Oxygen-glucose deprivation in neurons: implications for cell transplantation therapies

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Abstract

Cell replacement therapies hold the potential to restore neuronal networks compromised by neurodegenerative diseases (such as Parkinson’s disease or Huntington’s disease), or focal tissue damage (via a stroke or spinal cord injury). Despite some promising results achieved to date, transplanted cells typically exhibit poor survival in the central nervous system, thus limiting therapeutic efficacy of the graft. Although cell death post-transplantation is likely to be multifactorial in causality, growing evidence suggests that the lack of vascularisation at the graft site, and the resulting ischemic host environment, may play a fundamental role in the fate of grafted cells. Herein, we summarise data showing how the deprivation of either oxygen, glucose, or both in combination, impacts the survival of neurons and review strategies which may improve graft survival in the central nervous system.

1) Introduction

Neurodegenerative diseases are increasing in prevalence and most have no cure [1]. It is therefore important to seek novel intervention strategies. For some neurodegenerative disorders, cell therapies offer the potential to halt or even reverse the disease progression by replacing the affected neurons and repairing neural circuitry [2]. Cell replacement could be a particularly appropriate therapeutic strategy for Parkinson’s disease (PD) and Huntington’s disease (HD), both of which feature a relative loss of specific neuronal subgroups in defined brain regions. More acute injuries such as stroke [3], traumatic brain injury [4] or spinal cord injury [5] may also lend themselves to cell-based therapies due to the focal nature of the lesions.

Long-term follow-up of early clinical trials proved the concept that replacing lost dopamine neurons with neuronal progenitors derived from fetal ventral mesencephalon (VM) (the region of the developing brain in which the dopamine projection neurons are born) can provide remarkable recovery in a subset of PD patients [6, 7]. The many practical and ethical difficulties inherent to working with fetal tissue has led to a concerted effort across multiple labs to generate stem cell-derived sources of dopamine donor neurons [8], although studies with fetal-derived progenitors continue to be important for understanding factors that influence the reliability and robustness of cell replacement therapy in PD, thus paving the way for future stem cell-based strategies [9] [10].
One common theme associated with cell transplantation to the brain is the poor survival rates of the transplanted cells [11-17]. Taking PD as an example, grafted dopamine cells survive the transplantation procedure very poorly, with typically less than 5-10% of cells surviving to form a graft [15, 17]. Since transplanted cells are rarely pure populations, care must be taken when interpreting the survival rates, differentiating between total surviving cells and surviving neurons. However, even after taking into account the starting proportion of dopamine progenitors, dopamine neuron survival rates of ~4% are considered robust [17]. In a clinical setting, it is estimated that ~100,000 surviving dopamine neurons are required for functional benefit, necessitating a starting fetal cell number of ~4,000,000 [9]. By compensating for the inevitable neuron loss, remarkable behavioural recovery can be achieved in pre-clinical models [18] and in the clinic [6, 7], with good integration and synaptic connectivity of the graft and host [19, 20].

Despite such achievements, it is the authors’ view that improving cell survival post-transplantation is still a high research priority for the following reasons. First, transplanting excess cells to compensate for post-transplantation cell death results in a large physical space being occupied by the implanted cells, potentially causing additional host tissue disruption. Secondly, the higher antigen load (at least initially), and the presence of dying/non-integrated cells, may exacerbate both adaptive and innate host inflammatory responses [15, 21] and evoke an unnecessarily strong gliotic response, potentially creating a sub-optimal microenvironment for neuron maturation/integration [22]. Thirdly, critical progenitor populations in the graft may be disproportionately affected (dopamine neurons are particularly vulnerable [23]), leading to unwanted cell types dominating and reduced therapeutic potential. These downsides of a compensatory grafting approach are generally applicable to both fetal- and stem cell-derived grafts, so whilst improving grafting efficiency is particularly pertinent to fetal cell transplants, it is likely to have therapeutic benefits for all cell therapies, regardless of the cell source. Finally, with a view to the future and continual refinement of cell transplantation, this compensatory approach does not address the root cause of the problem, nor maximize the therapeutic efficiency of grafting.

