Developing a human cerebral organoid ischaemia model to investigate the response of oligodendrocytes to ischaemia

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Abbreviations

3D three-dimensional
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA analysis of variance
APC adenomatous polyposis coli
ATP adenosine triphosphate
BDNF brain-derived neurotrophic factor
BSA bovine serum albumin
CDM cerebral differentiation medium
CNS central nervous system
DAPI 4’-6-diamidino-2-phenylindole
DAPK1 death-associated protein kinase 1
dH2O distilled water
EBs embryoid bodies
EBM embryoid body medium
GABA gamma-aminobutyric acid
H&E haematoxylin and eosin
hiPSCs human induced pluripotent stem cells
IHC immunohistochemistry
IIM intermediate induction medium
MBP myelin basic protein
NG2 neuronal-glial antigen 2
NMDA N-methyl-D-aspartate
NSCs neural stem cells
VI
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>OPCs</td>
<td>oligodendrocyte progenitor cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>platelet-derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick-end labelling</td>
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Abstract

Stroke is the second leading cause of disability and the third leading cause of death globally. In Norway, there are 11,000 new cases of stroke each year, and 80–85% of these cases are caused by cerebral artery blood clots or emboli and are termed ischaemic. The current treatment options for ischaemic stroke are limited, and there is a need for new treatments that can reduce brain tissue damage after a stroke. Unfortunately, several neuroprotective agents shown to rescue the stroke-affected tissue in mice have had little to no effect when tested in humans. This suggests differences in stroke pathophysiology between humans and mice, urging the scientific community to produce new experimental models to bridge this gap. Ischaemic stroke leads to cell death distal to the affected blood vessel, including oligodendrocytes. The main job of oligodendrocytes is to myelinate the neuronal axons, working as electrical insulation. Previous studies suggest that oligodendrocytes are particularly vulnerable to ischaemia. Thus, saving oligodendrocytes may be a significant factor in protecting brain tissue after an ischaemic stroke.

The major aim of this thesis was to develop a human cerebral organoid ischaemia model to study the response of oligodendrocytes to ischaemia. To achieve this, I aimed to find the optimal ischaemia duration to achieve (1) a significant increase in dead cells after ischaemia compared with control, (2) a significant reduction in oligodendrocytes after ischaemia compared with control, and (3) a significant change in the number of proliferating oligodendrocytes after ischaemia compared with control. Human cerebral organoids were produced from human induced pluripotent stem cells and were used as a model for the human brain. In order to develop an ischaemia model, we tested different durations of incubation in glucose- and oxygen-free conditions. By TUNEL assay, I show that 1, 2 or 3 hours of ischaemia (i.e. oxygen- and glucose deprivation) is insufficient to cause a significant increase in cell death in the organoids. Only at 24 hours of ischaemia did we find significantly more cell death compared with control; however, more time points between 3 and 24 hours should be tested. I used immunohistochemistry to test the effect of ischaemia on oligodendrocyte lineage cells and to look at proliferation 3 days after ischaemia. My results show that there is a large variation between organoids in the number of oligodendrocyte lineage cells that are present. There was no significant difference in the number of oligodendrocytes after 2 or 24 hours of ischaemia compared with control. I found that there was a 45% and 56% reduction in the number of proliferating cells after 2 and 24 hours of ischaemia, respectively, compared with control.
However, there was no significant difference in the number of proliferating oligodendrocytes between 2 or 24 hours of ischaemia and control.
# 1 Introduction

## 1.1 Oligodendrocytes and myelin

Myelin is a lipid-rich substance that functions as an electrical insulator of the nerve cell axons. The first to describe myelinated fibres was arguably the Dutch trader and scientist Antonie van Leeuwenhoek in 1717 [1, 2]. The name myelin, after the Greek *myelos*, was later suggested by the German pathologist Rudolf Ludwig Virchow in 1854, long before its function was known [3]. Myelin is an extension of the cell membrane of myelin-producing cells and is wrapped around the axons, making *myelin sheaths* (Figure 1). The myelin sheath is not continuous but separated by unmyelinated regions called nodes of Ranvier [4]. When an action potential travels along a myelinated axon, the electrical impulse will “jump” from one node of Ranvier to the next. At the nodes of Ranvier, ions traverse across the membrane through voltage-gated sodium channels, and by this regenerating the action potentials and increasing the conduction velocity. This propagation of action potential is called *saltatory conduction*, by the Latin word *saltare* which means “to leap” [5].

Although discovered over a hundred and fifty years ago, recent research has given new insight into myelin structure and function. It has recently been discovered that the diameter of the axon fluctuates throughout the length of the axons, and the calibre decreases at the paranodes and nodes of Ranvier [6]. Myelin has shown to promote transport and phosphorylation of the neurofilaments in the connected axons, resulting in increased diameter of the axon at the myelinated area (internodes) [7]. Furthermore, myelin can also influence the density of axonal mitochondria, giving a lower density in the internodes compared with the nodes, which can be explained by a higher need for energy in the unmyelinated nodes [8]. Recently it was shown that myelin also supports axonal function by shuttling metabolites such as lactate [9, 10].
Figure 1: Schematic image of an oligodendrocyte myelinating two neuronal axons. Adapted from Rinholm et al. 2016 [11].

Myelin is produced by Schwann cells in the peripheral nervous system, and by oligodendrocytes in the central nervous system (CNS). Oligodendrocytes are a type of glial cell and can generate up to 60 myelinating processes [12]. These myelinating processes can have intermodal lengths from 20-200 µm and up to 100 turns around the axon. Outstretched, the oligodendrocytes’ surface area can span from $5 \times 10^3$ to $50 \times 10^3 \text{µm}^2$, which is larger than any other cell type in the body [12].

Oligodendrocytes arise from neural stem cells and subsequently differentiate through a series of developmental stages from progenitor cells, to immature oligodendrocytes, and finally to mature oligodendrocytes. Throughout the differentiation, different proteins are expressed and can be used as markers of the developmental stages (Figure 2). Early oligodendrocyte progenitor cells (OPCs) express A2B5, a polysialoganglioside, on the cell surface [13]. Throughout the body, many different cell types can express A2B5, but in the brain, it is relatively specific for the oligodendrocyte lineage. The transcription factors Olig1, Olig2 and Sox10, are expressed from early OPCs and throughout the oligodendrocyte lineage to mature cells [14]. As the early OPCs differentiate, they lose A2B5 expression and start expressing neuron-glial antigen 2 (NG2) and platelet-derived growth factor receptor alpha (PDGFRα) [15]. By further differentiation towards immature oligodendrocytes, the cells start to express O4,
while losing the expression of NG2 and PDGFRα [14]. O4 is a mixed antigen of sulfatide and other antigens on the oligodendrocyte surface and in myelin and is therefore also expressed in the mature myelinating oligodendrocyte [16]. Immature oligodendrocytes also express the marker O1, which is a glycolipid found in myelin. As the cells become mature myelinating oligodendrocytes, they express high levels of myelin basic protein (MBP) and proteolipid protein (PLP). Mature oligodendrocytes can also be localised by the marker adenomatous polyposis coli (APC), or often referred to as its clone name, CC-1. APC is a tumour suppressor gene found in multiple tissues, but in CNS it is localised in the oligodendrocyte cytoplasm [17].

Figure 2: Schematic diagram of oligodendrocyte differentiation displaying transcription factors and other markers specific for the different developmental stages. Adapted from Siegel et al. 2011 [18].
1.2 Cerebral organoids

The human brain is a tremendously complex system of heterogeneous regions and cell types. The development of the human brain exhibits several unique aspects including increased complexity and expansion of neuronal output, which have been proven difficult to study in model organisms [19]. Consequently, creating an in vitro model to study human brain development and disease has lately been an intense area of research. Previous models include several variants of human cells in culture, usually containing one or two different cell types (e.g. neurons and oligodendrocytes), but these cultures usually only consist of one or a few cell layers and lack the complex three-dimensional (3D) organisation of cell layers and regions of an intact brain. Recently, a new method was described to develop 3D-structures of brain tissue, called cerebral organoids, also referred to as brain organoids [20]. Cerebral organoids are artificially grown miniature organs that resemble the brain and develop various discrete brain regions. Human 3D brain organoids pose a huge advantage over previous 2D models because they can to a much larger extent recapitulate the tissue architecture as well as the cellular diversity and molecular complexity of the human brain [21]. The principles of making cerebral organoids are based on culturing human pluripotent stem cells (hPSCs) through a series of different culturing media, containing specific growth factors and hormones to facilitate neural differentiation. The stem cells are cultivated to form so-called embryoid bodies (EBs) and will eventually start forming neuroectoderm. The EBs are embedded in Matrigel droplets and are at the end placed in a spinning bioreactor to grow (see complete protocol in chapter 3.2 Cerebral organoids). The organoids can live in the bioreactor for several months, but since the organoids lack a vasculature system, the nutrients in the media will not be able to diffuse to the centre as the organoid grows more substantial, which in turn leads to necrosis of the core tissue [19].

The most significant advantage of using cerebral organoids as research model is that one can investigate diseases and developmental disorders in living human tissue. Even though animal models, such as rodents, are highly valuable for studying the brain, many neurological phenotypes involve heterogeneous combinations of many alleles of small effect, which are extremely difficult to recreate in animal models [22]. It would be ideal to study disease and development in the human brain, but access to living human brain tissue is limited. Furthermore, post-mortem studies reflect an end-point of disease, and it can be hard to determine the relationship between histological findings and clinical history [22]. The new in vitro model also contributes to the first of the 3Rs for research animal welfare: replacement,
reduction and refinement [23], which means that use of research animals can in some cases be avoided.

