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Desenvolvimento de um novo modelo para o estudo do acidente vascular cerebral perinatal

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"Alguém disse que os grandes acontecimentos do mundo se passam dentro do cérebro."

Oscar Wilde

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Resumo

O acidente vascular cerebral perinatal é um evento patológico que ocorre entre a 20ª semana de gestação e o 28º dia após o nascimento. Apesar da sua incidência ser comparável à do acidente vascular cerebral na idade adulta, e de ser uma das causas mais comuns de paralisia cerebral hemiplégica, entre outros sintomas incapacitantes, o acidente vascular cerebral em idade perinatal é um tópico pouco explorado na literatura. A fisiopatologia subjacente não é ainda totalmente compreendida, apesar de mecanismos inflamatórios e pro-trombóticos parecerem ter um papel prevalente.

Neste sentido, o objetivo deste estudo foi estabelecer um modelo válido para melhor compreensão do acidente vascular cerebral perinatal, que seja relacionável com a fisiopatologia no humano.

Deste modo, foram utilizadas fatias organotípicas de hipocampo de murganhos com 2 a 3 dias de idade, e posteriormente quantificada a expressão de *ionised calcium binding adaptor molecule-1* (um marcador da microglia), de arginase-1 (um marcador da microglia M2) e do recetor do fator de crescimento vascular endotelial, após um período de privação de oxigénio e glucose. Esta privação mimetiza *in vitro* as condições de isquemia. Verificou-se um aumento de todas estas moléculas após privação de oxigénio e glucose, em comparação com o controlo (células não expostas a isquemia), sugerindo um aumento da população microglial e uma resposta protetora por parte destas células face ao dano.

Em suma, a inflamação parece ser um mecanismo subjacente ao acidente vascular cerebral perinatal, assim como a microglia parece ser um importante mediador deste processo. No entanto, é necessária mais investigação nesta área, de modo a compreender melhor a fisiopatologia inerente, e deste modo contribuir para o desenvolvimento de novas abordagens terapêuticas.

Palavras-chave

Acidente vascular cerebral perinatal, fisiopatologia, isquemia, fatias organotípicas de hipocampo, microglia.

Resumo Alargado

O acidente vascular cerebral perinatal é um evento patológico que ocorre entre a 20ª semana de gestação e o 28º dia após o nascimento, definido como uma interrupção focal da circulação cerebral arterial ou venosa, que geralmente é secundário a tromboembolismo.

Apesar da sua incidência ser comparável à do acidente vascular cerebral na idade adulta, e de ser uma das causas mais comuns de paralisia cerebral hemiplégica, entre outros sintomas incapacitantes, o acidente vascular cerebral em idade perinatal é ainda um tópico pouco explorado na literatura. A fisiopatologia subjacente não é totalmente compreendida, apesar de mecanismos inflamatórios e pro-trombóticos parecerem ter um papel prevalente. De facto, estes mecanismos parecem estar relacionados com a maioria dos fatores de risco identificados e que se podem agrupar nas seguintes categorias: maternos, fetais/neonatais, relacionados com a placenta e relacionados com outras causas. No entanto, devido à sua complexidade, atualmente a etiologia do acidente vascular cerebral perinatal é considerada multifatorial.

Os modelos animais são uma ferramenta valiosa para o estudo de mecanismos fisiopatológicos e consequentemente para o desenvolvimento de novas terapêuticas. Ao longo dos últimos anos tem-se verificado interesse crescente na área do acidente vascular cerebral perinatal. No entanto, as abordagens de modelos animais para o estudo desta patologia são ainda muito limitadas.

Neste sentido, o objetivo deste estudo foi estabelecer um modelo válido para melhor compreensão do acidente vascular cerebral perinatal, que seja relacionável com a fisiopatologia no humano. Modelos *in vitro* são muito úteis neste contexto, uma vez que permitem fácil acesso aos níveis de atividade celular, expressão e libertação de proteínas e propriedades de barreira.