2) Cell death post transplantation

There is limited published data on the time course of post transplantation cell loss, although the majority of cells (~80%) typically die within the first week [17, 24-27] and there is some evidence that the greatest cell loss occurs within the first 1-4 days, with further cell loss continuing until approximately 14 days post-transplantation [17, 24-27]. The cause of cell death following transplantation into the brain is likely to be multifactorial. The injection process itself has been hypothesized to cause cell damage [28, 29], although slow infusion rates typically used for implantation of cells into the brain helps to reduce shear stresses. Adil et al., showed that the injection process was not the principle cause of neural progenitor death, but that the process of lifting cells from a 2D culture surface prior to injection substantially reduced viability [30]. Regarding the events of the first days post-grafting, the loss of transplanted cells appeared to precede the microglial and astroglial invasion of the graft site [27], thus suggesting that the innate immune rejection was not the major cause of implanted cell death at this early stage. Moreover, a hypoxic core was already present in the graft by 4-6 hours post-transplantation (Figure 1), and this remained detectable for at least three days, along with the presence of apoptotic cells within the hypoxic region [27, 31].
This data indicate that a lack of oxygen reaching the graft could be responsible for rapid cell death in the graft. CD31\(^+\) endothelial cells, which are responsible for the angiogenetic process, can be observed near the graft within 24 hours [31], but fully functioning vascularisation probably takes much longer. For example, following ischaemic stroke, angiogenesis and functional vascularisation can take up to 21 days [32], hampering neurogenesis and neurite outgrowth [33]. Thus, it is likely that there is a period immediately following cell implantation when the graft is insufficiently vascularised.

We propose that the hypoxia seen in the graft core is due to inadequate vascularisation/blood perfusion, and is therefore accompanied by a lack of nutrients, including glucose. The graft site therefore represents a condition of oxygen and glucose deprivation (OGD), which might be responsible for the massive cell loss seen within the first days post-transplantation. Herein, we aim to review the effect of OGD on neurons, by looking at both brain ischemic injury and cell culture models of OGD, and to determine the mechanisms of cell death and the relative impact of a paucity of either oxygen, glucose, or both in conjunction, on cell viability.

3 The effect of oxygen and glucose deprivation on neurons

3.1) A lesson from brain ischemic stroke

While the association between OGD and transplanted cells is a recent topic of investigation [34, 35], ischemia and its effects have been well described as a result of stroke [36]. Ischemic stroke, characterized by insufficient blood supply to a part of the brain, represents 87% of stroke cases [37]. It occurs due to the occlusion of a brain artery (the middle cerebral artery is a prevalent site) resulting in focal neuronal cell death and neurological impairments such as motor, learning/memory and speaking deficits [37, 38]. A common but less severe form of brain ischemic stroke is the transient ischemic attack (TIA) that resolves within 24 hours [39].

During an ischemic stroke, the blood circulation is altered, leading to the formation of an ischemic core at the centre of the ischemic area (more severely affected region), and an ischemic penumbra where the collateral blood flow results in less ischemic damage [40]. The different area, duration and severity of ischemia account for the different outcomes which, especially in the core, result in neuronal cell death by necrosis [41]. This happens as the prolonged lack of blood flow to the ischemic core, leads to a lack of oxygen and the depletion of ATP in cells, in turn triggering a wave of depolarization and Ca\(^{2+}\) accumulation in the cytosol resulting in necrosis (also described as excitotoxic cell death) [37, 38, 41]. The paradigm of neuronal cell death in stroke is a complex and multifactorial event that is not limited to necrosis, but also includes apoptosis resulting from...
inflammation, ROS production and reperfusion damage [37]. To help understand the mechanism of neuronal cell death in ischemic stroke, simplified in vitro models of oxygen and glucose deprivation have been created, allowing individual effects of ischemia to be more easily distinguished than in the complex in vivo situation.

Ischemic stroke has been heavily investigated and reviewed elsewhere [36, 37], so here we will focus on the in vitro models used to understand ischemia and how OGD effects neuron viability. Since the core of grafts in the brain are hypoxic, cell health in in vitro conditions of extreme nutrient paucity may parallel that of neurons in the early stages post-transplantation, thus elucidating mechanisms of cell death post-transplantation. Figure 2 shows a graphical representation of a variety of cell models and how the length of time they are exposed to OGD correlates with their viability.

A strong pattern emerges from the in vitro OGD studies: neuron viability is affected by OGD in a temporal manner. The longer neurons are deprived of oxygen and glucose, the less their ability to maintain adequate ATP levels, resulting in greater cell death. Although there are some discrepancies amongst different protocols (cell types, duration of OGD exposure and reperfusion), Figure 2 highlights a clear temporal response to OGD [42-47]. Moreover, for the majority of studies, 12 hours of OGD induces severe damage (<50% cell viability) [42, 45, 48-51]. The time course of cell death, the apoptotic features and caspases activation of in vitro OGD models [44, 48, 52-54] resembles the fate of cells after transplantation [28, 55], supporting the idea that OGD is a major cause of death in the early phase post-transplantation. Such data, when coupled with the findings of Praet et al., showing an oxygen deficit in the graft core followed by apoptosis [31], may shed light on why cells die so rapidly post-transplantation.

