Research Article

In Vitro Oxygen-Glucose Deprivation-Induced Stroke Models with Human Neuroblastoma Cell- and Induced Pluripotent Stem Cell-Derived Neurons

Miia Juntunen, Sanna Hagman, Anaick Moisan, Susanna Narkilahti, and Susanna Miettinen

1 Adult Stem Cell Group, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
2 Research, Development and Innovation Centre, Tampere University Hospital, Tampere, Finland
3 Neuro Group, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
4 Cell Therapy and Engineering Unit, EFS Auvergne Rhône Alpes, 38330 Saint Ismier, France

Correspondence should be addressed to Miia Juntunen; miia.juntunen@tuni.fi

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1. Introduction

Stroke is a devastating neurological disorder and one of the leading causes of mortality and disability. To understand the cellular and molecular mechanisms of stroke and to develop novel therapeutic approaches, two different in vitro human cell-based stroke models were established using oxygen-glucose deprivation (OGD) conditions. In addition, the effect of adipose stem cells (ASCs) on OGD-induced injury was studied. In the present study, SH-SY5Y human neuroblastoma cells and human induced pluripotent stem cells (hiPSCs) were differentiated into neurons, cultured under OGD conditions (1% O₂) for 24 h, and subjected to a reperfusion period for 24 or 72 h. After OGD, ASCs were cocultured with neurons on inserts for 24 or 72 h to study the neuroprotective potential of ASCs. The effect of OGD and ASC coculture on the viability, apoptosis, and proliferation of and axonal damage to neuronal cells was studied. The results showed that OGD conditions induced cytotoxicity and apoptosis of SH-SY5Y- and hiPSC-derived neurons, although more severe damage was detected in SH-SY5Y-derived neurons than in hiPSC-derived neurons. Coculture with ASCs was protective for neurons, as the number of dead ASC-cocultured neurons was lower than that of control cells, and coculture increased the proliferation of both cell types. To conclude, we developed in vitro human cell-based stroke models in SH-SY5Y- and hiPSC-derived neurons. This was the first time hiPSCs were used to model stroke in vitro. Since OGD had different effects on the studied cell types, this study highlights the importance of using several cell types in in vitro studies to confirm the outcomes of the study. Here, ASCs exerted a neuroprotective effect by increasing the proliferation and decreasing the death of SH-SY5Y- and hiPSC-derived neurons after OGD.
preclinical animal models [5, 6]. The high failure rate in clinical trials may be due to the numerous macrostructural, cellular, and molecular discrepancies that exist between rodent and human brains [7]. In vitro human cell-derived models are considered to be advantageous for overcoming these challenges, revealing the pathological mechanisms of stroke and therefore developing new therapeutic drugs. Currently, the oxygen-glucose deprivation (OGD) induced model is the most relevant and commonly used in vitro model mimicking stroke [7]; however, studies have shown wide variability in the degree of injury [8]. The majority of OGD studies have used either primary rodent neuronal cells or neuroblastoma cell lines, such as the SH-SY5Y cell lines [9]. The SH-SY5Y cell line is a human-derived cell line that has been used for research on various neurological disorders, such as Parkinson’s disease, Alzheimer’s disease, ischemia, and amyotrophic lateral sclerosis [8, 10–12]. Since SH-SY5Y cells are of cancerous origin, they have several genetic aberrations; therefore, their use in in vitro models has been criticized [7]. Thus, in vitro nonneoplastic human cell-based models need to be developed. Human induced pluripotent stem cell (hiPSC) derived neural cells show great promise for studying neurological diseases because they are expendable cellular sources of neuronal cells, which are naturally hard to access [13, 14]. To our knowledge, there have been no reports on using hiPSC-derived neurons to model stroke in vitro.