Foi utilizado um novo modelo com fatias organotípicas de hipocampo, que oferece inúmeras vantagens face a outros modelos, sendo uma delas a possibilidade de utilizar animais com menos dias de vida. Tanto quanto se sabe, apenas um grupo recorreu a este modelo para o estudo do acidente vascular cerebral perinatal, no entanto, utilizaram animais com 7 a 10 dias de idade. Para o nosso estudo, foram preferidos animais com 2 a 3 dias de vida, de forma a corresponder às 23 a 32 semanas de gestação fetal, ou seja, a um bebé em idade prematura. Esta correlação foi preferida por a prematuridade poder estar associada ao acidente vascular cerebral perinatal, e, para além disso, pelo facto de diferentes fases no desenvolvimento da barreira hematoencefálica e do sistema imune representarem diferentes *outcomes* pós- acidente vascular cerebral, o que é determinado pela idade gestacional: em

crianças pré-termo os sistemas imunológico, glial e vascular ainda se encontram em desenvolvimento.

Tendo em conta o papel dos pericitos, astrócitos e microglia no desenvolvimento, regulação e manutenção do sistema nervoso central, considerou-se importante o estudo da variação quantitativa de *ionised calcium binding adaptor molecule-1* (um marcador da microglia), de arginase-1 (um marcador da microglia M2) e do recetor do fator de crescimento vascular endotelial (que tem um papel na permeabilidade da barreira hematoencefálica e na estimulação da angiogénese), após um período de privação de oxigénio e glucose, o que mimetiza *in vitro* as condições de isquemia.

Verificou-se um aumento de todas estas moléculas após o dano, em comparação com o controlo (células não expostas a isquemia), sugerindo um aumento da população microglial e uma resposta protetora por parte destas células face ao dano.

Em suma, o nosso estudo propõe um novo modelo para a abordagem do acidente vascular cerebral perinatal. Este modelo permite mais investigação neste âmbito, clarificando melhor a fisiopatologia inerente a este evento patológico e, consequentemente, contribuindo para o desenvolvimento de novas abordagens terapêuticas.

Abstract

Perinatal stroke is a pathologic condition that occurs between the 20th week of gestation and the 28th day after birth. Despite its incidence being comparable to stroke in adults and being one of the most common causes of hemiplegic cerebral palsy, among other incapacitating symptoms, perinatal stroke remains a poorly studied subject. The evident pathophysiology underlying perinatal stroke is still not well understood, although inflammation and prothrombotic mechanisms seem to have a prevalent role. In this way, the aim of this study was to establish a valid model to study perinatal stroke that would more closely relate to the human pathophysiology.

We used oorganotypic hippocampal slice cultures from 2-3-day old mice and then quantified the expression of ionised calcium binding adaptor molecule 1 (a microglia marker), arginase-1 (a M2 microglia marker) and vascular endothelial growth factor receptor, after oxygen and glucose deprivation. The induction of oxygen and glucose deprivation mimics ischaemic conditions. All three molecules were increased after this deprivation, comparing to control (cells not exposed to injury), suggesting that microglia proliferate and adopt a protective phenotype in response to injury.

In summary, inflammation seems to be an underlying mechanism in perinatal stroke, as well as microglia seems to be an important mediator. Further investigation is needed in this area in order to better understand the pathophysiology of perinatal stroke and to improve the development of new therapies.

Keywords

Perinatal stroke, ischaemia, pathophysiology, organotypic hippocampal slice cultures, microglia.

Contents

Acknowledgements iv
Resumo v
Resumo Alargado vi
Abstract viii
List of figures x
List of Abbreviations xi
Introduction 1
Methods 5
Organotypic hippocampal slice cultures (OHSC) 5
Oxygen and glucose deprivation (OGD) 5
Cell death 5
Western Blotting (WB) 6
Statistical analysis 6
Results 7
Discussion 11
Conclusion and Future Perspectives 14
References 15

List of figures

Figure 1 - Cell death quantification. Tissue was incubated with PI to assess cell death caused by OGD. Organotypic slices exposed for 60 minutes to OGD displayed significant cell death (n=1-5). 7
Figure 2 - Quantification of Iba-1 on control and ischemic OHSC. The expression of Iba-1 was increased in ischemic brain tissue (n=2). 8
Figure 3 - Quantification of Arg-1 on control and ischemic OHSC. Ischemic brain tissue produced more Arg-1 in response to injury (n=2). 9
Figure 4 - Quantification of VEGFR on control and ischemic OHSC. VEGFR expression was increased in ischemic brain tissue (n=2-3). 10