Since stroke, not cell transplantation, was the focus of the original studies shown in Figure 2, many of them analysed a period of reperfusion following OGD. Reperfusion plays an important role after a stroke, as it is necessary to restore an adequate level of oxygen and nutrients but it can also be detrimental to ischemic cells [56]. However, the data in Figure 2 shows that the OGD period alone causes the reduction in cell viability, with neither large beneficial nor detrimental effects observed from subsequent reperfusion.

Some limitations of interpreting the data in Figure 2 with regards to cell transplantation are as follows. Firstly, these in vitro models limited the OGD period to 24 hours, whereas OGD in grafts is likely to continue long past this (hypoxia can be detected up to 3 days post-transplantation [31]). Secondly, cells/neurons of different types will have different energetic requirements and so different susceptibilities to nutrient deprivation. In light of this, further studies should be carried out to investigate the impact of OGD on cells and neuron progenitors of interest for cell therapies.
Figure 2: Duration of oxygen-glucose deprivation correlates with reduced cell viability in a variety of in vitro stroke models. a) A schematic of data extracted from in vitro models showing the effect of different durations of OGD (red) and reperfusion (blue) on cell viability (green). OGD duration may vary from 1 h to 24 h, while the following reperfusion varies from 24 to 48 h (either calculated from the OGD onset or its end) or might be absent as cell viability was assessed immediately after the OGD incubation. The variability between the protocols also includes different cell types as reported in brackets. b) A scatterplot analysis of the data extracted from the studies shown in panel A, showing the linear relationship between the duration of oxygen-glucose deprivation and the percentage of dead cells, with the $R^2$ values given alongside each study. Cell viability is reported as stated or, when not present in the main text, approximated from the figures in the original articles [42-46, 49, 54].
3.2) The relative importance of glucose in oxygen-glucose deprivation

As both oxygen and glucose deprivation are detrimental to neurons, it is important to determine whether one of the two components has more effect than the other.

As Figure 3 illustrates, oxygen and glucose are two key elements in cell metabolism, used to produce and store energy in molecules such as adenosine triphosphate (ATP). In the cytosol, cells use glycolysis to convert glucose to pyruvate, generating ATP in the process. In the presence of oxygen, pyruvate enters mitochondria to be coupled with Coenzyme A forming acetyl-Coenzyme A (Acetyl-CoA) which enters the tricarboxylic acid (TCA) cycle to produce ATP, NADH, FADH$_2$ and succinate. Those molecules are then used in oxidative phosphorylation to produce ATP at a higher yield compared to glycolysis: 36 ATP molecules in comparison to just 2 ATP molecules produced via glycolysis [57].

However, Petite and co-workers found that human MSC survival relied on glucose much more than oxygen [58]. When cultured in anoxic conditions, cells were able to switch to glycolysis and survive in absence of oxygen for over two weeks, provided that sufficient glucose was present ($\geq 1$ mg/mL, similar to euglycemia or physiological glucose level in blood) [59]. Moreover, it was glucose rather than other nutrients such as glutamine, serine, or pyruvate, that was responsible for this prolonged viability [35] [60]. However, these studies did not determine the differential effects of how low and high oxygen concentrations effect OGD mediated cell loss. Since oxidative phosphorylation is a more efficient way to produce ATP one could hypothesize that glucose paucity is less detrimental to cells cultured in normoxia. This may especially be the case in the human brain due to its high metabolic demands, synthesizing and consuming up to 8 kg of ATP per day [61], with neurons/glia essentially depending on oxidative phosphorylation to produce that energy [62]. One could argue that, during ischemia, providing enough oxygen to restore the more efficient oxidative phosphorylation would save neurons.