The organoids from this protocol will resemble tissue from an early developing human brain, approximately the first trimester. For this reason, cerebral organoids are a good model for studying developmental diseases, e.g. autism or epilepsy [19]. Cerebral organoids have been tested as a model for the neurodevelopmental disorder microcephaly, which has been challenging to study in a rodent model [20]. Microcephaly is a condition where the brain is underdeveloped, resulting in a markedly smaller head. The organoids were made from induced pluripotent stem cells (iPSCs) by reprogramming patient skin fibroblasts [20]. The patient skin fibroblast showed mutations in the CDK5RAP2 protein, which has been associated with primary microcephaly [24]. From this study, they showed that cerebral organoids could be used to model some aspects of microcephaly that has been difficult to model in mice due to a higher expansion of the founder population progenitor cells in humans before neurogenesis [20]. Zika virus (ZIKV) infections during the first trimester of pregnancy have been correlated with microcephaly in new-borns. By exposing human brain organoids to ZIKV, researchers have been able to investigate the early- and delayed-onset effects of ZIKV infections in the developing brain [25-28].

There are already several methods for cultivating neural stem cells (NSCs), but most of these methods make monolayer cultures. NSCs have been used for a long time to grow cell cultures for therapeutic purposes, but these methods are limiting because the cells will not orientate in the same manner as a developing brain [19]. A new method using neuronal rosettes have been developed to make organised 2D structures from pluripotent stem cells that can resemble neural tube epithelium [19]. The neuronal rosettes can be used to study many aspects of neuronal development, but with a 3D approach, as in the organoids, one can see more continuous neuroepithelia and different brain regions forming and capture the interplay of the different structures in the tissue.


1.3 Ischaemic stroke

The pathological condition where blood flow to a part of the brain is reduced or blocked entirely is termed stroke or cerebral infarction. The disruption of blood flow can be caused by occlusion (ischaemic) or rupture (haemorrhagic) of blood vessels. Stroke is the second leading cause of disability and the third leading cause of death globally [29]. In Norway, there are 11 000 new cases of stroke each year, and 80-85 % of these cases are ischaemic [30], and the rest of this chapter will focus on ischaemic stroke. Unfortunately, the options for treatment for this illness are limited. The treatments of ischaemic stroke in clinical practice include platelet inhibitors, thrombolysis and removing the thrombosis surgically (thrombectomy). Time is essential for treatment success; therefore, all the mentioned treatments should be given within a few hours after symptoms occur.

The attenuated blood supply in stroke leads to reduced supply of oxygen and nutrients and consequently to various degrees of cell damage and ultimately cell death. The area of the brain that is irrigated by the damaged artery will be most injured and is termed the ischaemic core. Around the ischaemic core, the tissue still has some blood flow to supply the cells and is called the penumbra [31]. In the penumbra, the cells do not die momentarily but can be viable for hours or days before they die of excitotoxicity, as described below [30]. Because the cells can stay alive longer, this region might be salvageable with post-stroke therapy [32].

Figure 3: Schematic diagram of glutamatergic synapses showing glutamate transporters in normal (A) and ischaemic (B) conditions. A: Glutamate is either taken up from the synaptic cleft or synthesised from glutamine.
by phosphate-activated glutaminase (PAG). Glutamate is packed into vesicles by vesicular glutamate transporter (VGLUT) and released to the synaptic cleft. From the synaptic cleft, glutamate can act on the postsynaptic glutamate receptors (GluR), opening the Na\(^+\) and Ca\(^{2+}\) permeable receptor channels NMDA and AMPA. Excessive glutamate can be taken up to astrocytes by the transporters GLT-1 and GLAST and converted to glutamine by glutamine synthetase (GS), or to the presynaptic neuron by GLT-1 or to the postsynaptic neuron by the transporter EAAC1. B: In ischaemic conditions, glutamate is initially released via vesicles, and later by reversal of glutamate transporters, into the synaptic cleft. Released glutamate acts on the glutamate receptors NMDA and AMPA, resulting in an influx of Na\(^+\) and Ca\(^{2+}\). Astrocytes can accumulate glutamate because insufficient ATP to convert it to glutamine. Presynaptic vesicles will lose glutamate due to ATP drop that leads to inhibition of VGLUT. Adapted from Allen et al. 2004 [33].

After ischaemic onset, adenosine triphosphate (ATP) levels drop rapidly due to nutritional shortage, as much as 20 % within 2.5 minutes [34]. The fall in ATP leads to dysfunction of the Na\(^+\)/K\(^+\)-ATPase, thereby disrupting the Na\(^+\) and K\(^+\) gradient across the cell membrane, resulting in membrane depolarisation. When the cell membrane depolarises at neuronal synapses, neurotransmitters are released through exocytosis and due to energy insufficiency, the neurotransmitter transporters are reversed [33] (Figure 3 B). Together, this causes excessive release of neurotransmitters including the most abundant excitatory and inhibitory transmitter molecules, glutamate and gamma-aminobutyric acid (GABA), which, in turn, disrupts neuronal function. Especially, extracellular glutamate concentration has to be under tight control as increased levels are highly toxic and have even been given its own name, excitotoxicity [35].

In the synaptic cleft, glutamate acts on three types of postsynaptic ionotropic receptors: N-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. Activation of these receptors leads to an exchange of ions across the cell membrane. To avoid over-activation of the receptors, released glutamate is quickly taken up from the synaptic cleft by the surrounding cells. Especially the NMDA receptor is highly involved in the mechanism of excitotoxicity due to its permeability to calcium. It was shown in a paper by Choi [36] that calcium influx is an essential part of glutamate excitotoxicity by showing that removal of extracellular calcium gave markedly less neuronal death in cortical cell cultures exposed to glutamate. When the NMDA receptor is activated by glutamate, the ion channel opens which allows passage of Ca\(^{2+}\) and Na\(^+\) into the cell and K\(^+\) out of the cell. In physiological conditions, Ca\(^{2+}\) levels are tightly regulated, and prolonged rise in Ca\(^{2+}\) levels can permanently alter a range of cellular functions. Stimulation by glutamate gives an initial Ca\(^{2+}\) rise that will descend to basal level within a few minutes. Although a short period of Ca\(^{2+}\) elevation is not harmful, longer challenges give a secondary phase of sustained Ca\(^{2+}\) rise that can reach a critical threshold from which cells rarely recover [37]. This prolonged elevation in Ca\(^{2+}\) levels will induce a signalling cascade, leading to cell death through either apoptosis or necrosis, depending on the cells mitochondrial function [38].
The brain glial cells, microglia, astrocytes and oligodendrocytes, are all necessary for normal neuronal function in CNS and can be indirectly involved in neuronal damage after ischaemia [39, 40]. Oligodendrocytes are highly vulnerable to the major pathological components of ischaemia, namely, hypoxia [41] and hypoglycaemia [42]. Since oligodendrocytes are in close relation to neurons, oligodendrocyte death can result in damage or loss of neuronal axons [43]. Studies from mice suggest that oligodendrocytes are particularly vulnerable to ischaemia compared with other glial cells and neurons [44], but the mechanisms leading to oligodendrocyte death are still controversial. Although oligodendrocytes are vulnerable to ischaemia, recent research has shown that oligodendrocyte precursor cells exposed to sublethal injury from ischaemia tend to proliferate more, giving rise to new oligodendrocytes, possibly in an attempt to remyelinate the peri-infarct area [45, 46]. Oligodendrocytes contain a high level of protein-bound iron, needed for the synthesis of myelin components [47]. In addition to being important for myelination, ferrous iron (Fe\(^{2+}\)) can be a potent cytotoxin by catalysing the conversion of hydrogen peroxide to hydroxyl radicals through the Fenton reaction [48, 49]. In ischaemia, lactic acidosis, a result of oxygen deficiency, causes mobilisation of protein-bound Fe\(^{2+}\), resulting in free cytosolic Fe\(^{2+}\) that can participate in the Fenton reaction and promoting oxidative stress [50]. Oligodendrocytes also have a low content of reduced glutathione, which is an important electron donor for the peroxide scavenger glutathione peroxidase, resulting in an amplified damaging effect [51]. Ceramide, a constituent of the myelin membrane, can promote apoptosis when released to the cytoplasm, e.g. through caspase-mediated pathways [52]. Furthermore, oligodendrocytes are very sensitive to disruption of the intracellular Ca\(^{2+}\) level, and ATP- and glutamate-mediated Ca\(^{2+}\)-elevation are major components of oligodendrocyte and myelin damage in cerebral ischaemia [53]. Importantly, oligodendrocytes are thought to express Ca\(^{2+}\)-permeable NMDA receptors that presumably contribute to their death in ischaemia [33], although this is controversial. Lately, it was suggested that Proton-gated Ca\(^{2+}\)-permeable TRP channels contribute to myelin damage in ischaemia [54].
1.4 Stroke therapy

The primary aim of stroke therapy is to restore blood flow to the affected area without causing more damage to the tissue. The secondary aim is to modulate factors that can exacerbate this damage and repair the damage if possible [39]. New treatment strategies seek to target both primary and secondary causes of stroke damage to reduce mortality and morbidity. Consequently, the research for neuroprotective agents to preserve neurons in the penumbra has been ongoing for a long time. When tested in animal stroke models, many of these substances reduced stroke lesion size and significantly improved recovery of the animals [39]. Surprisingly, despite the success of these preclinical studies, few of the substances have passed clinical trials, and to date, there is no agent approved for neuroprotection after ischaemic stroke in Europe or the United States [55-57]. A key challenge in this therapeutic field is, therefore, to be able to develop treatments that work in humans and not only in lab animals.

The most investigated treatment strategy has been to antagonise or modulate the NMDA receptor. The NMDA receptor can be inhibited by for example preventing glutamate from binding the active site, by blocking the co-agonist glycine from binding, or working as the channel blocker magnesium [58]. Loss of function of the NMDA receptor could theoretically result in less glutamate-mediated Ca\(^{2+}\) influx and possibly less damage on the affected cell. A long list of NMDA receptor antagonists and modulators have been tested, but none have passed clinical trials [59].

