (blue) 48 hours after control and spray delivery respectively. Scale bars = 50µm. Scatter graph (c) illustrates the proportions of cells expressing NG2 in control and sprayed cells with no significant difference detected (p-
value=0.240, Mann-Whitney test, n=6). Scatter graph (d) shows the proportions of proliferating OPCs in control and sprayed cells at 48 hours with no significant difference detected (p-value=1.0000, Mann-Whitney test, n=3).

OPCs retained their differentiation capacity following spray delivery
OPCs differentiated for 8 days post spraying showed the range of expected oligodendrocyte lineage cell (OLC) morphologies and increased cellular branching with greater developmental age (Figure 4a). Depending on the stage of maturation, cells were observed to express NG2 or the mature oligodendrocyte marker MBP (Figure 4b-e). At this time point, OPCs represented ca 12% of cells, with immature phenotypes representing ca 70% and mature phenotypes representing the remainder in sprayed populations. For controls, OPCs represented ca 10% of cells, immature phenotypes ca 60% with mature phenotypes making up the remainder of cells. There was no statistical difference in the proportions of mature cells expressing MBP or the proportions of each cell type at different stages of the lineage, between control and sprayed cells (Figure 4f-g).
Sprayed OPCs are capable of differentiating into oligodendrocytes. Cartoon (a) demonstrates the maturation stages in the oligodendrocyte lineage; OPC, pre-oligodendrocyte, immature oligodendrocyte and mature oligodendrocyte. Fluorescent images (b-e) illustrate the different stages of maturation following spray delivery of OPCs where scale bar = 10µm. Representative images (f) and (g) show day 10 differentiated OLCs control and spray delivered respectively with scale bars = 50µm. Scatter graph (h) shows the proportion of MBP expressing control and spray cells at 10 days with no difference noted (p=0.200, Mann-Whitney test, n=3). Graph (i) shows the relative proportions of oligodendrocyte lineage cell morphologies between the spray and control at day 10. There was no significant difference in any categories of
oligodendrocyte lineage morphology between the spray and control tested by individual Mann-Whitney tests (p-values > 0.05, n=3).

OPCs retained a high viability following RT (room temperature) or 4°C storage in Hibernate-A™. Viable OPCs were observed following 72 hours of storage at control, RT and 4°C conditions (Figure 5a-c). Quantification revealed that OPC viability remained high following 72 hours of storage in Hibernate-A™ at RT (76.26% ± 6.92) and 4°C (83.34% ± 3.71) and was similar to controls (76.18% ± 8.47; Figure 5d). Cells stored at the lower temperatures exhibited rounded morphologies (Figure 5a-c). However, following return to 37°C and differentiation for 10 days, viable differentiated cells were clearly observed. These showed the branched/multipolar phenotypes characteristic of OPCs/immature oligodendrocytes with no obvious differences between experimental and control conditions (Figure 5e-g).

**Figure 5. OPC viability and differentiation after storage in Hibernate-A™.** Live cells were stained with calcein (green arrow), dead cells with EH (red arrow) and cell nuclei stained blue with Hoechst dye. (a-c) Representative, tripe-merged fluorescent images showing OPCs following 72-hour storage in a) control, b) RT Hibernate-A™ and c) 4°C Hibernate-A™ conditions. (d) Scatter graph displaying percentage viabilities of OPCs at 3 days with no significant differences between conditions (p > 0.05, Kruskal-Wallis test, n=4). (e-g) Triple-merged fluorescent images showing viable and mature OLC morphologies after differentiation for 7 days following 72 hours storage in e) control conditions, f) RT Hibernate-A™ and g) 4°C Hibernate-A™ respectively. All scale bars = 50µm.