Transplantation of mesenchymal stem cells (MSCs) represents a new potential therapeutic strategy for stroke [15]. In previous in vivo animal studies, transplantation of MSCs has been shown to promote functional recovery and reduce lesion size [16, 17], and these cells have already been utilized in clinical phase studies with varying results [18]. The mechanisms of action of MSCs are not known, but their restorative functions are suggested to be mediated by a paracrine effect. MSCs secrete various neurotrophic, angiogenic, and immunoregulatory factors, thereby suppressing inflammation and promoting angiogenesis, neurogenesis, remyelination, and axonal plasticity [19]. It is also noteworthy that endogenous neural stem cells can secrete multiple factors that are able to beneficially regulate neurogenesis and modulate inflammatory responses after CNS damage [20].

Adult MSCs can be harvested from the bone marrow (BM-MSCs) and adipose tissue, among other tissues of mesenchymal origin [21]. Adipose tissue-derived stem cells (ASCs) present multiple advantages due to their higher yield from donors and because they require less invasive harvesting methods than BM-MSCs [21]. In addition, MSCs can be expanded with human platelet lysate instead of fetal bovine serum to avoid immunological reaction to xenogenic substances [22, 23]. ASCs have been shown to have a beneficial effect on stroke recovery in animal models [24–26]. We, among others, have shown that ASCs improve behavioral recovery [24, 26] and reduce the death of neural cells in in vivo models [24]. However, ASC therapy has different results, especially in animal models with induced comorbidities such as hypertension [27, 28] and diabetes [27], in which beneficial effects of ASCs are not observed.

In this study, we developed two in vitro human cell-based models of ischemic stroke using neurons differentiated from the neuroblastoma cell line SH-SY5Y and hiPSCs and optimized an OGD protocol for use in cell models. In addition, we studied the paracrine neuroprotective effect of human ASCs against OGD-induced injury in these two in vitro models.

2. Materials and Methods

2.1. Differentiation of SH-SY5Y Neuroblastoma Cells into Neurons. The human neuroblastoma cells SH-SY5Y (ATCC) were thawed and cultured in basic medium including Eagle’s minimum essential medium (EMEM, Sigma-Aldrich) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific), 1% penicillin-streptomycin (P/S, Lonza), and 1% HyClone L-glutamine (GE Healthcare Bio-Sciences Austria GmbH). SH-SY5Y cells were differentiated into neuronal cells by using the protocol described by Shipley et al. [9] with moderate modifications. Briefly, SH-SY5Y cells were detached from cell culture flasks with TrypLE Select (Thermo Fisher Scientific), suspended in basic medium and plated into 6-well plates at a density of 10,000 cells/cm² (Nunc). The next day, the medium was changed to neuronal differentiation (ND) 1 medium supplemented with 2.5% FBS and 10 μM retinoic acid (RA, Sigma), and the cells were cultured for 7 days. Thereafter, the cells were cultured in ND2 medium supplemented with 1% FBS and 10 μM RA for 3 days. After that, the SH-SY5Y cells were detached with TrypLE Select and plated in 15 μg/ml laminin (LN521, Biolamina)-coated 24-well plates (Nunc) at a density of 25,000 cells/cm². The next day, the medium was changed to ND3, which was composed of neurobasal medium (Thermo Fisher Scientific) supplemented with 2% B-27 (Thermo Fisher Scientific), 20 mM potassium chloride (Merck), 1% P/S, 2 mM GlutaMAX (Thermo Fisher Scientific), 50 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems), 2 mM dibutyryl cyclic AMP (db-cAMP, Sigma), and 10 μM RA. SH-SY5Y cells were cultured under these conditions for 11 days; thus, altogether, differentiation was performed for 20 days prior to the start of OGD treatment. Thereafter, differentiated SH-SY5Y cells are referred to as SH-SY5Y-neurons.