List of Abbreviations

Arg-1	Arginase-1
BBB	Blood-Brain Barrier
CT	Computerized Tomography
Iba-1	Ionised Calcium Binding Adaptor Molecule 1
MCA	Middle cerebral artery
MRI	Magnetic Resonance Imaging
NOS	Nitric Oxide Synthase
OGD	Oxygen and Glucose Deprivation
OHSC	Organotypic Hippocampal Slice Cultures
OPCs	Oligodendrocyte Progenitor Cells
PBS	Phosphate-Buffered Saline
PI	Propidium Iodide
P*n*	*n*-day old
PVL	Periventricular leukomalacia
RIPA	Radioimmunoprecipitation
RT	Room temperature
TBS-T	Tris-buffer saline containing Tween-20
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WB	Western Blotting

Introduction

Perinatal stroke is a pathologic condition that occurs between the 20th week of gestation and the 28th day after birth (1-4). It can be either ischaemic or haemorrhagic, with the ischaemic being the most common (1). The estimated incidence is 1 in 2300 to 1 in 5000 live births (1, 2, 4), considering that these are likely underestimations given the difficulty to diagnose and the limited data currently available (2, 4). Nevertheless these numbers are comparable to the incidence of stroke in the elderly (2, 5-7).

Perinatal ischaemic stroke is defined as a focal interruption of arterial or venous cerebral blood flow usually secondary to thrombosis or embolism. Confirmation by image or pathologic studies is always mandatory (2-4). Relatively to arterial ischaemia, this interruption is more frequently observed in the left middle cerebral artery (MCA) (3, 4). Importantly, in the foetal circulatory system, placental or systemic venous emboli may pass through a patent ductus arteriosus or *foramen ovale* directly to the left carotid artery and subsequently to the left MCA, facilitating occlusion (8). Considering venous ischaemic events, they mainly include periventricular venous infarction and venous sinus thrombosis (1, 3).

Ischaemic injury observed is largely influenced by brain maturity. Therefore, term newborns will be differently affected from preterm newborns. In preterm newborns, oligodendrocyte progenitor cells (OPCs), which are responsible for the dynamic process of myelination and efficient neurological function, are more sensitive to ischaemia which interferes with their maturation and predisposes to periventricular white matter diffuse injury, also known as periventricular leukomalacia (PVL), resulting in cerebral palsy. Accordingly, the risk for PVL has already been defined as high between 23rd to 32nd weeks of gestation. Subplate neurons, a transient neuronal population important for the formation of mature neuronal networks, are another vulnerable target to ischaemia in preterm newborns, also contributing to white matter diffuse injury. Moreover, diminished quantities of pericyte and astrocyte along blood vessels are one of the reasons preterm brain is more susceptible to ischaemic injury. Oppositely, in term newborns grey matter is predominantly affected, in a focal pattern (more frequently the striatum, thalamus and cortex) greatly impacting on motor function (2, 5, 9, 10).

The most common symptoms following a stroke during the perinatal age are seizures, cognitive impairment, language and visual deficits and behavioural problems. Furthermore, it leads to severe long-term neurological deficits, with hemiplegic cerebral palsy being the most frequent long-term motor outcome (1, 2, 4, 6, 11). However, there are several aspects that may delay the suspicion of diagnosis, such as (i) neonates with seizures may appear clinically

well between episodes (2, 6); (ii) in the first days, newborns may present with discrete non-specific symptoms like lethargy, apnoea, difficult feeding and chewing movements (1, 2, 4, 6); (iii) some cases may be asymptomatic during the neonatal period, presenting with lateralized symptoms around the 5th month (1, 2, 4). Lateralized symptoms are rare in neonates who suffered a perinatal stroke, contrarily to children or adults who suffer a stroke (2, 6).

Magnetic resonance imaging (MRI) is the standard method to stablish the diagnosis of perinatal stroke (1-4). Nevertheless, ultrasound, computerized tomography (CT) and the Prechtl's method (12) can also be useful for this purpose (1, 3, 4).