Other targets than the NMDA receptor have been tried for reducing the neuronal damage in stroke, including opioid receptor antagonists [60], Na\(^{+}\)-channel antagonists [61], Ca\(^{2+}\)-channel antagonists [62] and GABA receptor agonists [63], but all with sparse or negative results in clinical trials. Another target in the cascade of excitotoxicity is using NA-1, a peptide that disrupts NMDA receptor subunit interactions, neuronal nitric oxide synthase (nNOS) and postsynaptic density (PSD)-95 [64, 65]. Present data show promising neuroprotective effects of NA-1, but larger clinical trials are needed. Another approach to target excitotoxicity is by inhibiting death-associated protein kinase 1 (DAPK1) from coupling with the 2B-subunit of the NMDA receptor [66, 67]. During stroke conditions, DAPK1 is recruited to the NMDA receptor subunit 2B, leading to the initiation of the apoptosis pathway. Inhibiting this coupling can prevent the cell from going into apoptosis, but clinical trials have not yet been done to determine its effect in stroke. During the ischaemic cascade, free radicals in the form of reactive oxygen...
and nitrogen species (ROS/RNS) are produced. Preclinical research has shown that prevention of ROS/RNS-production or improvement of the cells antioxidative properties gives less damage after ischaemia [39]. Unfortunately, recent clinical trials for this treatment method have failed [68, 69]. However, Edaravone, another scavenger drug, is used clinically in parts of Asia and studies suggest that Edaravone might be efficacious to reduce neuronal impairment after stroke, but more data is needed on this matter [70].

To this day, most neuroprotective treatment has focused on salvaging the neurons by targeting the neurons themselves. However, within recent decades, researchers have hypothesised that one might have to target other brain cells, herein astrocytes, oligodendrocytes and microglia, to obtain an effective stroke treatment [71-73]. A major point here is that the human brain has a much higher number of glial cells, and particularly myelinating oligodendrocytes, than that seen in rodents [74]. This could potentially explain why drugs that mainly target neurons have protective effects in lab rodents but fail when tested on human subjects. Further, as the pathways to apoptosis are so complex, one target will not be enough to reduce post-ischaemic damage clinically.

AMPA and NMDA receptor antagonists have also been preclinically tested for ischaemia protective effect in oligodendrocytes. The AMPA receptor antagonist NBQX has shown reduced ischaemia-induced injury in oligodendrocytes [75, 76]. Another AMPA receptor antagonist is the clinically used antiepileptic, topiramate, which showed a protective effect on oligodendrocytes when administered post-insult [77]. The NMDA receptor has multiple target points to modulate, and one example tried for oligodendrocyte protection is memantine. Memantine is clinically used as a treatment for Alzheimer’s disease by blocking the channel with higher affinity than magnesium but has also shown effective in protecting oligodendrocytes after ischaemia [78]. Antioxidants and radical scavengers are other possible therapeutic agents for reducing ischaemia-induced injury in oligodendrocytes. The natural polyphenols mangiferin and morine have shown, in addition to the antioxidative effect, an ability to attenuate intracellular Ca^{2+} levels in oligodendrocytes [79]. Other agents with possible beneficial antioxidative and radical scavenging effect include Ebselen [80, 81], vitamin K [82], and melatonin [83]. In addition to attempting to reduce damage to oligodendrocytes after ischaemia, a therapeutic goal could be to increase the number of new oligodendrocytes by targeting oligodendrocyte proliferation.
In summary, no targeted cell-protective treatments after stroke have passed clinical trials. Two possible reasons for this could be that previous studies have ignored the importance of glial cells in stroke pathophysiology and that there are differences between the neural tissue of humans and laboratory animals (that are yet unknown). Consequently, there is a need for improved in vitro human stroke models that take all brain cells into consideration. The new development of human 3D cerebral organoids now makes it possible to make more advanced in vitro human stroke models that include most brain cells.
2 Aims and hypotheses

The overall aim of this thesis was to develop a human cerebral organoid ischaemia model to investigate the response of oligodendrocytes to ischaemia.

Sub-aims: to achieve the overall aim, I aimed to find the optimal time of ischaemia duration in order to achieve:

1. A significant increase in dead cells after ischaemia compared with non-ischaemic controls.
   - I hypothesise that one hour of ischaemia will give a significant increase in cell death compared with control.

2. A significant reduction in oligodendrocytes after ischaemia compared with non-ischaemic conditions.
   - I hypothesise that one hour of ischaemia will give a significant reduction of oligodendrocytes compared with control.

3. A significant change in the number of proliferating oligodendrocytes after ischaemia compared with non-ischaemic controls.
   - I hypothesise that that one hour of ischaemia will give a significant increase in the number of proliferating oligodendrocytes, while a longer duration of ischaemia, such as 24 hours, will give a significant decrease in the number of proliferating oligodendrocytes.
3 Materials and methods

3.1 Experimental setup

This thesis makes use of human cerebral organoids developed from induced pluripotent stem cells. Firstly, the cerebral organoids were prepared and cultured for three months. Then, the cerebral organoids were split into different treatment groups: 1 hour ischaemia (1h), 2 hours ischaemia (2h), 3 hours ischaemia (3h), 24 hours ischaemia (24h), and control (Ctrl, no ischaemia). After the treatment, the organoids were cut into sections, and three different experiments were performed:

1) TUNEL assay and fluorescence microscopy were done on the sections. Quantitative analysis of cell death was performed.

2) Immunostaining and confocal microscopy were done on the sections. Qualitative and quantitative analysis of oligodendrocytes were performed.

3) H&E staining and light microscopy were done on the sections. Qualitative analysis of cell morphology was performed.

An overview of the experimental setup is given in Figure 4.
Figure 4: Schematic display of experimental setup.
3.2 Cerebral organoids

Figure 5: Simplified presentation of the cerebral organoid culturing process. Modified from Lancaster et al. 2014 [19].

The following protocol for culturing cerebral organoids is based on the well-established method by Lancaster et al. [19], with minor modifications. An overview of the protocol is given in Figure 5. Since the original protocol by Lancaster gives a relatively low percentage of oligodendrocytes, we tried different protocols with different supplements to promote oligodendrocyte differentiation ([84, 85]). The best results were achieved by adding brain-derived neurotrophic factor (BDNF) (data not shown). Therefore, after the establishment of the organoids, BDNF was added from 30 days to promote oligodendrocyte differentiation.

Making embryoid bodies (EBs)

To make EBs, the human induced pluripotent stem cells (hiPSCs) (received from Magnar Bjørås, NTNU) were grown in one well of a six-well plate in the incubator (37 °C, 5 % CO₂) until 70-80 % confluency. The cells were then washed with 2 mL phosphate-buffered saline
1 mL Accutase was added, and the plate was put back into the incubator for 4-8 minutes. To help detach the cells from the plate, a pipette with 1 mL pipette tip was used to triturate and wash the plate. Cell suspension was transferred to a 15 mL conical tube with 5 mL of pre-warmed DMEM/F12 and mixed by trituration with a 1 mL pipette tip. The cells were centrifuged at 200g for 5 min at room temperature and aspirated and resuspended in 2 mL of embryoid body medium (EBM) with ROCK inhibitor (50 µM) and bFGF. A pipette with a 1 mL pipette tip was used to triturate up and down to ensure a single-cell suspension. 20 µL of cell suspension was taken out for counting of live cells. Another calculated volume of EBM with ROCK inhibitor was added to give 2,500 live cells per 150 µL. 150 µL of the cell suspension was plated out in each well of a 96-welled plate of low-attachment and put back in the incubator.

Feeding EBs and initiation of germ layer differentiation

Small EBs with clear borders should be visible after 24 hours. The EBs were continued to culture in the tissue incubator at 37 °C with 5 % CO₂. Every other day, the EBs were fed by removing approximately half of the medium and adding 150 µL fresh medium. ROCK inhibitor (1:100) and low-bFGF medium (4 ng/mL) were included the first four days.

Induction of primitive neuroepithelia

As the EBs began to brighten and have smooth edges, and the diameter was 500-600 µm, they were transferred to a low-attachment, flat-bottom 24-welled plate. Each EB was moved by a 200 µL cut-off pipette to wells containing 500 µL intermediate induction medium (IIM). 48 hours after the transfer, the EBs were fed with new 500 µL neural induction medium (NIM). After further two days, neuroectodermal differentiation should have started, and the aggregates could be transferred to Matrigel droplets.

Transferring neuroepithelial tissues to Matrigel droplets

Matrigel was thawed on ice at 4 °C for 1-2 hours. A dimpled Parafilm sheet was prepared (4x4 dimples) and placed into a 60 mm tissue culture dish. One by one neuroepithelial tissue was transferred to each of the dimples in the Parafilm sheet using a cut-off pipette. Excess medium was removed before droplets of ~30 µL Matrigel were added to each dimple. The tissue was then immediately moved to the centre of the droplet with a pipette tip. The dish containing the Parafilm sheet of Matrigel droplets was placed back into the 37 °C incubator.
and incubated for 20-30 minutes. 5 mL of cerebral organoid differentiation medium (CDM) without vitamin A were added to the dish. The Matrigel droplets were then removed from the Parafilm sheet and placed directly into the dish, and the culturing was continued in a CO₂ incubator.

**Stationary culture of expanding neuroepithelial buds**

After 24 hours the embedded tissues were observed under the microscope, and the tissue should be forming buds within 1-3 days. The droplets were incubated for another 24 hours before the medium was replaced with 5 mL of fresh CDM without vitamin A and put back in the incubator for another 48 hours.

**Growth of cerebral tissue**

After four days in static culture, the embedded organoids were transferred to a 125 mL spinning bioreactor. The organoids were cultured in 75-100 mL of CDM with vitamin A. The bioreactor was placed on a magnetic stir plate in the incubator. The media was changed every six days and morphology was monitored until the desired developmental stage. On day 30, freshly reconstructed BDNF was added to the flasks at a concentration of 14 ng/mL. We continued changing the medium every six days, but now with added BDNF.

**3.3 Human cerebral organoid ischaemia model**

Human cerebral organoids were used as a model of the human brain in our ischaemia experiments. Since the organoids lack blood vessels, an ischaemic stroke was simulated by inducing glucose deprivation and hypoxia as is done in other *in vitro* models [86-88]. The organoids were split into two groups: ischaemia and control. Briefly, the ischaemia group was treated with glucose-depleted media and put in a gas chamber with 95 % N₂ and 5 % CO₂, while the control received no treatment intervention (see Setup for full description).