3.2.1) A focus on oxygen replacement

To this end, we formulated oxygen producing microspheres aimed at rescuing transplanted cells from OGD [34]. We cultured rat VM cells, human MSCs and SH-SY5Y neuroblastoma cells (a model for dopamine neurons [63]) either in normoxia or near anoxia and in a variety of glucose concentrations. Whilst the oxygen producing microspheres were able to provide a marginally better outcome for MSC and SH-SY5Y in OGD, the key finding emerging from this study was the high dependency these cells had on glucose, also in the case of normoxic incubation.
Figure 3: Glucose plays a vital role in neuron survival, especially in hypoxic conditions. Schematic diagram showing neuronal metabolism under normal oxygen tension and euglycemia (physiological glucose concentration) (black arrows). During an episode of OGD (red arrows), the lack of both oxygen and glucose causes a depletion of ATP and scavenger molecules such as glutathione (GSH). This triggers both the influx of Ca$^{2+}$ from the NMDA receptor, causing excitotoxicity, and endoplasmic reticulum (ER) Ca$^{2+}$ depletion. This perturbation of homeostasis will further trigger intrinsic apoptosis through the calpain-catepsine pathway, while the influx of calcium in mitochondria will act as a stress signal. Moreover, the mitochondria will start producing reactive oxygen species (ROS) aggravating the overall oxidative stress caused by the lack of oxygen. These events will activate death receptors by FAS and TNF-α signalling, triggering the BID cascade which results in permeabilization of mitochondria and release of cytochrome C (cytC) and apoptosis-inducing factor (AIF). CytC will start the apoptosome resulting in further cleavage of procaspase-3 in caspase-3, while the translocation of AIF into the nucleus will initiate chromatin condensation. Both these events will eventually cause cell death by apoptosis. Notably, if the glucose concentration is sufficient, but oxygen is lacking, then the absence of oxygen will stabilize the hypoxia inducible factor 1α (HIF-1α, green arrows), which would result in enhanced transcription of glucose and monocarboxylate transporter (GLUT and MCT) families, glycolytic enzymes and pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1). This adaptation mechanism would provide the neurons with ATP, NADH and NAPDH via enhanced glucose influx and glycolysis. While this less efficient production of ATP will dampen the energetic crisis, NADPH will preserve the pool of reduced glutathione during its ROS scavenging activity. These combined effects, made possible by the presence of glucose, will result in a better outcome for the hypoxic neuron.
Depriving MSCs of glucose for four days reduced viability to less than 60%, even when cultured in normoxic conditions. In contrast, provision of 1 mg/mL of glucose resulted in over 80% viability in either anoxia or normoxia [34], thus confirming the findings from Petite’s group [35]. The effect of glucose on SH-SY5Y cell viability was more dramatic: in both normoxia or anoxia, the viability decreased to less than 40% after one day of glucose deprivation, and less than 15% on the second day [34]. Again, the provision of 1 mg/mL glucose for the two days resulted in viability of over 65% (anoxia) and over 90% (normoxia). Interestingly, VM derivatives (a mixed population of cell types) did not suffer from the extreme conditions of either (or combined) oxygen and glucose deprivation. These cells, extracted from E14 embryonic rat VMs, exhibited a very low oxygen consumption rate and less rescue upon treatment with oxygen producing spheres in OGD when compared to the other cells. With such low oxygen consumption, they may have switched to a metabolically quiescent state whereby the glucose contained in the serum component of the medium may have provided a sufficient energy source. Alternatively, the glial cells in their population may have mediated the reduced susceptibility [47].

The fact that SH-SY5Y were able to survive 48 hours of hypoxia when sufficient glucose was present but died within 24 hours in the presence of 21% oxygen but no glucose, led us to reconsider the role of glucose in OGD and in neural cell transplantation. Concomitantly, Petite and co-workers have speculated whether the provision of extracellular glucose may improve MSC viability in grafting and other tissue engineering applications [35]. Other studies that have analysed the effect of glucose on neuron viability are presented in Table 1 and highlight the relative importance of glucose in the maintenance of cell survival in ischemic conditions.