2.2. Human Pluripotent Stem Cells and Differentiation of Neurons. The hiPSC line 10212.EURCCs [29] was generated with Sendai virus technology (Life Technologies) [30] at the Faculty of Medicine and Health Technology (MET), Tampere University, Finland. MET has received supportive statements from the regional ethics committee of Pirkanmaa Hospital District for the derivation, culture, and differentiation of hiPSCs (R08070). Informed consent was obtained from patients who provided cell samples. hiPSCs were expanded in a feeder-free culture system as described previously [31]. Cortical neurons were differentiated as previously described [32]. Briefly, the basal medium consisted of 1:1 DMEM/F12 with GlutaMAX, neurobasal medium, 0.5% N2, 1% B-27 with RA, 0.5 mM GlutaMAX, 0.5% NEA, 50 μM 2-mercaptoethanol (all purchased from Thermo Fisher Scientific), 2.5 μg/ml insulin (Sigma), and 0.1% P/S (Thermo Fisher Scientific). For neural induction, the basal medium...
was supplemented with 100 nM LDN193189 (Sigma) and 10 μM SB431542 (Sigma) for 12 days. Thereafter, the cells were cultured in basal medium supplemented with 20 ng/ml fibroblast growth factor-2 (FGF2, R&D Systems) for 15 days. Then, the cells were cultured in maturation medium, which consisted of basal medium supplemented with 20 ng/ml BDNF, 10 ng/ml glial-derived neurotrophic factor (R&D Systems), 500 μM db-cAMP, and 200 μM ascorbic acid (Sigma) for 7 days to promote the maturation of neurons. On day 32, the cells were plated in 24-well plates (Nunc) at a density of 100,000 cells/cm² and cultured for 7 days prior to OGD treatment. Differentiated hiPSCs are hereafter referred to as hiPSC-neurons.

2.3. Adipose Stem Cells. To study the effect of ASCs on neuronal cells, human ASCs (Master Cell Bank/Stock no. 1—Donor RESSTORE01, Batch no. 591133643763) were cultured in Alpha MEM (Gibco) supplemented with 5% human platelet lysate (STEMulate, Cook Medical) and 1% P/S. ASCs were isolated and cultured as previously described [26, 27]. The ASC phenotype was analyzed by flow cytometry (FACSAria Fusion Cell Sorter, BD Biosciences) and was found to reflect a typical MSC immunophenotype featuring expression (>95%) of the surface markers CD73, CD90, and CD105 and no expression (<2%) of CD11a, CD34, CD45, and HLA-DR [26].

2.4. Oxygen-Glucose Deprivation and Reperfusion. To model ischemic stroke in vitro, cells were first washed with glucose-free medium. Thereafter, SH-SY5Y-neurons were incubated in glucose-free ND3 medium, and hiPSC-neurons were incubated in glucose-free DMEM in a humidified oxygen control CO₂ incubator (HeraCell, Thermo Fisher Scientific) with 1% O₂, 5% CO₂, and 94% N₂ for 24 h at 37°C. Immediately after OGD, cells were reperfused by removing the medium and replacing it with ND3 for SH-SY5Y-neurons and maturation medium containing glucose for hiPSC-neurons, and the cells were incubated for 24 or 72 h at 37°C in 95% air/5% CO₂ (Figure 1). Control SH-SY5Y-neurons and hiPSC-neurons were washed and incubated in ND3 medium and maturation medium containing glucose, respectively, under normoxic conditions for 24 h in 95% air/5% CO₂ (HeraCell).

2.5. Coculture of Neurons and ASCs. The effects of ASCs on OGD-treated SH-SY5Y- and hiPSC-neurons during reperfusion were assessed in cocultures. ASCs (10,000 cells/insert, density of 30,000 cells/cm²) were plated on ThinCert™ TC inserts (pore size 0.4 μm, Greiner Bio-One) and incubated with SH-SY5Y- or hiPSC-neurons for 24 or 72 h at 37°C and 95% air/5% CO₂ (HeraCell). The experimental design is presented in Figure 1.