OPCs retained specific cellular markers, proliferation and differentiation capabilities following storage at RT or 4°C in Hibernate-A™. Following recovery from lower temperature storage and then 24 hours culture in OPC maintenance medium at 37°C, there was no obvious differences in cellular morphology between the control and experimental conditions (Figure 6a-c). The proportion of cells positive for NG2 did not change.
significantly differ between OPCs controls (84.33% ± 9.01), storage at RT (78.02% ± 9.71) or 4°C (85.51% ± 8.32; Figure 6a-d). OPCs continued to divide during the 24-hour period of cell culturing immediately following removal from lower temperature storage, with cells in all experimental conditions showing positive EdU staining. There was no significant difference between the proportion of dividing OPCs in the controls (25.47% ± 5.72), following storage at RT (12.87% ± 5.49) or 4°C (27.92% ± 3.86; Figure 6a-c, i).

OPCs removed from lower temperature storage and differentiated for 7 days showed the range of expected OLC morphologies whilst expressing early (NG2) or late (MBP) surface markers depending on developmental age (Figure 6e-g). No difference in the expression of MBP was seen following differentiation in the controls (46.19% ± 7.79) compared to cells previously stored at RT (46.96% ± 3.95) or 4°C (46.54% ± 8.62; Figure 6e-h). Quantification at this timepoint also revealed no differences between the proportions for each cell type at different stages in the lineage across the control and experimental conditions (Figure 6e-g, j). Immature phenotypes constituted the majority of total OLCs contributing over ca 60% in populations previously stored at low temperatures and the control. Mature phenotypes accounted for the second most abundant lineage stage and OPCs remained the least, accounting for less than ca 14% of OLCs across experimental conditions and the controls.
Figure 6. OPCs retain key cellular characteristics following lower temperature storage in Hibernate-A™. OPCs are stained for NG2 (green top images, red bottom images), oligodendrocytes for MBP (green), proliferating cells with EdU (red) and cell nuclei with hoechst (blue). (a-c) Representative, triple-merged fluorescent images showing OPCs cultured for 24 hours following 72h storage in (a) control conditions, (b) Hibernate-A™ at RT and (c) Hibernate-A™ at 4°C. (d) Scatter graph displaying the proportion of NG2 positive cells with no significant differences between cells at RT, 4°C and the control (p-value>0.05, Kruskal-Wallis test, n=4). (e-g) Representative, triple-merged fluorescent images showing OLCs differentiated after storage in e) control conditions, f) RT Hibernate-A™ and g) 4°C Hibernate-A™ respectively. All scale bars = 50µm. (h) Scatter graph showing proportions of MBP expressing cells in day 10 OLCs controls and cells previously stored at RT and 4°C with no significant differences detected (p=0.865, Kruskal-Wallis test, n=3). (i) Scatter graph displaying the proportion of proliferating cells following storage in Hibernate-A™ with EdU application over a 24 hour period with no significant differences detected (p-value=0.067, Kruskal-Wallis test, n=3). Scatter graph (f) shows quantification for the proportion of different OLC morphologies in each experimental condition following differentiation post-recovery at day 10. There were no significant differences between the control and cells previously stored at RT and 4°C for all phenotypes of OLCs (p>0.05, Kruskal-Wallis test, n=3).

Discussion

To our knowledge, this is the first ‘proof-of-concept’ that a cell spray/aerosolized delivery format is feasible for cell therapy using the major neural transplant population of OPCs. Second, we show that Hibernate-A™, a CO₂ independent nutrient medium approved for human use can support OPC survival at low temperatures, without the need for specialised storage conditions. Cells retained viability and characteristics essential to their therapeutic potential post-storage, whilst exhibiting healthy morphologies and cell marker expression. Taken together, we consider these dual lines of evidence provide a strong case for the development of a novel class of advanced spray devices to facilitate remote delivery of OPC transplant populations in neural cell therapy.