To set up the first protocol, articles from similar experiments with brain slices in cultures were used as a base [87, 88]. We started with 40 minutes as ischaemia duration and different recovery periods (24h, 3d and 10d), and modified this through several pilot experiments. Even though reference articles did not need ischaemia treatment over 60 minutes, our organoids required longer time in ischaemic conditions to achieve a notable increase in the number of dead cells.
In this thesis, I show data from 1h, 2h, 3h and 24h ischaemia treatment with 24h and 3d recovery time.

**Setup**

![Diagram of the human cerebral organoid ischaemia model](image)

**Figure 6**: Schematic diagram of the human cerebral organoid ischaemia model.

Different media were prepared for each group (see chapter 3.12 *Solutions*) and distributed into a 24-welled plate. Each organoid needed two wells with media in addition to one cleaning well per plate. The plate with the medium for the ischaemia group was placed in the ischaemia chamber (MIC-101, Billups-Rothenberg) for equilibration (Figure 6). An equilibration step was done to ensure that the media did not contain any oxygen taken up from the atmosphere. When used, the media will be both completely glucose- and oxygen-free. After placing the plate in the chamber, the chamber was flushed with \( N_2 \)- and \( CO_2 \)-gas for three minutes (in order to remove oxygen) before closing the valves. The chamber was then placed in the incubator at 37 °C for 45 minutes to obtain physiological temperature for the organoids.

After equilibration, the organoids were transferred to the ischaemia chamber. Briefly, the organoids chosen for the ischaemia treatment were moved to wells containing the equilibrated ischaemia medium. This was done to rinse off any oxygen- and glucose-containing medium (Figure 6). The chamber was then re-flushed for three minutes with \( N_2 \)- and \( CO_2 \)-gas to remove
oxygen, then closed, and put in the incubator. After the wanted incubation time in ischaemic conditions was ended (1h, 2h, 3h or 24h), the ischaemia chamber was taken out, and the organoids were prepared for the recovery period. In this experiment, the organoids were allowed to recover for 24 hours and three days before further analysis. During the recovery period, the organoids need a good supply of oxygen, glucose and other nutrients. Therefore, the organoids were taken out of the ischaemia chamber and put in Petri dishes with ordinary medium for recovery. The dishes were placed on a shaker in the incubator at 37 ºC for the recovery period (5 % CO₂ added to room air in incubator). In the control group, the organoids were placed in normal growth medium and put in the incubator, with access to oxygen, and taken out at the end of the recovery period. To ensure that the organoids had enough nutrients during the recovery period, the media was changed after 24 hours.

3.4 Fixation

When tissue dies, the cellular structure will start to break down. To allow immunocytochemical analysis of the tissue, it must, therefore, be treated such that the cellular architecture and proteins can be preserved. This can be achieved by the use of fixatives. The fixative used for this project is paraformaldehyde (PFA). PFA is one of the most common fixatives and is a polymer composed of four units of formaldehyde. PFA conserves cytoarchitecture by forming covalent cross-links, called methylene bridges, with the nitrogen entity of proteins in the tissue [89]. Methylene bridges prevent lysis of the cells and give rigidity to the tissue. The organoids in this project were first added PFA at 4 % concentration (in phosphate buffer, see chapter 3.12 Solutions) for 2 ½ hours on a shaker at room temperature. After 2 ½ hours, the PFA was changed to 0.4 % concentration until further processing. The organoids can be stored in 0.4 % PFA for longer periods.

3.5 Cryo-preservation and microtomy

For cutting histological slides of the organoids, a frozen-section procedure was performed using a cryostat-microtome (CryoStar NX70, Thermo Scientific). Microtomy on frozen tissue is advantageous for organoids because the tissue is highly water-rich, which hardens when frozen and can give thin clear sections. The procedure is also less time-consuming than for example paraffin-embedded sections. The cryostat-microtome is a semi-automatic machine that cuts histological sections at micrometre level. The cryostat keeps a temperature at approximately -
17 °C while cutting the organoids. Before the organoids can be frozen down and histologically cut, they must be saturated with sucrose to prevent ice crystal formation that can negatively affect the cytoarchitecture of the tissue. The sucrose saturation is done by adding a sucrose solution to the organoids and stepwise increase the concentration of up to 30 %. Completely saturated organoids will sink to the bottom of the well. It should be ensured that the organoids are saturated before increasing the sucrose concentration. In this experiment, the organoids were incubated in 10 % sucrose (diluted in phosphate buffered saline, PBS) for one hour (1h), 20 % sucrose for three hours (3h) and finally in 30 % sucrose (overnight), all at 4 °C.

The sections for this project were cut 16 µm thick. They were mounted onto glass slides and stored at -20 °C.

### 3.6 Immunohistochemistry

Immunohistochemistry (IHC) is a technique for detecting specific antigens and their location in the tissue, using labelled antibodies. The technique was first introduced in 1941 and is now widely used to investigate a broad range of disease processes [90]. IHC can be done either directly or indirectly. When done directly, the antibody that binds to the wanted antigen is also conjugated to a reporter label, e.g., a fluorophore. The labelled antibody can then be detected in a microscope. To amplify the signal strength, the indirect method is more often used [91], which relies on the use of two antibodies: a primary and secondary antibody (Figure 7). The primary antibody is specific to the wanted antigen and binds to this, while the secondary antibody is specific to the host organism from which the primary antibody is made. The secondary antibody is conjugated to a reporter label and can be detected in a microscope.

The procedure for the IHC was as follows (see chapter 3.12 Antibodies): the frozen organoid sections were taken out to room temperature to thaw. A small container with 10 mM citrate buffer (pH 6) was put in the water bath and heated to 85 °C. The organoid sections were first washed for 10 minutes in PBS, then put in the container with preheated citrate buffer and heated in the water bath for 25 minutes before taken out to cool down to room temperature. All the following steps were carried out at room temperature unless otherwise specified. The organoid sections were washed in PBS 2x10 minutes and dried with tissue paper (Kimwipes). A hydrophobic barrier-pen (PAP-pen) (ImmEDGE, Vector Laboratories) was used to draw a circle around the sections on the object glass. The PAP-pen border makes a lipophilic barrier
around the sections so the applied water-based solutions will not float out during incubation. Blocking solution was applied inside the PAP-circles on the slides and incubated in a humidity chamber for one hour. The organoid sections were then washed 3x10 minutes in PBS. Solutions with primary antibodies were applied inside the PAP-circles, and the sections were incubated in a humidity chamber overnight. The next day the sections were washed 3x10 minutes in PBS before solutions with secondary antibodies were applied in the PAP-circles and incubated in a humidity chamber for one hour. The sections were washed 3x10 minutes in PBS. 4′-6-diamidino-2-phenylindole (DAPI) was applied in the PAP-circles and incubated in a humidity chamber for 15 minutes, followed by washing 3x10 minutes in PBS. The sections were dried, and mounting media (ProLong) was applied to the slides and cover glasses put on top. The cover glass was sealed with nail polish to prevent the sections from drying out. The mounted sections were stored at 4 °C until pictures were taken (see below) and then at -20 °C for long-term storage.

IHC was performed to asses what effect ischaemia had on oligodendrocytes and cell mitosis. Sections from 3.5 months old organoids from the ischaemia experiment were stained for the oligodendrocyte-specific markers Sox10, O4 and APC. In addition, the mitosis marker Ki-67 was used to examine differences in mitotic cells between ischaemia treated and control organoids. The OPC-specific antigen NG2 was also used to test the presence of OPCs in control organoids. Confocal microscopy was performed on the stained sections, and for Sox10 quantification, pictures were taken systematically of each organoid section. The pictures were taken of the area with most Sox10 positive cells within cortical regions (see last paragraph chapter 3.7 Cell death detection assay (TUNEL assay) for explanation of regions). Two pictures were taken of each section (four sections were analysed for each of the 2h ischaemia and control organoids, three sections were analysed for each of the 24h ischaemia organoids), and the unrepresentative images were removed before statistical analyses were performed.
Figure 7: Illustration of the principle of IHC. The primary antibody binds the target antigen on the cell. The secondary antibody, with an attached fluorophore, binds the primary antibody and can be detected in a fluorescence microscope.

3.7 Cell death detection assay (TUNEL assay)

After exposing the organoids to ischaemia, a viability test was done to determine the severance and extent of the damages after ischaemia. DNA degradation is one of the hallmarks of apoptosis [92]. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) is an assay that localises apoptosis in the tissue. The method is based on the binding of TdT to 3’OH-ends of broken DNA and attaching deoxynucleotides tagged with a fluorochrome [93]. The signal for apoptosis can then be detected and quantified through a fluorescence microscope.

The procedure for TUNEL assay was as follows (see chapter 3.12 Solutions): the frozen organoid sections were taken out to thaw and washed in PBS for 10 minutes. The sections were circled with PAP-pen. Permeabilisation solution was applied and incubated for 2 minutes on ice. Permeabilisation solution was removed, and the sections were washed twice with PBS. The reaction solution was applied to the sections and incubated in a humidity chamber for one hour in 37 °C. The slides were washed 2x10 minutes in PBS and dried before DAPI was applied and incubated in a humidity chamber for 15 minutes in room temperature. After the incubation with
DAPI, the slides were washed in PBS 3x10 minutes, dried and mounted with mounting medium and coverglass. Slides were kept at 4 ºC until pictures were taken and then at -20 ºC.

TUNEL assay was performed after each ischaemia experiment as a test for how the different durations of ischaemia conditions affected cell death. The TUNEL results from the two latest ischaemia experiments, including 3-3.5 months old organoids, were combined for statistical analysis. Fluorescence microscopy was done on the stained sections, and 1-2 pictures of the area with most TUNEL staining were taken systematically from the region of interest. Not using specific markers of brain regions, I found it only possible to distinguish between three main structural regions of the organoids, namely, those that resemble ventricles and the ventricular zone, the cortical and deeper layers of brain parenchyma, and lastly fluid-filled cysts, all described in the literature [20, 94]. In this model, the cortical area was chosen to analyse as it inhabits high quantities of mature cells, while the ventricular zone-looking area resides radial glia and significant numbers of stem and progenitor cells [20]. The two areas are depicted in Figure 12 A.