2.6. Immunocytochemical Staining. Immunocytochemical staining was performed as previously described [33]. The primary antibodies included dendritic marker microtubule-associated protein 2 (MAP2, chicken, 1:4000, NB300-213, Novus), microtubulin marker β-tubulinIII (mouse, 1:1000, T8660, Sigma), apoptosis marker cleaved caspase-3 (c-Casp3, rabbit, 1:400, 9664, Cell Signaling), and proliferation marker Ki-67 (rabbit, 1:800, AB9260, Millipore). The secondary antibodies included Alexa Fluor 488-conjugated donkey anti-rabbit (1:400), Alexa Fluor 568-conjugated goat anti-mouse (1:400), and Alexa Fluor 647-conjugated goat anti-chicken (1:200, all from Thermo Fisher Scientific). Cell samples were mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were acquired with an Olympus IX51 microscope equipped with an Olympus DP30BW camera (Olympus Corporation). CellProfiler [34] and CellProfiler Analyst [35] were used for image analysis.
genes was calculated using a mathematical model as previously described [38].

2.9. Viability Staining. A LIVE/DEAD Cell Viability/Cytotoxicity kit for mammalian cells (Thermo Fisher Scientific) was used to evaluate the viability of neuronal cells. The cells were incubated for 30 min at 37°C with 0.1 μM green fluorescent calcein-AM to detect live cells and with 0.5 μM red fluorescent ethidium homodimer-1 (EthD-1) to detect dead cells. The samples were imaged immediately with an Olympus IX51 microscope equipped with an Olympus DP30BW camera. For all staining experiments, four images per well were taken and used for image analysis. CellProfiler [34] software was used to perform image analysis as previously described [39], where the areas of calcein-AM- and EthD-1-positive cells in the images were determined from.

2.10. Statistics. An independent t-test was used for normally distributed data. A p value less than 0.05 was considered significant. If Bonferroni correction was applied, a p value less than 0.025 was considered significant. All data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using IBM SPSS Statistics 25 (IBM), and graphs were generated using GraphPad Prism 5.02 (GraphPad Software, Inc.).

3. Results

3.1. ASC Treatment Reduced OGD-Induced Cytotoxicity in SH-SY5Y- and hiPSC-Neurons. To model stroke in vitro, SH-SY5Y- and hiPSC-neurons were exposed to OGD for 24 h followed by reperfusion for 24 h or 72 h. Additionally, the paracrine effect of ASCs on injured neurons and control cells was studied. First, differentiated SH-SY5Y-neurons and hiPSC-neurons were characterized by staining for the neuronal marker β-tubulinIII and the dendritic marker MAP2, which were both expressed at high levels after neuronal differentiation in both cell types. After differentiation, SH-SY5Y cells and hiPSCs adopted a more neuronal cell-like morphology with long and branched neurites (Figure 2).

Then, the viability and cytotoxicity were studied with live/dead staining, and the percentages of areas with live cells and with dead cells were quantified. Overall, live/dead staining showed that SH-SY5Y-neurons were viable after OGD treatment (Figure 3(a)). However, there was a significant decrease in the percentage of live cells in the OGD group compared to the control group (p = 0.002) (Figure 3(b)). Similarly, after OGD (p = 0.001) and 24 h (p < 0.001) and 72 h (p = 0.012) after reperfusion, an increase in the number of dead cells compared to that in the respective control group was observed (Figure 3(c)). ASC treatment significantly increased the percentage of live SH-SY5Y-neurons after 72 h of culturing (p = 0.015) in control conditions (Figures 3(a) and 3(b)) but not after OGD at any of the time-points. Instead, upon coculture with ASCs, a decrease in the number of dead cells was observed in both control and OGD conditions at 24 (CTRL: p < 0.001; OGD: p = 0.002) and 72 h (CTRL: p < 0.001; OGD: p = 0.008) after reperfusion (Figures 3(a) and 3(c)). CyQuant analysis showed that fewer SH-SY5Y-neurons were detected at all timepoints in OGD conditions than in control conditions (0 h: p = 0.036; 24 h: p < 0.001; 72 h: p < 0.001). Coculture with ASCs significantly increased the number of SH-SY5Y-neurons after 24 h of reperfusion (p < 0.001) in OGD conditions, while ASCs did not have an effect on the number of cells in control conditions (Figure 3(d)). Next, apoptosis was studied by cl-Casp3 staining. More cl-Casp3-positive SH-SY5Y-neurons were detected in OGD conditions than in control conditions, while fewer were detected after ASC coculture, especially after 72 h of reperfusion (Figure 4).