Numerous studies indicate that OPC transplantation can improve functional outcomes in pre-clinical models of SCI and traumatic brain injury (TBI).\(^6,7,21,29\) We demonstrate that post-spraying, cells retained high viability, proliferative and differentiation capabilities with healthy morphologies and cell marker expression. Our data firstly therefore indicate that aerosolization per se, does not exert detrimental effects on neural cell populations. Recent clinical trials adopted intra-parenchymal cell delivery routes, however, no stereotactic FDA approved injection cannula exists for cell transplantation and custom designed cannulae are adopted in clinical trials.\(^2–4,9\) In addition to the reduced viability on cell passage through fine bore needles, the potential for blockages within cannulae increase the complexity of such approaches in clinical practice. Increased pressures required for injection through a blocked cannulae may further damage transplant populations. The alternative would be to use a different cannula carrying the risk of a separate insertion path and direct brain injury. Spray delivery offers a clear advantage in this regard and is well suited to

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neurological injuries such as SCI and TBI, where the areas of pathology may be extensive or in a relatively superficial anatomical area. For penetration into deeper tissues, the inherent migratory abilities of transplanted cells to sites of pathology (‘pathotropism’) could provide a route for cells to reach deeper target tissues. However, this is speculative, and the spray transplantation method may not be well suited to delivering cells directly to very specific, localised regions of the CNS such as the substantia nigra in Parkinson’s disease.

A reduction in OPC viability was observed post-spraying at the early time but not later following cell differentiation. Physical parameters such as droplet size and viscosity, spray velocity, nozzle bore size and design can all impact cell viability.\[30,31\] The initial reduction may be due to the inexpensive, commercially available spray device used in this study (with limited potential to modify delivery parameters). This spray device is pump operated and therefore a degree of user dependence also exists meaning there is likely to be variability in each pump pressure and outcomes. Developing bespoke, tuneable spray devices will likely be required to achieve optimal cell spray delivery (for example, increasing droplet size to increase cushioning on impact; using lower droplet viscosity and increasing nozzle bore diameter Dijkstra et al).\[31\] Shear force damage can also be limited during spraying by cell shrinkage, for example using hyperosmolar sugar solutions as demonstrated for macrophages sprayed into the respiratory tract,\[32\] but neural cell viability would need assessment. Additionally, our experiments sprayed cells onto hard, glass substrates, but delivery into soft CNS tissues with high water content can be predicted to better cushion the mechanical impact, increasing resultant cell viability. Although a reduced viability following spray delivery exists in the current study (and requires device optimisation), spraying could still offer a comparative advantage versus the widely used techniques of intraparenchymal injection or systemic delivery which result in far greater magnitude of cell death/loss. A small volume of fine bubbles were found concentrated around the circumference of the wells after the spray device had been dispensed, however, this dissipated within seconds after the spraying process. We do not consider this impacted the viability or the distribution of the cells post-spraying. However, we expect that a better spray design potentially with a wider bore nozzle could further limit the issue.

In relation to the use of chemical transport media, given the immediacy to administer cell therapy in acute neurological injuries, hospitals require a stock of ‘ready to use cells’ but the infrastructure to support remote cell delivery does not currently exist, restricting cell therapies to centres attached to manufacturing facilities. The feasibility of implementing a stock system would be increased with simple, inexpensive transportation and storage systems. Our data suggest the storage of cells in
Hibernate-A™ is safe between 4 and 25°C, potentially providing the capability to transport cells at ambient temperatures. Removing the necessity of cold-chain transport is particularly desirable as it removes complexity, is inexpensive and compatible with changing environments. The chemical composition of Hibernate-A™ can be cross referenced with other cell storage media to expand the range of excipients for use in neural cell therapy. While we have shown Hibernate-A™ has potential for use as an OPC storage medium, future transport solutions may be combined with gel matrices which provide mechanical protection during transit. Such an approach is being developed for non-neural transplant transportation where a range of cells have been encapsulated in polymers.\textsuperscript{33,34} This technology could be integrated into a spray device enabling sterile transportation and spraying using a single medical product. Further refinements to develop novel neurosurgical devices seem feasible. For example, some neurosurgical products such as dural sealants are dual-chamber spray devices where the contents are mixed immediately prior to delivery. Multiple cell types, growth factors or pharmaceutical agents such as antimicrobial agents could also be tested in such ‘multi-purpose’ sprays. These refinements and future optimisation of a neural cell spray device can be achieved through appropriate collaboration with the pharmaceutical industry.