3.8 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining is an old and well-established staining method in histology. The principle for this method is to dye the tissue with two types of dye: haematoxylin and eosin. Haematoxylin stains chromatin in the nucleus and other acidic cellular elements [95]. The nucleus should obtain a blue stained nuclear membrane and chromatin, and unstained nucleoplasm. Eosin is the cytoplasmic counterstain and gives a red colour to the tissue. After staining with eosin, one can see different shades of red for different tissue structures, e.g. blood cells will be darker than smooth muscle [95]. Unlike IHC and TUNEL-staining, H&E is a dye and cannot be viewed in a fluorescent microscope but needs a conventional light microscope. Even though H&E staining is incompatible with immunofluorescence, haematoxylin can be used as a counterstain for immunohistochemical procedures that uses colourimetric substrates [96]. H&E staining was performed on organoid sections to examine the morphological changes in the tissue in control and ischaemia treated organoids. The examination was purely qualitative with a focus on live and dead cells. In the stained cerebral organoid sections, we looked for morphological hallmarks of necrosis, including a condensed nuclear appearance (pyknosis), fragmentation of the nucleus (karyorrhexis), complete breakdown of the nucleus (karyolysis) and disintegration of the cell membrane [97].
The procedure for H&E staining for frozen tissue sections was as follows (see chapter 3.12 Solutions): the organoid sections were air dried for several minutes to remove moisture. Haematoxylin was applied to the sections and removed after 1-10 minutes. The sections were rinsed in cool running distilled water (dH\textsubscript{2}O) for 5-30 minutes. If needed, 0.3 % acid alcohol was used to differentiate the sections. Eosin solution was applied to the sections for up to 30 seconds before the sections were dipped in dH\textsubscript{2}O to stop the staining. If needed, 0.3 % acid alcohol was used to differentiate the sections. To dehydrate the tissue, the sections were dipped 10 times in 50 % ethanol, 10 times in 70 % ethanol, placed in 95 % ethanol for 30 seconds and finally 1 minute in 100 % ethanol. The sections were dipped a few times in xylene to extract the ethanol, then dried with Kimwipes and mounted with Cytoseal XYL and coverslip.

3.9 Fluorescence microscopy

Unlike a conventional light microscope, where light passes through the specimen, the fluorescence microscope uses fluorescence or phosphorescence to generate an image of the sample. The specimen that is to be examined must be marked with substances that emit light when irradiated with a specific wavelength, also known as fluorophores [98]. The word fluorescence was coined by Stokes in 1852 when he observed red light emitted from the mineral fluorspar when illuminated with ultraviolet radiation [99]. This imaging technique is widely used in biology because of its ability to identify multiple fluorescent markers in tissue simultaneously, due to different excitation and emission wavelengths of the fluorophores. In this study, the fluorescence microscope was used for detecting viable and dead cells in the organoid sections after performing the TUNEL assay.

The principal of fluorescence is based on excitation and emission of light from the fluorophore. The prepared specimen is irradiated with multispectral light from a xenon or mercury source, which is filtered through an excitation filter. When the filtered light hits the specimen, it excites the fluorophore, which then emits light of a longer wavelength that can be detected through oculars, camera or other detectors. To ensure the unwanted excitation wavelengths do not reach the detector, an emission filter is used to allow passage of only the emitted wavelengths.

For imaging the results of the TUNEL staining we used a Zeiss Axioplan 2 fluorescence microscope with Zeiss Axiovision software. The images were taken with 100x and 200x magnitude using 10x (aperture 0.30) and 20x (aperture 0.8) objectives.
3.10 Confocal microscopy

Confocal microscopy is an imaging technique within fluorescence microscopy for improving optical resolution and contrast using a pinhole. Most confocal microscopes use lasers which emit light with highly specific wavelengths, thereby increasing the accuracy of fluorophore excitation. This concept was developed by Marvin Minsky in the 1950s [100] and is now widely used in biomedicine to image both fixed and living cells. In a conventional widefield fluorescence microscope, secondary fluorescence is often detected and can interfere with the resolution of the area in focus. By using a pinhole, the out-of-focus signals can be eliminated or reduced, and thin optical sections can be achieved. These optical sections can be processed to make 3D-images of the structures or combined to make 2D-stacks. In addition to reducing out-of-focus glare, the confocal microscope lets us control the depth of field, which is useful in sections thicker than 2 μm [101]. The organoid sections used in this study were 16 μm thick, which made the confocal technique ideal for imaging different cell types and structures within the tissue. By using different lasers and fluorophores, the confocal microscope can be used for detecting co-localization of structures in the specimen.

To image the organoid sections stained by IHC, a Leica TCS SP8 STED confocal microscope with Leica LASX software was used. The images were taken with 400x magnitude with a 40x (aperture 1.30) oil objective.

3.11 Image analysis

For all the image analyses, the Java-based open-source image processing program, ImageJ, was used. ImageJ was developed at the National Institute of Health and the Laboratory for Optical and Computational Instrumentation [102]. The pictures taken by the microscope are raster images, meaning they are built up by picture elements (pixels). The pixels of an image are assigned a specific location and colour value. The image resolution specifies the number of pixels per inch (PPI), while the bit depth describes how much colour information is available in each pixel. A greyscale image with bit depth 1 will only have two possible pixel values: black and white. If the image has a bit depth 8, the possible values will be $2^8 = 256$. Therefore, an 8-bit picture will have a value range from 0-255, 0-65535 for 16-bit and 0-4294967295 for 32-bit. The microscope pictures contain greyscale images from each laser channel that can be segmented by intensity and shape to give positive and negative regions. For the quantitative
analysis of cells, we used predefined threshold algorithms to define the positive regions of interest. For the analysis of dead cells after the TUNEL assay the threshold algorithms Renyientropy, Moments and Intermodes were considered for each image. Renyientropy and Intermodes are both histogram-based algorithms, where Renyientropy uses entropy and Intermodes uses the mean of two local maxima in the histogram to threshold [103, 104]. Moments is a threshold method where it attempts to preserve the moments of the image in the result [105]. For the analysis of the IHC results, we used the Trainable Weka Segmentation to threshold and segment the images. The Trainable Weka Segmentation is a free ImageJ-plugin that can be trained by the user to extract positive regions of interest and later perform the segmentation on new unknown images [106]. On the segmented images, we performed a particle analysis, with the ImageJ-plugin, to extract information about the number, size, shape and intensity of the cells in the image. To analyse the particles automatically, we made JavaScripts for each operation. The picture-taking and the processing were blinded to avoid bias.

3.11.1 Statistical analysis

For the statistical analyses, Microsoft Excel and GraphPad Prism 7 were used. For normally distributed data, one-way Analysis of Variance (ANOVA) with Dunnett’s test for multiple comparisons as posthoc analysis was conducted. For data with non-normal distribution, Kruskal-Wallis one-way ANOVA on ranks was carried out with Dunn’s test for multiple comparisons as posthoc analysis. All results are shown as mean ± standard deviation. To reduce influence from inter-experimental variation, all results were normalised to the mean of the control group, if not otherwise specified. Outliers were found with Grubbs’ test and removed before statistical analysis.

In the TUNEL experiment, results from two experiments are combined. Four sections were included from each organoid in each treatment group, and 1-2 pictures were systematically taken from each section. The data for each organoid was produced by the mean of the data from its associated pictures. In the combined analysis the treatment groups are 1h (n=2), 2h (n=7), 3h (n=7), 24h (n=3) and control (n=10), all with three days recovery, where n is the number of organoids.

In the IHC experiment, organoid sections from the last ischaemia experiment were used, with six organoids in both 2h ischaemia and control group, while 24h ischaemia had three organoids.
Four sections from each organoid were included, except the 24h ischaemia group where three sections were included. Pictures were systematically taken of each section.

### 3.12 Solutions and antibodies

<table>
<thead>
<tr>
<th>Antibodies for immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigen</strong></td>
</tr>
<tr>
<td>APC/CC1</td>
</tr>
<tr>
<td>NG2</td>
</tr>
<tr>
<td>O4</td>
</tr>
<tr>
<td>Sox10</td>
</tr>
<tr>
<td>Ki-67</td>
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</tbody>
</table>

**Solutions for immunohistochemistry:**

- PBS (0.01 M, pH 7.4)
- Citrate buffer (10 mM, pH 6.0)
- DAPI (1:5000, Sigma, #D-9542-1MG))
- ProLong mounting medium (Invitrogen, #P36981)

**Block solution:**
- 10 % Normal goat serum (NGS) (Sigma, #69023)
- 1 % Bovine serum albumin (BSA) (Saveen Werner, #B2000-500)
- 0.5 % Triton X-100 (Sigma, #T8787)
- Diluted in PBS
Block solution for O4:
10 % NGS
Diluted in PBS

Primary and secondary antibody solution:
3 % NGS
1 % BSA
0,5 % Triton X-100
Diluted in PBS

Solutions for TUNEL assay:

PBS (0,01 M, pH 7,4)

ProLong mounting medium (Invitrogen, #P36981)

In Situ Cell Death Detection Kit (TUNEL) (Roche, #11684817910)

Permeabilization solution:
0,1 % Triton X-100
Diluted in PBS

Solutions for H & E staining:

Heamatoxylin solution (from dept. of pathophysiology)

Eosin solution (from dept. of pathophysiology)

Acid alcohol (0,3 % HCl in ethanol)

Ethanol (50 %, 70 %, 95 % and 100 %)

Xylene

Cytoseal XYL mounting medium (Thermo Fischer, #22-050-262)
Solutions for culturing organoids:

Geltrex, LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific, #A1413301)

DMEM/F-12 medium (ThermoFisher Scientific, #11330-057)

Essential 8 medium (Invitrogen, #A1517001)

Accutase (Stemcell Technologies, #07920)