Similarly, as for SH-SY5Y-neurons, the viability, cytotoxicity, and apoptosis of hiPSC-neurons were studied after OGD and coculture with ASCs. Quantification of the area (Figures 3(e) and 3(f)) showed that the percentage of live cells was significantly decreased in the 24 h reperfusion group compared to the control group (p = 0.015), while no differences were found in the percentage of dead cells between OGD and control conditions (Figures 3(e) and 3(g)). ASC coculture significantly increased the percentage of live cells at the 72 h timepoint in OGD (p = 0.017) and control (p = 0.002) conditions and decreased the percentage of dead cells at the 72 h timepoint in control conditions (p < 0.001). CyQuant analysis of hiPSC-neurons illustrated that the number of these cells was decreased immediately after OGD (p = 0.016) and 24 h after reperfusion (p = 0.003) compared to after culture in control conditions, but no differences were noticed between the OGD group and the control group after 72 h of reperfusion (p < 0.05). Compared to control culture, coculture with ASCs increased the number of cells 72 h after reperfusion (p = 0.008; Figure 3(h)). However, under OGD conditions, ASC coculture decreased the number of hiPSC-neurons 24 h after reperfusion (p = 0.019; Figure 3(h)). In hiPSC-neurons, a higher number of cl-Casp3-positive cells were detected after OGD than after ASC treatment (Figure 4).

3.2. ASC Coculture Enhanced the Proliferation of SH-SY5Y- and hiPSC-Neurons in Both OGD and Control Conditions. To investigate the effect of OGD and ASC coculture on
Figure 3: Continued.
proliferation, cells were stained with the cell cycle marker Ki-67 and quantified. The number of Ki-67-positive SH-SY5Y-neurons was decreased after OGD \((p = 0.011)\) and 24 h after reperfusion \((p = 0.017)\) compared to that after culture in control conditions. Coculture with ASC significantly increased the percentage of Ki-67-positive cells in both control and OGD conditions after 24 \((\text{CTRL: } p < 0.001; \text{OGD: } p < 0.001)\) and 72 h \((\text{CTRL: } p < 0.001; \text{OGD: } p < 0.001)\) of reperfusion (Figures 5(a) and 5(b)). A decreased number of Ki-67-positive hiPSC-neurons were detected after OGD \((p = 0.013)\) compared to that after control culture, whereas after 24 and 72 h of reperfusion, the number of Ki-67-positive cells was the same as that in the control group. Coculture with ASC increased the percentage of Ki-67-positive cells in both the control and OGD groups after 24 \((\text{CTRL: } p = 0.002; \text{OGD: } p = 0.001)\) and 72 h \((\text{CTRL: } p = 0.012; \text{OGD: } p = 0.014)\) of reperfusion (Figures 5(a) and 5(c)).

3.3. Axonal Damage to SH-SY5Y- and hiPSC-Neurons Was Increased in OGD Conditions. To examine whether OGD disrupts the integration of microtubules in axons, the expression of \(\beta\)-tubulinIII was studied at the gene and protein levels. Furthermore, gene expression of the neuronal growth cone marker \(\text{GAP43}\) was used to detect regenerative responses after OGD. In SH-SY5Y-neurons, the relative expression of \(\text{TUBB3}\) was decreased after OGD and 24 h of reperfusion compared to after control culture and then returned to the control level. Coculture with ASC did not influence the expression of \(\text{TUBB3}\) (Figure 6(b)). \(\beta\)-TubulinIII was expressed along the cytoskeleton of the neurons under control conditions. After OGD, \(\beta\)-tubulinIII staining became fragmented, showing disintegration in axons. Upon coculture with ASCs, less axonal disintegration of \(\beta\)-tubulinIII SH-SY5Y-neurons was observed in OGD conditions compared to control conditions (Figure 6(a)). The relative expression of \(\text{GAP43}\) was decreased after OGD and 24 h of reperfusion compared to after control culture, whereas no effect of ASCs on its expression levels was detected (Figure 6(c)). In hiPSC-neurons, the relative gene expression of \(\text{TUBB3}\) was not different between the OGD group and the
control group (Figure 6(e)), while β-tubulinIII was expressed at a lower level around the cell body and along the axons (Figure 6(d)) in OGD conditions compared to control conditions. Axons were denser in ASC and hiPSC-neuron cocultures than in OGD conditions. The relative gene expression of GAP43 was similar between the control and OGD groups, whereas its expression increased upon ASC coculture in OGD conditions (Figure 6(f)) compared to that in OGD conditions alone.