If the feasibility of optimising spray delivery devices (with appropriate supportive chemical media) is proven successful, then this approach warrants investigation for cell delivery in locations remote from cell manufacturing facilities. Neural cell therapy development has prioritised chronic neurological conditions such as degenerative pathologies, where cell therapy timings can be planned in advance. By contrast, conditions such as TBI have a limited time window post-injury for cell therapies to be effective with most pre-clinical trials adopting early administration with a view to suppressing the initial immune response.\textsuperscript{6} This would present logistical challenges in existing healthcare structures. Patients require stabilisation and prevention of secondary injuries, due to the frequent multiple organ injuries associated with major trauma conditions, before transferring to facilities offering manufacture and delivery of cell therapies. This obstacle is especially pronounced in the military context with a high TBI incidence,\textsuperscript{35} where patient evacuation may take days to weeks.\textsuperscript{36} Further development of the spray device strategy therefore appears warranted for OPCs and other neural transplant populations. This can be predicted to be of high clinical benefit in the surgical management of serious neurological injuries overcoming translational barriers associated with current cell delivery routes. Delivery of neural cells in a spray format to remote locations has added potential for increasing collaboration between laboratories and resource poor environments, benefiting scientific and clinical scientific communities with limited access to cell manufacturing facilities.

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Conclusions
Aerosolised delivery of neural transplant populations is feasible. Storage of cells in a CO₂ independent nutrient medium is also feasible, with important regenerative properties being retained by the ‘stored’ cells post-recovery. Together, these lines of evidence support the development of a novel class of medical devices based on spray delivery and transport in suitable supportive excipients for neural cell therapy.

Experimental Section
Reagents
All cell culture reagents and cell culture grade plastics were from Thermo Fisher Scientific (Loughborough, UK) or Sigma-Aldrich (Dorset, UK) unless otherwise stated. For media components, items purchased elsewhere were fetal bovine serum from Biosera (Sussex, UK, catalog no. 11573397) and human recombinant basic Fibroblast Growth Factor (FGF-2, catalog no. 100-18B) and platelet derived growth factor AA (PDGF-AA, catalog no. 100-13A) from Peprotech (London, UK). For assays calcein was from VWR (Pennsylvania, USA, catalog no. 89139-470), normal donkey serum (NDS, catalog no. 017-000-121) was from Stratech Scientific (Cambridgeshire, UK) and mounting medium with 4’6-diamidino-2-phenylindole (DAPI, catalog no. H-1000) was from Vectashield (Petersborough, UK). Primary antibodies were anti-MBP from Bio-Rad (California, USA, catalog no. aa82-87) and anti-NG2 from Dako Omnis (USA, catalog no. AB5320). Secondary antibodies for both fluorescein isothiocyanate (FITC) and cyanine 3 (Cy3) included donkey anti-rabbit (catalog no. GTX26701-GTX) and donkey anti-rat (catalog no. LS-C351180-LSP) from Stratech Scientific (Cambridgeshire, UK). Mist plastic pump spray bottles (10ml) were utilised as spray devices and sourced from SelfTek (UK). All animal use was in accordance with the Animals (Scientific Procedures) Act of 1985 (UK). Hibernate-A™ was purchased from Thermo Fisher Scientific (UK).

Preparation of primary mixed glial cultures (MGCs)
MGCs were derived from cortices of postnatal day one to three CD1 mice and propagated until stratification into a bed layer of astrocytes with loosely adherent OPCs and microglia as previously described. Cells were cultured for 10 days in D10 medium comprising of DMEM supplemented with sodium pyruvate (100 mM), GlutaMAX-I (1 mM), penicillin (5000 U/ml), streptomycin (5000 µg/ml) and 10% FBS with a 50% medium change every 2-3 days. Specific neural cell types can be isolated from these cultures through sequential shaking (all at 220 rpm) and subsequent enzymatic treatments. To derive OPCs, microglia were first removed from the MGCs by shaking for 2 hours.
and removing the medium. Fresh medium was added and the cultures placed on the orbital shaker for 16-18 hours during which time OPCs detach and can be collected. This method has been previously described to derive a high purity of OPCs (>85%).[38]