ROCK inhibitor Y-27632 (EMD Millipore, #SCM075)

KnockOut Serum Replacement (ThermoFisher Scientific, #10828-028)

Hyclone Fetal Bovine Serum (ThermoFisher Scientific, #SH30070.03)

GlutaMAX Supplement (ThermoFisher Scientific, #35050-061)

MEM Non-Essential Amino Acids Solution (Invitrogen, #11140-050)

2-mercaptoethanol (ThermoFisher Scientific, #21985023)

Penicillin Streptomycin Solution 100X (VWR, #30002C1)

Recombinant human FGF-basic (Peprotech, #100-18B)

N-2 supplement (100X) (Life Technologies, #17502048)

Heparin (Sigma, #21103-049)

Neurobasal Medium (Invitrogen, #21103-049)

Insulin (Sigma, #I9278)

B27 without Vitamin A (Invitrogen, #12587010)

B27 (Invitrogen, #17504044)

Matrigel matrix gel (Corning, #356234)
Recombinant Human BDNF (Peprotech, #450-02B)

**Preparation of medium for culturing organoids:**

**Embryoid body medium – EBM (~500 mL):**
- DMEM-F12 (400 mL)
- KOSR (100 mL)
- Foetal Bovine Serum (15 mL)
- GlutaMAX (5 mL)
- MEM-NEAA (5 mL)
- 2-mercaptoethanol (3.5 μL)
- Penicillin-Streptomycin (5 mL)

**Intermediate induction medium – IIM (~500 mL):**
- DMEM-F12 (500 mL)
- KOSR (30 mL)
- FBS (4.5 mL)
- N2 supplement (3.5 mL)
- GlutaMAX (5 mL)
- MEM-NEAA (5 mL)
- Heparin (350 μg)
- Penicillin-Streptomycin (5 mL)

**Neural induction medium – NIM (~500 mL):**
- DMEM-F12 (480 mL)
- N2 supplement (5 mL)
- GlutaMAX (5 mL)
- MEM-NEAA (5 mL)
- Heparin (500 μg)
- Penicillin-Streptomycin (5 mL)

**Cerebral differentiation medium – CDM (~500 mL):**
- DMEM F-12 (250 mL)
- Neurobasal medium (250 mL)
N2 supplement (2.5 mL)
GlutaMAX (5 mL)
MEM-NEAA (2.5 mL)
Insulin (125 μL)
2-mercaptoethanol (1.75 μL)
Penicillin-Streptomycin (5 mL)
B27 with or without vitamin A (5 mL)

**Solutions for the ischaemia experiment:**

**Ischaemia medium (10 mL):**
DMEM/F-12 Flex media, ÷ glucose, + phenol red (5 mL)
Neurobasal -A medium, ÷ D-glucose, ÷ sodium pyruvate added penstrep and phenol red (4.85 mL)
N2 supplement (0.05 mL)
MEM-NEAA (0.05 mL)
B27 supplement + Vit A (0.1 mL)
2-mercaptoethanol (0.0034 mL)
NaCl (0.05 mL from 2 M stock)

**Control medium (10 mL):**
DMEM/F-12 Flex media, ÷ glucose, + phenol red (5 mL)
Neurobasal -A medium, ÷ D-glucose, ÷ sodium pyruvate, added penstrep and phenol red (4.6 mL)
N2 supplement (0.05 mL)
MEM-NEAA (0.05 mL)
B27 supplement + Vit A (0.1 mL)
2-mercaptoethanol (0.0034 mL)
Glucose (0.3 mL)

Paraformaldehyde (4% and 0.4 %) (Santa Cruz, #281692)
Sucrose in PBS (10 %, 20 % and 30 %)
4 Results

4.1 The effect of ischaemia on cell viability

TUNEL cell death assay

The TUNEL cell death assay was performed to study the viability of the cells after the ischaemia experiment (see chapter 3.7 Cell death detection assay (TUNEL assay)) was performed. Overall, there was a large variation of TUNEL staining between organoids within the same treatment group: some organoids had nearly no TUNEL-positive cells, while other organoids had high numbers of TUNEL-positive cells (Figure 9). In the combined analysis the treatment groups are 1h (n=2 organoids), 2h (n=7), 3h (n=7), 24h (n=3) and control (n=10), all with three days recovery. To ensure no TUNEL staining outside cells were counted, only double-positive TUNEL and DAPI cells were included in the analysis. The results of the analysis are presented in Figure 10. After 24 hours of ischaemia, there was a 12-fold increase of TUNEL-positive cell density compared with control (Ctrl: 1 ± 1.27. 24h: 12.08 ± 3.78, p=0.0036). In the other groups there was not a significant difference compared with control (1h: 2.72 ± 1.19, p=0.5027. 2h: 2.96 ± 2.51, p=0.2228. 3h: 1.8 ± 1.93, p>0.99).

To assess which recovery time would be used for future experiments, a small pilot experiment was done with 24 hours recovery (Figure 11). Because of a loss of organoids during the experiment, the dataset was too small to perform a robust quantitative analysis. However, a graphical representation of the data shows the distribution of cell death density among the organoids, but one should show caution when interpreting the data (Figure 11 F).

Cell death analysed with H&E staining

After the ischaemia treatments, H&E staining was done to examine qualitative differences between the treatment groups: 24h ischaemia, 2h ischaemia and control. The qualitative impression of this analysis was that it appeared to be some variation both among and within the organoids regarding the cell morphology. However, it seemed that the ischaemia groups tended to show more signs of cell death than the control (Figure 12). The overall impression of the ischaemic treated parenchyma was a change in the general cytoarchitecture with loss of the
normal cellular integrity, compared with the healthy tissue in control organoid sections (Figure 12 A-B). Organoid sections with much apparent necrosis would show clustered pyknotic nuclei with disrupted cell membrane (Figure 12 B and D). Karyorrhectic nuclei and karyolytic cells were also found in these organoid sections (Figure 12 D).

4.2 The effect of ischaemia on oligodendrocytes

To test how the different durations of ischaemia affected the number of oligodendrocytes, immunohistochemistry labelling for oligodendrocyte lineage cells was performed on organoid sections after the ischaemia treatment. Sections were taken from six organoids from the 2h ischaemia, three organoids from the 24h ischaemia and six organoids from the control group.

O4 and APC

For the oligodendrocyte markers APC (mature oligodendrocytes) and O4 (immature and mature oligodendrocytes), our organoid sections did not contain enough positive cells to perform quantitative analysis. Thus, qualitative analyses were carried out for these markers.

Only a few organoids showed mature APC-positive oligodendrocytes, and in these organoids the density of APC-positive cells was low (Figure 13 A-B). A similar impression was achieved with the O4 marker, which labels immature and mature oligodendrocytes (Figure 13 C-D). In all, our impression was that there seemed to be no relation between which organoids that stained for mature and immature oligodendrocytes and those exposed to ischaemia, but due to the low number of positive cells and the variation between organoids we cannot make any conclusions.

As a control to test that the antibodies are specific and functional, we have performed similar immunostaining with APC and O4 on mouse brain slices in which the antibodies showed strong staining of oligodendrocytes (data not shown). Further, the cytoplasmic labelling shown in Figure 13 A-B is typical for APC-labelling observed in oligodendrocytes in vivo [107]. Moreover, the labelling in Figure 13 C-D of the membrane-bound marker O4 shows similar morphology to what is reported in oligodendrocytes [108].
NG2
We tested organoids from the control group multiple times for the presence of NG2 (oligodendrocyte progenitor cells), but no NG2-positive cells were found (Figure 14 A). Although no NG2-positive cells were found in the 3.5 months old organoids, some NG2-positive progenitors were observed in 9 months old organoids. As evidence of a functional antibody, images from 9 months old organoids of NG2 progenitors are shown in Figure 14 B.

Sox10
The cerebral organoid sections were also labelled for the oligodendrocyte-specific transcription factor Sox10 (Figure 15 C). Sox10 is located in the cell nucleus in oligodendrocytes in all stages of development [14]. After 24 hours of ischaemia, there were on average 76 % fewer Sox10-positive cells compared with control, but this difference was not statistically significant (Ctrl: 1 ± 0.77. 24h: 0.24 ± 0.09, p=0.5545). The organoids treated with 2 hours of ischaemia showed a 137 % higher mean number of Sox10-positive cells compared with control, but this difference was also not statistically significant (Ctrl: 1 ± 0.77. 2h: 2.37 ± 1.59, p=0.1043). The data are presented in Figure 15 E. In Figure 15 C the nuclear staining of Sox10 is shown.

Cells in mitosis
The same organoid sections were also stained for the mitosis marker Ki-67 to determine if there were differences in number of cells undergoing mitosis between the ischaemia treated and control organoids (Figure 15 B). Ki-67 is a nuclear marker that stains the perichromosomal compartment [109]. The ischaemia treatment groups showed a statistically significant lower number of mitotic cells compared with the control group. After 2 hours and 24 hours of ischaemia there was a 45 % and 56 % reduction of Ki-67-positive cells compared with the control group, respectively (Ctrl: 1 ± 0.36. 2h: 0.55 ± 0.19, p=0.0477. 24h: 0.44 ± 0.26, p=0.0354) (Figure 15 F).

Oligodendrocytes in mitosis
An analysis of the number of cells positive for both Sox10 and Ki-67 was done to see if there was a difference in the number of mitotic cells from the oligodendrocyte lineage between the treatment groups. The organoids treated with 24 hours of ischaemia had on average 84 % fewer mitotic oligodendrocytes compared with control (Ctrl: 1 ± 1.24. 24h: 0.16 ± 0.085, p=0.0904)
(Figure 15 G), but this was not statistically significant. There was also no significant difference between the 2 hours ischaemia and control group (Ctrl: 1 ± 1.24, 2h: 0.73 ± 0.72, p>0.99). Cells positive for Ki-67, Sox10 and DAPI is shown in Figure 15 D.

**Cell density**

There was an observable variation in cell density among the organoid sections. Therefore, the mean number of DAPI-positive cells over area was calculated for each organoid used in immunohistochemistry (Figure 8). There was not found a significant difference between the treatment groups. However, to account for individual differences in cell density, data from immunohistochemistry has been presented as number of cells per DAPI-positive cell.