4. Discussion

Stroke has been modeled in vitro by removing oxygen and glucose from the cells or by chemical or enzymatic inhibition of cellular metabolism [7]. Most frequently, ischemia-like conditions are produced with OGD conditions, which involve replacing O2/CO2 equilibrated medium with N2/CO2 equilibrated glucose-free medium and maintaining cells in a hypoxic atmosphere [7, 40]. Since neurons have high glucose and oxygen demands [41], the removal of glucose and oxygen leads to impairment in maintaining normal ionic gradients followed by excitotoxicity, oxidative stress, and eventually apoptosis, autophagocytosis, and necrotic cell death [42, 43]. Commonly, OGD is followed by a reperfusion period, in which the restoration of glucose and oxygen to the cells causes the production of reactive oxygen species, which further induce cellular damage [44] and neuronal degeneration [45]. There is no standardized OGD protocol to mimic stroke in vitro, and in previous studies, the duration of OGD has ranged from 1 to 24 h [46] with or without a subsequent reperfusion period. The O2 concentration in hypoxic environments varies from 0% [47–53] to 8% [54]. In our in vitro stroke model, 24 h of OGD with 1% O2 and 24 or 72 h of reperfusion period was used.

Cellular platforms that have been used to model stroke in vitro include brain slices, organotypic cell cultures, primary neuronal cells, immortalized cell lines, and stem cells of human and rodent origin [7]. Human brain slices and primary cells are highly physiologically relevant; however, they are extremely limited in availability [7]; therefore, most OGD studies are performed with primary rodent neuronal cells or human neuroblastoma cell lines such as the SH-SY5Y cell line [9]. Prior studies in SH-SY5Y cells have used either undifferentiated cells or cells differentiated into neuronal cells to more precisely mimic a mature neuronal phenotype [9, 11]. However, high passage numbers and oncogenes limit the physiological relevance of neuroblastoma cell lines [7]. Human stem cells have a high potential to be utilized for in vitro stroke models because they are unlimited and have the potential for efficient neuronal differentiation [55]. hiPSC-derived neuronal networks have shown similar patterns of functionality as primary rat cells [32]. Currently, no studies have used hiPSC-derived neurons to model stroke in vitro. Here, two different cell types of human origin, SH-SY5Y cells and hiPSCs, were differentiated into neurons and used to model stroke in vitro. SH-SY5Y cells were differentiated into neurons using gradual serum starvation and the addition of extracellular matrix proteins and neurotrophic factors to yield more homogenous and mature neuronal cultures [9], while hiPSCs were differentiated into cortical cultures with small molecules to yield mature, functionally active neurons [32].