3.2.2) Glucose is critical for the survival of neural cells

Studies examining neural cells used fetal mouse cortical neurons, glia, and co-cultures of both, to assess the cell viability after an ischemic insult (OGD) and the effect of either oxygen or glucose deprivation alone [47, 52, 64, 65]. Goldberg and Choi found that in the case of combined oxygen and glucose deprivation, 70 minutes of exposure were sufficient to cause complete neuronal death, which was extended to 8 and 14 hours, when 2 and 5.5 mM of glucose were present, respectively [47]. 20 mM of glucose resulted in a significant rescue of viability to 40% even after 24 hours [47]. In contrast, another experiment with a predominantly glial population necessitated more than six hours of OGD exposure to detect cell injury [47]. However, in the presence of glucose (5.5 mM), the glia cells remained viable after five days in hypoxia [47]. In summary, cortical neurons required less hypoxic exposure than glia to produce widespread loss, even in the presence of glucose which suggests a relatively greater vulnerability of these neurons under ischemic conditions. This finding is of particular importance to cell transplantation studies using heterogeneous populations, where a cell type of interest may not survive as well as other concomitantly grafted cell types.
<table>
<thead>
<tr>
<th>REFERENCE</th>
<th>CELLS</th>
<th>OXYGEN GLUCOSE DEPRIVATION</th>
<th>CELL VIABILITY</th>
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<tbody>
<tr>
<td></td>
<td>Type</td>
<td>pO₂ [glucose] Duration R* Method</td>
<td>Timepoint</td>
</tr>
<tr>
<td>Goldberg, 1993 [47]</td>
<td>Mice neocortical tissue (E14-17)</td>
<td>2.75-3.75 hemispheres (24-well) 0.5-2 hemispheres (24-well) 0.25-0.5 hemispheres (24-well)</td>
<td>&lt;2 mm Hg (&lt;0.28% pO₂) 0-20 mM up to 70 min (OGD), up to 24 h (hypoxia) up to 5 days</td>
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<tr>
<td>Bruckner, 1999 [70]</td>
<td>Primary rat neurons and astroglia (D1 or D21)</td>
<td>1.5 x 10⁴ 1.0 x 10⁵ cell/mL</td>
<td>~0% pO₂ 0-100 mM up to 24 h</td>
</tr>
<tr>
<td>Wang, 2002 [44]</td>
<td>SH-SY5Y</td>
<td>N/A</td>
<td>0-unknown mM up to 24 h</td>
</tr>
<tr>
<td>Almeida, 2002 [69]</td>
<td>Rat cortical neurons (E16-17) Rat neonatal astrocytes (D1)</td>
<td>2.5 x 10⁵ cell/mL 2.5 x 10² cell/mL</td>
<td>6.7 +/- 0.5 µM (in medium) (&lt;0.67% pO₂) 0 mM</td>
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<tr>
<td>REFERENCE</td>
<td>CELLS</td>
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<tr>
<td><strong>Cater, 2003 [68]</strong></td>
<td>Rat organotypic hippocampal slice (D8-10)</td>
<td>N/A</td>
<td>0, 5, 30 mM either glucose or lactate</td>
</tr>
<tr>
<td><strong>Fordel, 2007 [67]</strong></td>
<td>SH-SY5Y</td>
<td>8 x 10^4 cell/cm^2 (up to 16 h OGD)</td>
<td>0-0.2% pO_2</td>
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<tr>
<td><strong>Wohnsland, 2010 [66]</strong></td>
<td>Rat embryonic cortical neurons (E18)</td>
<td>600,000 cell/mL</td>
<td>N/A</td>
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<tr>
<td><strong>Newland, 2018 [34]</strong></td>
<td>SH-SY5Y</td>
<td>10,000 cell/well</td>
<td>0.1% pO_2</td>
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<tr>
<td></td>
<td>Embryonic rat ventral mesencephalic cells (E12-14)</td>
<td>50,000 cell/well</td>
<td>0.1% pO_2</td>
</tr>
<tr>
<td></td>
<td>hMSC</td>
<td>10,000 cell/well</td>
<td>N/A</td>
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</table>
The effect of anoxic incubation in the presence of glucose was also described by Bruckner et al. while investigating the expression of glucose transporters in neurons exposed to OGD [70]. They reported that 24 hours of hypoxic incubation in the presence of glucose resulted in 82% viability for primary neurons from 21-day old rats. That is the same percentage obtained after 6 hours of complete OGD incubation, and it dropped to 43% following 24 hours of OGD. They also found that hypoxia and, especially OGD, increased GLUT1 mRNA levels and stability (41 and 128-fold higher after 24 hours of hypoxia or OGD respectively). GLUT3 mRNA level increased only by 7-fold after 24 hours in hypoxic condition and was detected in neurons but not in glial cultures. Unsurprisingly, normoxic incubation at different glucose concentrations had little effect on mRNA expression. This is in line with the role of hypoxia inducible factor-1α (HIF-1α) stabilization and its effect on the GLUT family during hypoxic and ischemic injury (Figure 3), as a mechanism to increase glucose supply and maintain cell functions via the less energetically efficient glycolysis pathway [71]. Murine cortical neurons cultured with glucose at a concentration of 1 mg/mL were also able to survive in near anoxia for 18 hours with over 90% cell viability [72].