![Image of cell density analysis](image)

**Figure 8:** Analysis of cell density between treatment groups. Shown as mean number DAPI-positive cells over area. Each data point (filled circle) represents one organoid. Data is not normalised.
Figure 9: A-F: Images showing the large variation in TUNEL staining between organoids. Images of 2 typical organoid sections with low (A-C) and high (D-F) number of TUNEL-positive cells (green). DAPI staining (all cell nuclei, blue) shown in A and D, TUNEL staining shown in B and E, and merged image shown in C and F. Scale bar: 200 µm.
Figure 10: A-E: Images of organoid sections from TUNEL assay of 3 days recovery, showing DAPI (all cell nuclei, blue) and TUNEL-positive cells (green). Treatment groups are 1h ischaemia (A), 2h ischaemia (B), 3h ischaemia (C), 24h ischaemia (D) and control (E). F: Graph shows TUNEL assay where each data point (filled circle) represents one organoid. Normalised to the control mean. Scale bar: 100 µm.
Figure 11: A-E: Images of organoid sections from TUNEL assay of 24 hours recovery, showing DAPI (all cell nuclei, blue) and TUNEL-positive cells (green). Treatment groups are 1h ischaemia (A), 2h ischaemia (B), 3h ischaemia (C), 24h ischaemia (D) and control (E). F: Graph shows TUNEL assay where each data point (filled circle) represents one organoid. Normalised to the control mean. Scale bar: 100 µm.
Figure 12: Images of cerebral organoid sections stained with H&E from control (A and C) and ischaemia treated (B and D) organoids. A: Organoid section from the control group showing cortical and deeper brain parenchymalike regions (open arrows) and ventricular zone looking structures (filled arrow) (see chapter 3.7 Cell death detection assay (TUNEL assay) for background information). B: Organoid section from 2h ischaemia treatment showing clustering of condensed nuclei (filled arrow). C: Organoid section from the control group showing normal nuclei (filled arrow) and processes (open arrow). D: Organoid section from 24h ischaemia treatment showing fragmented nuclei (orange filled arrow), leakage of cytoplasm (orange open arrow) and complete breakdown of the nucleus (white filled arrow). Scale bar: 100 µm (A-B) and 15 µm (C-D).
Figure 13: Images of sections from control (A and C) and 2h ischaemia treated (B and D) organoids immunostained with APC (A-B) and O4 (C-D). DAPI is blue. Scale bar: 50 µm.
Figure 14: Images of sections from 3.5 months old organoid (A) and 9 months old organoid (B) immunostained for NG2 (green). DAPI is blue. Scale bar: 50 µm.
Figure 15: A-D: Immunostaining of section from 2h ischaemia treatment, with DAPI (all nuclei, blue, A), Ki-67 (red, B) and Sox10 (green, C). D: Merged image showing Sox10- and DAPI-positive nuclei (turquoise, blue arrow), Ki-67- and DAPI-positive nuclei (magenta, pink arrow) and Sox10-, Ki-67- and DAPI-positive nuclei (yellow, white arrows). E-G: Graphs display mean number of Sox10- (E), Ki-67- (F), and both Ki-67- and Sox10-positive cells (G) divided by the mean number of DAPI-positive cells. Normalised to the control mean. Each data point (filled circle) represents one organoid. Scale bar: 80 µm.
5 Discussion

In this study, we have aimed to establish a model for treating human brain organoids with ischaemia. We have shown that a surprisingly long period of ischaemia is required to kill a significant number of cells compared with control organoids, however, more experiments are needed to find the optimal conditions for the ischaemia. From this model, we showed that cells from ischaemia-treated organoids are significantly less mitotic than cells from control organoids. We have found the presence of oligodendrocytes in the organoids, although, their quantity was low and did not change by the ischaemia treatment. With more research, this model has the potential of being used for in vitro testing of post-stroke treatment methods.

5.1 Discussion of methods

Cerebral organoids as a model of the human brain

The use of cerebral organoids allows us to study brain development and disease directly in artificially grown human brain tissue, which has been difficult to do before. Many results from clinical trials do not correspond with the preclinical studies, which suggest differences between humans and animals that are yet unknown to us. Because of this low correspondence between preclinical studies and clinical trials, an in vitro model with human tissue can be valuable for verifying the findings in animal models before proceeding to clinical trials. For many research purposes, using cerebral organoids alone will not be sufficient to give a complete answer to disease and developmental issues. The amount of research on brain organoids as research models has escalated in the last few years, but there are still several limitations to this model.

Even though cerebral organoids have derived from human stem cells, some essential aspects distinguish them from a human brain. For instance, they lack vascularisation and blood supply. The absence of a vascular system limits the growth and differentiation of the organoid by nutrients not being able to diffuse to the centre of the tissue. The centre of the organoid will, therefore, be necrotic, and healthy cells will mostly be found in the outermost ~300 μm of the organoid (personal observations). Researchers are currently investigating the problem of vascularisation, and a recent paper by Mansour et al. showed that cerebral organoids could be transplanted into mouse brains and be supplied by the mouse’s vascular system [110]. Vascularisation by adapting the organoid to a living host would probably solve some issues
regarding cell survival and differentiation, but the model would no longer be purely human, which is one of the desired aspects of this model. Furthermore, to be able to implant a human organoid into a mouse Mansour et al. used immunodeficient SCID mice (Jackson laboratory 001303 [111]) which could be suboptimal due to lack of a physiological immune response.

When an ischaemic stroke occurs, cells from the innate and acquired immune systems are recruited to the site of injury. Ischaemic brain cells release damage-associated molecular patterns, including reactive oxygen species (ROS), high mobility groups box 1 (HMGB1) and ATP, which influences microglia and infiltrating leucocytes to produce cytokines to further influence stroke pathology [112]. Microglia are local macrophage-like cells and are a crucial part of the innate immune system in the CNS. Following an ischaemic stroke, microglia are rapidly activated and release both pro- and anti-inflammatory mediators [113, 114]. The immune responses following ischaemic stroke occur from minutes to weeks after the injury, but whether their role is beneficial or detrimental to the tissue damage are still not entirely clear [112, 115]. We need to recognise that these are effects the ischaemia model with cerebral organoids are missing, both during ischaemia and recovery. However, it can also be useful to examine the brains cellular responses to ischaemia without the influence of the immune system.

The culturing of cerebral organoids is a highly time-consuming process that stretches over several months. Due to many critical steps and quality control, many organoids are lost during the process (typically ~75 % of the starting number). Therefore, to achieve enough organoids for statistical analyses, we need to make huge batches. This makes working with organoids more difficult and time-consuming than many other models.

As the research on brain organoid develops, ethical questions will appear. Today, cerebral organoids have no moral status, but as research advances, a moral status must be defined at some point before the organoids become human brains on a dish. Questions regarding when this moral status shall occur and to what criteria it shall be based on are currently under discussion. It has been presented three elements to follow considering if and what kind of moral status cerebral organoids should achieve: human origin, biological threshold and the potential to generate human beings [116]. Since the organoids have derived from human cells, they are considered having a human origin. Setting a biological threshold for when a moral status should be generated is more difficult to agree with. Several suggestions have been made, including measuring consciousness, ability to feel pain and learning capacity [116-118]. The last element regards the ability to generate a human being, which would apply for cerebral organoids, even
though we are not making a complete body. These are all suggestions from researchers and should be considered as the morality debate goes on.

**Ischaemia model**

The low correlation between animal stroke studies and human clinical trials suggest that there may be (yet unknown) differences between the neural tissue of humans and laboratory animals. Thus, there is a need for human tissue models that can be used to verify the findings from animals. The recent development of human cerebral organoids now allows the study of molecular mechanisms and drug effects in 3D human brain models. To this date, there are no available protocols that model ischaemic stroke in cerebral organoids.

Stroke is a vascular disease caused by blockage or rupture of an artery in the brain. With this model, we are trying to simulate an ischaemic stroke, but without any blood vessels to block. Since oxygen and glucose deficiency is the most critical aspects of a stroke, this is the principle for our ischaemia model. In the recovery period following an ischaemic stroke, the different circulating inflammatory mediators can still affect the cells in the stroke area, which also will not happen in our ischaemia model. Even though this is a well-established method for simulating ischaemia, the lack of a vascular system in the model will make it less physiological.

**Cell death analyses**

Regarding the method of using TUNEL assay for the detection of cell death, one must consider the data in light of a few methodological considerations. As the assay works by TdT binding to broken DNA-fragments (see chapter 3.6.1 *Cell death detection assay (TUNEL assay)*) one of the limitations with this method is that DNA damage occurs not only in apoptosis but also to some extent in necrosis, which affects the specificity and sensitivity of the assay. In our case, a marker for detecting cells in apoptosis and necrosis is beneficial, because we can expect both during ischaemic treatment, but there will be some uncertainty around the exact cell death number. However, in this study we focused on establishing a coarse timeframe of ischaemia needed for perturbation of cell viability, leaving the exact cell death number subordinate in this respect. One of the advantages of the TUNEL assay is that it is highly sensitive. However, regarding its specificity, it is unknown how many DNA strand breaks are required to give a positive signal, which may give an overestimation of cell death. It has also been observed that living cells undergoing DNA repair have been stained with the TUNEL assay [119].
preparation of the tissue, e.g. fixation and permeabilisation, can also affect the detection of damaged DNA. Therefore, consistency in the preparation is of importance [120]. Nevertheless, for this study, TUNEL assay is a well-suited method, and we can confidently measure the differences in cell death between treatment groups.

In addition to TUNEL assay, H&E staining was used to give an impression of the cell viability. To accurately describe the extent of cell death, H&E staining should not be used alone, and morphological examination to determine cell viability is mostly replaced by quantitative methods [105]. However, H&E allows describing cell morphology of all cells and cellular architecture, often lost in other methods like immunohistochemistry, flow cytometry or TUNEL assay. The combination of H&E and TUNEL staining gives more strength to the analyses and allows us to better characterise cell death in the ischaemia treated organoids. In the future, we will also try other cell death assays, e.g. caspase-3 staining or flow cytometry of annexin V-binding, to further optimise the cell death analysis.