Several risk factors for perinatal stroke have been recognized and can be grouped into the following categories: maternal, foetal/neonatal, placental and other causes (**Table 1**) (2, 4, 11, 13, 14). Most of them appear to be related to a propensity for a pro-thrombotic state. Pregnancy is itself a natural prothrombotic and pro-inflammatory state; there is a physiological activation of the coagulation cascade both on foetus and mother near delivery. In this context, foetus has an augmented haematocrit and higher pro-coagulant proteins and blood viscosity (2, 4, 6, 8). Additionally, when other blood and lipid disorders are present, such as polycythaemia, prothrombin mutation, increased factor VIII and disseminated intravascular coagulopathy, there is a potentiation of the risk for thromboembolism (8, 15). In fact, thromboembolism from the placenta passing through the patent neonatal *foramen ovale* has been identified as the main cause for perinatal ischemic stroke (8). Given its complexity, it is widely accepted that the aetiology of perinatal stroke is multifactorial (1, 2, 5).

Moreover, infections and/or inflammation seem to be a prevalent underlying mechanism in stroke, in several ways (11, 13): inflammation (i) is associated to several risk factors for perinatal stroke (e.g. chorioamnionitis which is an inflammation of amniochorionic membranes) (11, 13); (ii) is strongly triggered during stroke, with elevation of inflammatory cytokines (13); (iii) is associated with a worse prognosis in the period after stroke (7). As a result, several pro-/anti-inflammatory molecules and growth factors are released and have been studied as part of the impactful secretome unleashed by ischaemia, such as vascular endothelial growth factor (VEGF) (16, 17).

The treatment of perinatal stroke is still very restricted, since it is essentially based on symptomatic control (e.g. antiepileptic drugs for seizures) (1, 2, 4). Hypothermia is the only established effective measure to improve global outcome, reducing the risk of long-term neurologic disabilities (2, 5, 14). Notwithstanding, new therapies are being tested, including the application of growth factors (e.g. erythropoietin), allopurinol, antioxidants, anti-inflammatory approaches, stem cell transplantation and electrical stimulation (1, 2, 14).

Animal models are a valuable tool to understand the mechanisms underlying a disease and to advance the design of more effective treatments. Fortunately, over the years, there has been growing interest on the study of neonatal and perinatal stroke. Nevertheless, the number of articles detailing experimental animal approaches is still very low and they favour a later period, since current animal models tend to use 7-day-old or older animals for *in vivo* experiments. A week old rat, the most commonly used species, would represent a 2-month-old infant (well beyond the 28th day post-birth) if considering peripheral organ systems (18, 19), and a term infant, if considering brain development (19). Importantly, the majority of models use O_2 rates much higher (5 to 12%) than those believed to occur after an ischaemic event (20).

Considering these findings, our goal was to establish a valid model to study perinatal stroke that would more closely relate to the human pathology, when stroke occurs in earlier stages of the perinatal period.

Table 1 - Identified risk factors for perinatal stroke (2, 4, 11, 13, 14).

Risk factors for perinatal stroke
Maternal
Autoimmune disorders
Coagulation disorders: protein C or S deficiency, factor V Leiden, factor V G1691A or factor II G20210A mutation
Acquired antiphospholipid antibodies
Twin-to-twin transfusion syndrome
Cocaine abuse
Infection: central nervous system infection, systemic infection
Pre-eclampsia/eclampsia
Gestational diabetes
Fever
Foetal/Neonatal
Cardiac disorders: congenital heart disease, patent ductus arteriosus, pulmonary valve atresia
Blood and lipid disorders: polycythaemia, prothrombin mutation, factor V Leiden, factor VIII, protein S deficiency, hyperhomocysteinemia, increased lipoprotein(a), disseminated intravascular coagulopathy
Vascular malformation or defect
Uterine growth restriction
Hypoglycaemia
Infection: central nervous system infection, systemic infection
Hyperthermia
Nephrotic syndrome
Placental
Placental thrombosis
Placental abruption
Placental infection
Feto-maternal haemorrhage
Other
Trauma and catheterization
Birth asphyxia
Dehydration
Extracorporeal membrane oxygenation

Methods

All experiments were performed in accordance with protocols approved by national ethical requirements for animal research, the European Convention for the Protection of

Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU) for the care and use of laboratory animals.