In our study, cytotoxicity and apoptosis of SH-SY5Y-neurons were increased, and viability SH-SY5Y-neurons was decreased after OGD followed by 24 or 72 h of reperfusion. This is in line with previous studies in nondifferentiated SH-SY5Y cells showing decreased cell viability and activation of apoptosis after OGD [53, 56]. Gao et al. showed that cell viability decreases, and the apoptosis rate increases after 16 h of OGD and 9 h of reperfusion. Lee et al. showed that cell viability decreases after 20 h of OGD and 24 h of reperfusion in SH-SY5Y cells. Similar to SH-SY5Y-neurons, hiPSC-
neurons showed increased cytotoxicity and apoptosis after OGD and 24 h of reperfusion, although the magnitude of damage was not as robust as in SH-SY5Y-neurons. In hiPSC-neurons, the cytotoxic effect of OGD was detected by determining the number of cells with CyQuant analysis but not with live/dead staining; however, OGD-induced damage was detected by both methods in SH-SY5Y-neurons. Altogether, our results show that both cell models respond to the same OGD parameters rather similarly, suggesting that the OGD paradigm can be standardized between different cell types used to model stroke in vitro.

Ischemic stroke causes rapid and significant loss of axons in the brain [57]. Thus, we explored in vitro axonal damage after OGD in both cell models. SH-SY5Y-neurons showed more disintegrated axons than hiPSC-neurons, although the morphology of hiPSC-neurons was changed after OGD compared to after control culture. Consistent with our studies, others have reported severe axonal damage in primary rodent neurons after OGD [50, 52]. Liu et al. showed degradation and disappearance of axons, a decrease in the length of the axons, and a change in the morphology of primary rat neurons after 90 min of OGD [52]. A similar effect was also shown in primary hamster neuronal hippocampal cells after 2 h of OGD and 48 h of reperfusion; neurons were severely injured, and axonal processes disappeared [50]. It seems that in primary neurons compared to SH-SY5Y- or hiPSC-neurons, OGD-induced cell damage is more rapid and more destructive to axons. We also studied the effects of OGD-induced axonal damage on the gene expression of the microtubule protein TUBB3 and the neuronal growth cone marker GAP43, which is considered an essential player in regenerative responses in the CNS [58]. In our study, SH-SY5Y-
Figure 6: Axonal damage to SH-SY5Y- and hiPSC-neurons. (a, d) Representative images of \( \beta \)-tubulin III staining after 72 h of reperfusion in (a) SH-SY5Y- and (d) hiPSC-neurons (blue = nuclei, red = \( \beta \)-tubulin III), scale bar, 50 \( \mu \)m (representative images of two to four separate experiments for SH-SY5Y-neurons and two separate experiments for hiPSC-neurons). (b, c) Relative expression of TUBB3 and GAP43 in SH-SY5Y-neurons (\( n = 1 \) from one experiment). (e, f) Relative expression of TUBB3 and GAP43 in hiPSC-neurons after OGD and 24 h or 72 h of reperfusion (\( n = 2 \) from two experiments).
neurons showed decreased expression of GAP43 and TUBB3 after OGD and 24 h of reperfusion. Similar effects were also reported for GAP43 protein expression in rat cortical neurons after OGD [52]. However, in hiPSC-neurons, a similar effect was not observed, as OGD did not influence the expression of GAP43 or TUBB3. Overall, SH-SY5Y-neurons were more prone to OGD-induced damage, while injury was less severe in hiPSC-neurons. The limited responsiveness of hiPSC-neurons to OGD might be due to the plasticity of hiPSCs. Most hiPSC-derived cells mimic the embryonic or fetal stage of development and therefore have a more robust ability to overcome damage [14]. Moreover, culture conditions, such as cell density and the composition of the medium used, may also influence the severity of damage.

After setting up the in vitro OGD-induced models in SH-SY5Y- and hiPSC-neurons, they were studied to the potential neuroprotective effect of ASCs. Studies in animal models of stroke have shown that MSC treatment has beneficial effects on stroke recovery; however, contradictory results have also been reported [16]. The majority of in vitro and in vivo studies have been performed on rodent and human BM-MSCs, while human-derived ASCs are less studied [15]. Here, ASCs were cocultured on inserts with SH-SY5Y- and hiPSC-neurons after OGD for 24 or 72 h. The results showed that ASC coculture increased cell viability and decreased cytotoxicity and cell death in both cell models. Similar findings have been reported for BM-MSCs [47, 48, 51, 54] and ASCs [59, 60] in various cell types, suggesting the overall beneficial effects of ASCs after OGD.