**Immunohistochemistry**

The use of immunohistochemistry (IHC) also requires considerations regarding its limitations and challenges. Preparation of the tissue, e.g. fixation and antigen retrieval, can affect the result of the staining. The cerebral organoids used were fixed with paraformaldehyde (PFA) to prevent degradation of the tissue. If the tissue is over-fixed, the antibodies may not be able to bind the target antigen because of excessive crosslinks, which can give false-negative results [90]. To overcome this problem, we have optimised the fixation procedure to the specific organoid tissue. Additionally, we use antigen retrieval as another strategy to overcome the issue of over-fixation. Antigen retrieval is achieved by treating the sections with either enzymes or heating. In this study, heat-induced epitope retrieval has been used. By heating the fixed tissue before IHC, the antigens are made more accessible for the antibodies by the hydrolysis of methylene crosslinks [90]. Even though the antibodies are made for specific antigens, unspecific labelling can occur, which means that the antibody can bind to other proteins in the tissue than the target antigen. Before applying the primary antibodies, the tissue is incubated with a solution with a blocking agent, e.g. serum, which covers up unwanted binding sites. In addition to using blocking agents, we can verify the staining by microscopy. The different antibodies label specific components of the cell; therefore, to check that our staining is correct we make sure our microscopic findings match the descriptions from the literature. We have also used older
organoids as a control for correct staining. If the antigens are located within the cell, the antibodies need to penetrate the cell membrane. To enable this, the tissue is permeabilised before and during incubating with antibodies. As a permeabilising agent, Triton X-100 was used, which is a non-ionic surfactant that dissolves the cell membrane so the antibodies can access the antigens inside the cell. Even though there are some possible obstacles with this technique, IHC is a well-established and reliable staining method.

**Statistical analyses**

All datasets were tested for normal distribution (not shown), as this determines the choice of analytical method. Where the distribution was normal, the one-way ANOVA was used for analysing the differences between the treatment groups. However, most of our data were not normally distributed, and in these cases, the non-parametric test Kruskal-Wallis one-way ANOVA was used for statistical analysis. The Kruskal-Wallis test does not require a normal distribution like the regular ANOVA because it is based on ranks instead of the data values. In this test, the dataset is ranked from 1 to the sum of N, and by summarising the ranks for each group, one can compute a statistic H and P-value to determine differences among population means [121]. If the results from the Kruskal-Wallis test (or ANOVA, for normally distributed data) is significant, one proceeds with posthoc tests to find which groups differ from each other. For this study, we were interested in comparing the ischaemia groups to the control and chose, therefore, Dunn’s test for the Kruskal-Wallis, and Dunnett’s test for the ANOVA, as is most commonly used and recommended [122].
5.2 Discussion of results

Cell viability

From the TUNEL assay, we found a significantly higher number of dead cells in organoids after 24h ischaemia compared with control. The TUNEL assay has been the most important method for testing the effect of the cerebral organoid ischaemia model. The main goal for this model was to be able to kill a significant amount of cells in ischaemia-treated organoids compared with control organoids. To our surprise, a significant difference did not appear until the organoids were treated with 24 hours of ischaemia, which does not resemble a typical clinical situation. Even though we are aiming for conditions resembling the physiological conditions in stroke, we might not expect to see the same response in the organoids, due to differences described above. We have not yet exposed the organoids to ischaemia treatments between 3h and 24h but will do this in the future to determine at what point a significant difference in cell death appear.

While reference articles with rodent brains did not need ischaemia treatment over 60 minutes, our organoids required longer time in ischaemic conditions to achieve results. As described above, the lack of a vasculature system and immune cells may contribute to cells being more resistant to ischaemia. This effect can also be related to the composition of cell types in the organoids. Cultured neurons and glial cells can show different levels of vulnerability to ischaemia. Astrocytes in culture are particularly resistant to ischaemia and can tolerate longer incubation periods in ischaemic conditions [123, 124], while oligodendrocytes and microglia show low survival after shorter periods of ischaemia [124]. Neural stem cells have shown to maintain viability after short periods of ischaemia, resulting in the promotion of differentiating [125]. Cerebral organoids at 3.5 months, as used in this study, contain a high level of neural stem, neuronal progenitors, and young glial cells, which can be one possible explanation that we observe such high viability after 1-3 hours of ischaemia.

Oligodendrocytes

To investigate how ischaemic conditions affected the number of oligodendrocytes in the organoids, IHC was performed with antibodies for different oligodendrocyte markers, and the number of cells positive for the marker Sox10 was quantified. Cells that express Sox10 can be oligodendrocytes in all stages of development, from stem cells to myelinating oligodendrocytes.
Sox10 is located in the nucleus, which makes it easily quantifiable. From this study, we could not see any significant difference in the number of Sox10-positive cells in ischaemia treated and control organoids. However, when doing immunohistochemistry on partly dead tissue, one needs a critical view on the accessibility of the epitopes in the tissue. As a cell dies, of either apoptosis or necrosis, the cell will start to degrade. It is hard to predict how many of the total epitopes are still immunoreactive after ischaemia [126, 127], which may affect the interpretation of the results. Even with no statistical significance between ischaemia and control organoids, there could still be a difference in the viability of the Sox10-positive cells. However, a marked reduction in Sox10-positive cells could suggest oligodendrocyte death. In future experiments, we will also co-stain with caspase-3, or other apoptosis markers, to achieve more information on the viability of oligodendrocytes after ischaemia.

We also used markers specific for the different developmental stages of oligodendrocytes, i.e. NG2 for oligodendrocyte precursor cells, O4 for immature and mature oligodendrocytes and APC for mature oligodendrocytes. However, due to no or few positive cells in each organoid and considerable variation between organoids within the same treatment group, the amount was not quantifiable. In future experiments, we will try to adapt the organoid culture protocol such that we can produce a higher number of oligodendrocytes in each organoid, thus making them more suitable for quantifying oligodendrocytes at different stages.

In addition to staining for oligodendrocytes, we added the antibody for Ki-67, which marks cells in mitosis. Oligodendrocyte precursor cells exposed to sublethal injury from ischaemia tend to proliferate more, giving rise to new oligodendrocytes, possibly in an attempt to remyelinate the peri-infarct area [45, 46]. One of the aims for this study was to find the optimal duration of incubation in ischaemic conditions to give an increased proliferation rate in oligodendrocytes. The results showed a significantly lower number of mitotic cells (all cell types) in the ischaemia treated organoids compared with control. We also checked for mitotic oligodendrocytes by co-staining of Ki-67 with Sox10. There was a lower number of mitotic Sox10-positive cells in organoids from 24h ischaemia, but the difference was not statistically significant compared with control, although the p-value was low (p=0.0904). A reduction of proliferating oligodendrocytes implies that 24 hours of ischaemia is a too harsh treatment. We are aiming for a treatment duration where oligodendrocytes are injured, but not killed, and will start to proliferate in the recovery period. This ischaemia treatment duration will probably be somewhere between 3 hours and 24 hours, which we intend to investigate further.
During the analyses of the cerebral organoids, we observed a noticeable variation between them. Some of the organoids that showed much cell death after ischaemia also expressed many oligodendrocytes and neural stem cells (data not shown). These individual differences make the results less reliable, especially with so few organoids in the experiments. A notably high variation was seen in the organoids stained for Sox10. Three of six organoids in the 2h ischaemia group showed vastly higher levels of Sox10 than the rest. Since this is half of the group data, we cannot assume these are outliers, and a larger sample size is necessary. With such big differences between organoids from the same batch, it will be hard to produce consistent results when the organoids are at different stages of development. The variation between organoids, and particularly between batches, is a general problem in organoid research [19]. Thorough quality control is important to make sure the organoids are similar regarding development. During the process of culturing the organoids, we have already implemented some quality control steps, according to the Lancaster protocol [19], but this control might have to get even stricter to reduce this variation. We must expect a certain degree of variation in experiments like this; therefore, we will in future experiments use larger sample sizes, in addition to strict quality control, to produce more robust and reliable data.

**Future work**

This study is merely the beginning of studying ischaemic stroke in cerebral organoids. Upcoming work will include adjustments of the existing protocols and trying out new additional methods for examining the effects of stroke on oligodendrocytes. As much research is currently going on in this field, the protocol for growing organoids will be optimised to give mature myelinating oligodendrocytes. The ischaemia model will be further tested and optimised regarding the duration of ischaemia treatment, especially between 3 and 24 hours. To validate the effect of the model, other viability assays will be carried out, including caspase-3 staining and flow cytometry, which will contribute to more robust test results. For testing the effect of ischaemia on oligodendrocytes, staining with oligodendrocyte markers combined with an apoptosis marker will be an easily quantifiable method to determine the viability after ischaemia. Once the ischaemia model has been optimised, it can be used to study the mechanisms that cause cell death in oligodendrocytes and other cell types. It can also be used to test the effect of various potential neuroprotective drugs after ischaemia.
6 Conclusion

The main aim of this master thesis was to develop a human cerebral organoid ischaemia model to study the response of oligodendrocytes to ischaemia. Through the work of this thesis, I have shown that 24 hours of ischaemia killed a significant number of cells in human cerebral organoids, but there was no significant difference in cell death from 3 hours of ischaemia or less. I used this model to study the change of oligodendrocyte number in organoids treated with 2 and 24 hours of ischaemia but did not find a significant difference compared with control. I also studied the cell proliferation rate in ischaemia treated organoids and found that 2 and 24 hours of ischaemia gave a significant reduction of proliferating cells compared with control. However, the number of proliferating oligodendrocytes was not significantly different between 2 or 24 hours of ischaemia and control.

This study provides data on how human cerebral organoids respond to experimental ischaemia that can be used in continuing studies. However, the methods used in this study need optimising, and more data is necessary to answer what effect ischaemia has on oligodendrocytes in human cerebral organoids.
References