Evaluating spray delivery of cells
OPCs were suspended at 2.0 x 10^5 cells/ml in OPC maintenance medium (DMEM, GlutaMAX-1 (2 mM), sodium pyruvate (1 mM), biotin (10 nm), insulin (5 µg/ml), hydrocortisone (10 nm), sodium selenite (30 nm), transferrin (5 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), 0.1% bovine serum albumin, PDGF-AA (10 ng/ml), FGF-2 (10 ng/ml)). Cell solutions were then sprayed or pipetted (control) onto nitric acid treated poly-D-lysine coated coverslips. For controls, 360 µL of cell solution was pipetted into each well. For spray delivered cells, three sprays were delivered to each well (calculated to deliver 360 µL in total) from a distance of approximately one centimetre. Before each discharge, the spray device was gently inverted to ensure an even cell suspension distribution throughout the canister. OPCs were cultured for 48 hours in OPC maintenance medium before a subset of wells was switched to OPC differentiation medium (DMEM, GlutaMAX-I (2 mM), sodium pyruvate (1 mM), 1% N2 supplement, penicillin (50 U/ml), streptomycin (50 µg/ml), triiodothyronine (30 nM), thyroxine (30 nM)) for 5-8 days. OPCs underwent 50% medium changes every 2-3 days.

Evaluating storage of cells in Hibernate-A™
OPCs were suspended at a density of 2.0x10^5 cells/mL in Hibernate-A™, pre-warmed to RT, and 300 µL of this suspension was added onto nitric acid treated PDL coated coverslips in each well of a 24 well plate. Different well plates were sealed with parafilm and stored undisturbed at 4°C or RT (21 to 25 °C) for 72 hours. The same cell density and volume per well was used for controls. However, here OPC maintenance medium was employed. Control samples were stored at 37°C, 5% CO2 with no medium changes for 72 hours. Upon removal from lower temperature storage or control conditions, cells were immediately warmed or maintained at 37°C. A subset of wells were used for analyses of viability and proliferation of OPCs and a subset of wells were switched to OPC differentiation medium for 7 days. OPCs in differentiation medium then underwent 50% medium changes every 2-3 days. Conditions of RT and 4°C were selected for cell storage as are desirable transportation temperatures. RT transportation does not require any specialised refrigerated cold chain transport equipment associated with the costs of purchase, installation, running and maintenance. Transportation at 4°C represents a more standard format and likely to be available due to established cold chain supply routes.
Characterisation of cell cultures

Cell viability was assessed using a live-dead assay where of cell specific culturing media (300 μL) with calcein (4 μM), Hoechst (1 mg/ml) and EH (6 μM) was added to each well for 30 minutes before imaging. Proliferation assays were performed using a Click-iT EdU imaging kit according to the manufacturer’s instructions where OPCs were incubated with component-A for 24 hours before fixation. For experiments of cells in Hibernate-A™, both live-dead and Click-iT EdU assays were initiated immediately following removal from low temperature storage.

Immunocytochemistry was performed to identify cell specific markers and assess cell morphologies. Samples were fixed in 4% PFA for 20 minutes at RT. Cells were then washed in PBS three times before addition of blocking solution (5% NDS in PBS-0.3% Triton X-100) for 30 minutes. Primary antibodies diluted in blocking solution were added to samples and incubated overnight. These were NG2 (1:150) to detect OPCs and MBP (1:200) to detect oligodendrocytes. Primary antibodies were then removed, samples washed three times with PBS and incubated for 30 minutes in blocking solution. Appropriate secondary antibodies diluted in blocking solution (1:200) were added for two hours before three PBS washes. Samples were mounted onto glass slides using mounting medium with DAPI and left for 30 minutes before imaging.

| Antibody         | Dilution |
|------------------|----------|
| Anti-MBP (rat)   | 1/200    |
| Anti-NG2 (rabbit)| 1/150    |
| Anti-rat         | 1/200    |
| Anti-rabbit      | 1/200    |

Table 1. Antibodies and dilution factors.