The presence of glucose during hypoxic incubation not only results in less cell death, but also in reduced mitochondrial dysfunction and oxidative stress in cortical neurons compared to OGD [69]. Glutathione (GSH) is depleted during OGD meaning that neurons fail to scavenge superoxide anions which inhibit mitochondrial complex I [69]. Instead, during hypoxia with 5.5 mM glucose, the glucose metabolism, through the pentose-phosphate pathway, produces NAPDH which is used to maintain the GSH pool and thus scavenge superoxide anions and reduce oxidative stress [69]. The role of GSH in neuronal survival after OGD has also been highlighted in another study [73], further suggesting that ROS scavenging might play a role in glucose neuroprotection during hypoxia. Although the authors did not see any change in glycolysis after one hour of incubation (either OGD or hypoxia) [69], glucose metabolites have been reported to act as a scavenger in other situations. In HT22 cells, a mouse hippocampal cell line, approximately 50% cell death was observed after 3-4 hours of OGD followed by reperfusion [42, 49], but the presence of pyruvate in the reperfusion media largely rescued cell viability [42]. Pyruvate, which is a product of glycolysis, was able to upregulate the monocarboxylate transport (MCT), dampen the ROS production and restore ATP levels [42].

3.3.3 A glucose paucity also negatively affects SH-SY5Y cells

Working with SH-SY5Y cells, Fordel et al. found that they remained viable when cultured in anoxia in the presence of glucose (up to 32 hours), and 24 hours of reoxygenation slightly reduced their viability, and it increased the levels of ROS [67]. In contrast, when SH-SY5Y cells were cultured in OGD, they showed an increased mortality (approximately 50% after 16 hours) [67]. Wang et al. used the SH-SY5Y cell line to study the non-excitotoxic component of ischemia induced neuronal death, as this line lacks functional ionotropic glutamate receptors [44]. When assessed at 24 hours, the viability of cells cultured in OGD was similar (approximately 80%) between samples that underwent 10-24 hours of ischemic insult, regardless the reperfusion period. From the same experiment, it appeared that 16 and 24 hours of OGD had the same effect on cell viability, both leading to severe cell loss when assessed at 48 hours.

In a second experiment, the effect of 16 hours of incubation in either anoxia or normoxia was compared at 48 hours, to investigate the effect of the medium composition [44]. When cultured in normoxia the lack of either cell medium (replaced with balanced salt solution), glucose,
serum, or their combination thereof, had no statistically significant effect on cell viability, although there was a trend suggesting that the lack of glucose and full media led to cell loss [44]. In contrast, when exposed to anoxia (16 hours), SH-SY5Y cells retained only 10% viability in glucose free medium, and further reduced in the absence of both glucose and serum [44]. The effect of serum (which contains approximately 0.6-1.4 mg/mL glucose), in OGD should not be overlooked, as it may cloud interpretation of this data. Glucose deprivation on this cell line affected its viability even when oxygen was present and, over prolonged incubation, it was possible to appreciate a temporal response in cell death upon glucose deprivation [34, 74].

3.3.4) Glucose consumption and lactate production

Wohnsland et al. demonstrated that cortical neurons exhibited a similar glucose-dependant behaviour, by culturing them for 24 hours either in normoxia or anoxia with different glucose concentrations [66]. Glucose consumption in the anoxic group was 5-fold higher compared to the normoxic control, resulting in a significantly higher cell loss in anoxia at <5 mM glucose, although at these concentrations neurons were also dying in normoxia [66]. This threshold effect provided by 5.5 mM of glucose was also reported also in other studies [34, 47, 72], although this value is almost 3.5-fold higher than euglycemia in the brain [75]. The anoxia group also showed ~4 times higher lactate production than the normoxia group [66]. Since Praet et al. showed a hypoxic core in grafts (Figure 1), additional lactate production could potentially lead to acidification of the graft, placing a further strain on transplanted cells.

This same study showed that cortical neurons or neural progenitor cells survived well in normoxic conditions so long as either glucose or L-lactate were present at equimolar or equienergetic concentrations [66]. The role of lactate as an energetic substrate for neurons has been described since the ‘80s [76] leading to the lactate shuttle hypothesis [77, 78] which has recently been challenged [79]. Although there is evidence of better outcomes when patients with TBI were treated with lactate [80], we hypothesise that during prolonged hypoxia it would have little improvement on neuron survival, as the lactate paradigm utilises oxidative phosphorylation to produce energy (Figure 3).

The role of lactate and glucose, both supplemented during either hypoxia or normoxia was evaluated by Cater et al., on hippocampal slices [68]. This study not only confirms that most of the damage occurs during the OGD period rather than reperfusion, but that the lack of glucose is responsible for that damage. Moreover, glucose appeared to have a better survival outcome in all the culture conditions when compared to lactate [68]. Hypoxic incubation with glucose, but with lactate present during reoxygenation, resulted in slightly better viability [68]. However, culturing with lactate and then glucose did not differ from the use of glucose only during the reperfusion following a complete OGD [68]. These results further demonstrate that during a hypoxic period, glucose is the key substrate for neurons and its absence is responsible for the widespread loss seen in OGD and reperfusion.