MSCs have been shown to provide neuroprotection by inhibiting apoptosis after OGD [47, 48, 50, 51, 54]. This anti-apoptotic effect has been observed in neurons treated either with human or mouse BM-MSCs or their conditioned medium prior to OGD [50, 51] or during reperfusion [47, 48]. In our study, apoptosis was evaluated with cl-Casp3 staining, which showed that ASC coculture decreased the number of cl-Casp3-positive SH-SY5Y- and hiPSC-neurons after OGD. Similar findings have been reported in rat primary neurons after treatment with ASC-conditioned medium or coculture with BM-MSCs for 24-48 h after OGD [48, 60].

MSCs secrete multiple soluble factors, such as cytokines, chemokines, and growth factors [19], which have an impact on the proliferation of neurons. Interestingly, in our study, ASC coculture increased the proliferation of SH-SY5Y- and hiPSC-neurons 24 and 72 h after OGD. Similar to our findings, increased proliferation of BM-MSC-treated human neuroblastoma M17 cells was observed after 24 and 48 h of reperfusion but not after 72 h of reperfusion [47]. Additionally, BM-MSCs have been shown to promote the proliferation of endogenous neural stem cells in a rat stroke model [61]. Here, increased proliferation after ASC coculture may also have been related to the presence of mitogenic factors in the ASC medium. Thus, the proliferative response of SH-SY5Y- and hiPSC-neurons seen here might have been due to the combined effect of mitogenic factors and secreted factors from ASCs.

Treatment with human BM-MSCs has been shown to promote axonal outgrowth, increase the length of axons [52], and diminish axonal disintegration [50] in primary rodent neurons after OGD-induced injury. In the present study, axons seemed less disintegrated after ASC coculture than after OGD, as demonstrated by β-tubulinIII staining. In SH-SY5Y-neurons, the gene expression of TUBB3 and GAP43 was not changed after ASC coculture, while GAP43 levels were increased 72 h after reperfusion in hiPSC-neurons, indicating ongoing regenerative processes. Similarly, increased GAP43 protein expression was observed by Liu et al. in rat cortical neurons after 48 h of exposure to both human BM-MSCs and their conditioned medium during reperfusion [52].

Overall, SH-SY5Y-neurons responded more strongly to OGD-induced injury and were more affected by subsequent ASC coculture than hiPSC-neurons. Surprisingly, ASC coculture appeared to be somewhat harmful to OGD-challenged hiPSC-neurons when cell numbers were compared using CyQuant analysis. In contrast, under control conditions, ASCs increased the number and viability of hiPSC-neurons. The harmful effect of ASCs after OGD injury might have been due to factors secreted by ASCs or because hiPSC-neurons were unable to adapt to the ASC microenvironment after OGD insult. This further suggests that OGD-treated hiPSC-neurons are more vulnerable to microenvironmental changes than control hiPSC-neurons. ASCs were grown on inserts that allowed trophic factors to diffuse between the cultures. Sheibe et al. reported that high concentrations (over 10%) of conditioned medium from mouse and human BM-MSCs and coculture with a high number of BM-MSCs have toxic effects on mouse primary neuronal cells [51]. In the present study, 10,000 ASCs were used for both neuronal cell types, which was twice the cell number that Sheibe et al. reported to be toxic. ASCs might also use the nutrients in the medium that are required by the neurons or secrete trophic factors that are harmful to OGD-treated hiPSC-neurons.

5. Conclusions

In conclusion, our data suggest that both cell types, SH-SY5Y- and hiPSC-neurons, are suitable for modeling stroke in vitro. Both cell types responded to OGD treatment; however, OGD had a stronger effect on SH-SY5Y-neurons than on hiPSC-neurons with immature phenotype. Our data also suggest that ASCs have neuroprotective effects after OGD injury in SH-SY5Y-neurons and hiPSC-neurons in vitro.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.
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