Image acquisition and quantification

Fluorescence micrographs were obtained on a Zeiss Axio Observer Z1 equipped with an AxioCam MRm camera powered by Zen 2 software. For each assay, five fields were selected from the middle and four corners of the coverslip. ImageJ software was utilised for quantification. For each assay, total cell nuclei were counted per field. Viability assay analysis consisted of counting live cells positive for calcein (green) and dead cells positive for EH (red) and expressing these as a proportion of total cell nuclei. Live dead assays report a current viability of a culture where following cell death, cells begin to detach and are washed away by media changes and are therefore undetectable in subsequent assays. Immunostained images were quantified by counting positively stained nuclei and total nuclei per field. Results were expressed as the total number of cells positive for a specific marker as a proportion of the total number of cells. Dividing OPCs where quantified by counting cells positive for both EdU (red) and NG2 (green) and expressing these as a proportion of total nuclei positive for NG2. Following differentiation of OPCs, all OLCs were categorised into one of
four groups namely OPCs (immature, bipolar), pre-oligodendrocytes (two or more processes with secondary and tertiary branching), immature oligodendrocytes (multiple processes with extensive secondary and tertiary branching), or mature oligodendrocytes (extensive membrane elaboration with ‘spider-web’ morphologies). Morphological categories were expressed as the total number of cells exhibiting a morphology as a proportion of the total number of OLCs.

**Statistical analysis**

GraphPad Prism 9.0.0 software was utilised for all statistical analyses. Data was analysed using non-parametric Mann-Whitney or Kruskal-Wallis tests. All results are expressed as the mean ± standard error of the mean. In results, ‘n’ refers to the number of primary cultures used per experiment, each derived from a separate litter of animals.

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**

[1] M. C. Jin, Z. A. Medress, T. D. Azad, V. M. Doulames, A. Veeravagu, *Neurosurg. Focus* 2019, 46, E10.
[2] D. Kalladka, J. Sinden, K. Pollock, C. Haig, J. McLean, W. Smith, A. McConnachie, C. Santosh, P. M. Bath, L. Dunn, et al., *Lancet (London, England)* 2016, 388, 787.
[3] G. K. Steinberg, D. Kondziolka, L. R. Wechsler, L. D. Lunsford, M. L. Coburn, J. B. Billigen, A. S. Kim, J. N. Johnson, D. Bates, B. King, et al., *Stroke* 2016, 47, 1817.
[4] H. Shichinohe, M. Kawabori, H. Iijima, T. Teramoto, T. Abumiya, N. Nakayama, K. Kazumata, S. Terasaka, T. Arato, K. Houkin, *BMC Neurol.* 2017, 17, DOI 10.1186/s12883-017-0955-6.
[5] E. Syková, P. Rychmach, I. Drahorádová, Š. Konrádová, K. Růžičková, I. Vořišek, S. Forostyak, A. Homola, M. Bojar, *Cell Transplant.* 2017, 26, 647.
[6] S. Gennai, A. Monsel, Q. Hao, J. Liu, V. Gudapati, E. L. Barbier, J. W. Lee, *Br. J. Anaesth.* 2015, 115, 203.
[7] A. Rolfe, D. Sun, *Stem Cell Therapy in Brain Trauma: Implications for Repair and Regeneration of Injured Brain in Experimental TBI Models*, 2015.
[8] S. B. Dunnett, A. Björklund, 2017, pp. 1–51.
[9] M. Kawabori, A. Tanimori, S. Kitta, H. Shichinohe, K. Houkin, *Stem Cells Int.* 2020, 2020,
First report showing that the major transplant population of oligodendrocyte progenitor cells (OPCs) can survive aerosolized delivery. Additionally, OPCs can survive in a CO\textsubscript{2} independent nutrient solution without specialized maintenance conditions. Cells retained regenerative features post-spray or post-storage in the nutrient solution. Together, the data support the feasibility of developing novel regenerative medical cell spray devices for neural transplantation therapy.

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