4) Rescuing neurons from oxygen and glucose deprivation – a means to improve graft survival?

Several strategies attempting to improve the survival of neurons after an episode of OGD have been explored, so here we focus on those that might be relevant for neural transplantation.
There have been considerable efforts made to investigate the role of N-methyl-D-aspartate (NMDA) receptor signalling during ischemic stroke and the subsequent neuronal death (Figure 3)[47, 64, 65, 81]. Overall, the neuronal injury caused by OGD exposure showed necrotic features compatible with NMDA receptor mediated excitotoxicity [47]. This is characterised by rapidly triggered necrosis followed by apoptosis [81] and, when neurons were treated with an NMDA antagonist, a delayed apoptotic event was shown [64]. An NMDA antagonist effectively improved the survival of neurons after OGD [47] (and have been recently reviewed as therapeutic target for stroke [82]). Furthermore, the presence of a delayed apoptotic process [64, 81] could potentially be prevented by caspase inhibitors such as Z-VAD-fmk and Z-DEVD-fmk [52]. Apoptosis is also a common event in grafting and when this caspase inhibition strategy (using Ac-YVAD-cmk) was applied to neural transplantation it showed improvement both in the survival of rat VM tissue (four days post transplantation: 31-41% treated cells versus 9-11% control) and subsequent functional recovery after transplantation [55]. Similar results, with the same inhibitors, have also been achieved when transplanting NSCs in the gastrointestinal tract of mice [83].

Oxidative stress and ROS production are commonly involved both in cell death post-transplantation and ischemia followed by reperfusion [28, 37, 84]. One strategy to prevent this could be to use compounds that can act as ROS scavengers or interact with the molecular cascade they evoke (Figure 3). Resveratrol has been investigated in neural OGD models for this reason [85-87]. Pre-treatment with 40 μM resveratrol, started 24 hours prior to OGD and continued during the OGD- and reperfusion- periods, decreased apoptosis (73.8% and 39.2% cell viability in treated and untreated group respectively)[85]. This antioxidant and pro-survival mechanism is probably due to activation of either AMPK [86] or PINK1/Parkin signalling [87]. Both signalling pathways are involved in mitophagy following episodes of brain ischemia, as a pro survival mechanism to eliminate the damaged mitochondria and regulate ATP catabolism [88]. Simvastatin, used at 1-10 μM, also ameliorates cell viability in a model of spinal cord injury reducing the ROS production [89]. Another potential avenue could be to inhibit coflin [90]. This cytoskeletal protein becomes dephosphorylated as a result of oxidative stress and ATP depletion in OGD, and is responsible for mitochondrial dysfunction, release of cytochrome C and caspase-3 cleavage [90]. Alternatively, the use of acetyl L-carnitine, a molecule involved in mitochondrial acyl-CoA/CoA balance, improves cell viability in association with increased expression of pAkt, Bcl-2 and pGSK3b which are inhibited by ROS [90].

Another strategy specifically designed to overcome OGD following neural transplantation is hypoxic conditioning of cells prior to grafting [91]. Short exposure to mild hypoxia leads to adaptation, resulting in a less severe response upon a second hypoxic insult (grafting), this is an attractive strategy as it can be combined with enhanced expression of pro-survival factors [92-95]. Briefly, this has been used to enhance the paracrine effect of bone marrow mesenchymal stem cells (BMSCs) on neurons. The hypoxic conditioning enriched the BMSC secretome with vascular endothelial growth factor (VEGF) as a result of HIF-1α activity [92]. This method also yielded good results in NSCs when Breast cancer susceptibility protein 1 (BRCA1) was upregulated during OGD and following reperfusion [93]. The overexpression of this pro-survival protein was able to reduce apoptosis and the oxidative stress of grafted NSCs and provide better functional recovery [93]. Similarly, the enhanced expression of brain-derived neurotrophic factor (BDNF) in GABAergic hippocampal neurons resulted in better conditioning and survival upon a second OGD experiment [95]. The survival of NSC might
also be improved by upregulating small ubiquitine modifier (SUMO) [94]. This enhanced NSCs ability to survive grafting in an ischemic environment by overexpressing Ubc9, which induces resistance to OGD/R. At five days post transplantation, SUMOylated NCS survived 1.9-fold more than the control (28.4% vs 15.2%) [94]. We are not aware of any attempt at OGD preconditioning, which would of course be detrimental to the cell’s viability if the glucose level is reduced too far.

A different strategy to enhance neuronal survival upon ischemic injury would be to provide nutrients to avoid energetic failure. One example used biomaterials composed of polylactic acid which served the double purpose of offering a biodegradable surface where NSCs could adhere and grow, and the release of energetically active metabolites (L-lactate) during the material degradation [96]. Although the authors reported good viability for neurons [96], as they are able to oxidize the L-lactate released to produce ATP [66], this mechanism would likely be ineffective in a situation where oxygen is the limiting factor [31, 68]. To provide the other key component for efficient ATP production via oxidative phosphorylation, our group formulated oxygen producing microspheres [34]. In this case, the problem was the opposite: while the spheres were able to provide the necessary oxygen, the paucity of glucose limited the rescue of SH-SY5Y cells, proving again the vital importance of this substrate for cells [34]. From in vitro evidence, glucose should ideally be maintained at a level of at least 5 mM (approximately 1 mg/mL), even though the glucose concentration in the brain is lower (approximately 1 mM) [75, 97, 98]. Sustained release of glucose from a delivery system represents a formulative challenge, as it is a small hydrophilic molecule without formal charges. However, using such a system could mean meeting the metabolic demands of the graft prior to vascularisation, thus improving not only the graft viability, but also its maturation rate and ultimate function.

Petite and co-workers, mentioned previously in relation to hMSC transplantation, came to this same conclusion: adequate glucose is important for transplanted cells [35, 58-60]. Indeed, they added glucose to either fibrin or hyaluronic hydrogels (1g/construct) which, when injected into an MSC-seeded scaffold, dramatically improved graft survival post-subcutaneous implantation of the cell/glucose loaded scaffold [59]. This group later revealed that the glucose release was brief and uncontrolled and sought to rectify this with glucose producing materials [99]. These consisted of a fibrin hydrogel containing a polymer of glucose such as starch, and an enzyme such as α-amyloglucosidase (either free or encapsulated in nanoparticles) capable to degrading the starch to glucose in situ. Subcutaneous implantation of the cell seeded constructs resulted in 7.5-fold increase in cell viability at 14 days compared to the non-glucose-producing hydrogel [99]. This not only showed the importance of a continuous glucose supply for cells cultured in near anoxia, but also proved the concept that a sustained release of glucose improves graft survival.

It should be noted that these glucose producing structures are too large for injection, so there are still obstacles and refinements required if such a strategy is to be made small enough to be used for assisting neural cell transplantation in the brain. Down-sizing glucose delivery systems for injection through fine needle canulae will likely require a rethink of the strategy, perhaps resulting in nano/microparticles with polymeric coatings tailored to control/prolong the glucose release. With this comes the obvious difficulties of an increasing surface-area-to-volume ratio with decreasing particle size making achieving controlled release non-trivial. Nevertheless, the
data amalgamated in this review provides a strong rationale for the consideration of glucose and glucose releasing devices in future neural transplantation paradigms.

5) Conclusions

For better functional recovery and more consistent outcomes from neural transplantation, one pressing challenge should be addressed: cell death post-transplantation. While several factors will clearly influence the efficacy of cell transplantation, we think that reproducible, robust and evenly distributed cell survival will contribute greatly towards better graft survival and maturation, culminating in improved integration and functional recovery.

The data reviewed herein indicate that the massive cell loss in the early phases post-transplantation occur with, and are probably caused by, the ischemic environment and specifically the paucity of glucose. Prolonged hypoglycaemia at the site of transplantation results in rapid ATP depletion and energetic failure. Moreover, it increases oxidative stress due to depletion of molecules such as NADPH that contribute to maintaining the pool of reduced glutathione. Together, these events cause apoptosis and necrosis of grafted cells, in a manner similar to that occurring in ischemic stroke. Although some strategies can enhance cell viability upon transplantation, we argue that the role of glucose and its metabolites have largely been overlooked as a possible solution to rescue cells.

We hope that this review will stimulate interest amongst the scientific community to develop solutions for the unmet need to engineer a glucose supply, at least in the days/weeks immediately post-transplantation, as better graft viability is likely to translate to better clinical outcomes in future transplantation studies.

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References

[54] D.A. Le, Y. Wu, Z. Huang, K. Matsushita, N. Plesnila, J.C. Augustinack, B.T. Hyman, J. Yuan, K. Kuida, R.A. Flavell, M.A. Moskowitz, Caspase activation and neuroprotection in


[87] M. Ye, H. Wu, S. Li, Resveratrol alleviates oxygen/glucose deprivation/reoxygenation-induced neuronal damage through induction of mitophagy, Molecular Medicine Reports 23(1) (2021) 73.