Organotypic hippocampal slice cultures (OHSC)

OHSC were obtained from 2-3-day-old (P2-3) C57BL/6 mice, as previously described by us (21). Brains were removed to isolate hippocampi in Gey's Balanced Salt Solution (Biological Industries, Israel), under sterile conditions. Hippocampi were cut into 350 µm-thick slices using a tissue chopper (McIlwain) and transferred to 0.4 µm porous insert membranes (Millipore Corp., Bedford, Massachussets, United States of America - USA), which were placed in six-well plates containing culture medium. Medium was composed of 25% heat-inactivated horse serum, 50% Opti-MEM minimal essential medium, 25% Hank's Balanced Salt Solution, 25nM D-glucose (Merck, Darmstadt, Germany) and 50 U/ml penicillin and 50µg/ml streptomycin (all from Invitrogen, Carlsbad, California, USA). Each membrane contained six slices and was kept in a humidified atmosphere (5% CO_2) at 37°C for 24 hours before oxygen and glucose deprivation. Subsequently, media were refreshed every 2 days, for 5 days.

Oxygen and glucose deprivation (OGD)

OHSC were kept at 37 °C in a 5% CO_2 and 95% N_2 gas environment (0.1% O_2) for 15, 30, 45 and 60 minutes, in a MIC-101 modular incubator chamber (Billups-Rothenberg Inc., Del Mar, California, USA). In these conditions, media were composed by 0.15M phosphate-buffered saline (PBS). Only 60 minutes of exposure induced significant cell death (n=3) and, for this reason, this time point was selected for subsequent studies.

Cell death

Propidium iodide (PI; 3 µM in serum Optimem; Sigma-Aldrich) was used to evaluate cell death (necrosis and late apoptosis). After having OHSC for five days in culture, PI was added for 24 hours and then slices were fixed with 4% paraformaldehyde and mounted in Dakocytomation fluorescent medium (Dakocytomation Inc., Carpinteria, California, USA). Percentage of PI-positive cells was calculated from cell fluorescence intensity, using an Axioskop 2 Plus fluorescent microscope (Carl Zeiss, Jena, Germany).

Western Blotting (WB)

Slices exposed to 60 minutes of OGD were incubated with Radioimmunoprecipitation (RIPA) lysis buffer (0.15 M NaCl, 0.05 M Tris-base, 5 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 10mM dichlorodiphenyltrichloroethane containing a cocktail of proteinase inhibitors). Total protein from cell lysates was quantified using the bicinchoninic acid assay (Thermo Scientific, Massachussets, USA). Then, samples were loaded onto 12% bis-acrylamide gel (Applichem, Darmstadt, Germany). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (120 V) using a running buffer (Tris-glicine with 10% sodium dodecyl sulfate; Acros Organics, Geel, Belgium; pH 8.3) and then transferred to polyvinylidene fluoride membranes (GE Healthcare, Little Chalfont, United Kingdom - UK) through semi-dry transfer: 300 mA, for 90 min at 4ºC in a solution containing 10 mM CAPS (Sigma) and 20% methanol (Fisher Chemicals, Waltham, Massachussets, USA). To block non-specific binding, membranes were incubated in Tris-buffer saline containing 0.1 % Tween-20 (Thermo Fisher Scientific, Waltham, Massachussets, USA) for 15 min, at room temperature (RT), and then incubated overnight at 4ºC with the primary antibody solution diluted in 0.1% gelatin (Fluka, St Louis, Missouri, USA) in Tris-buffer saline containing Tween-20 (TBS-T). Primary antibodies used were *ionised calcium binding adaptor molecule-1* (Iba-1), Arginase-1 (Arg-1) and vascular endothelial growth factor receptor (VEGFR), all at 1:5000. After rinsing three times with TBS-T, membranes were incubated for 1 hour at RT with respective secondary antibody conjugated to horseradish peroxidase. The detection of peroxidase was performed by enhanced chemiluminescence detection and densitometric analyses, using the software ImageLab (Bio-Rad, Hercules, California, USA). Tris-buffered saline with Polysorbate 20

Statistical analysis

Statistical significance was determined using the software GraphPad Prism 6 for Windows. The samples were randomly chosen and are independent, and normal distribution was assumed based on literature (22). Student's t-test was used to compare means of the two samples, with $p<0.05$ considered to represent statistical significance.

Results

OHSC were exposed to OGD for different periods of time (15, 30, 45 and 60 minutes) in order to mimic significant ischemic cell damage. First, cell death was quantified by incorporation of PI, a compound that permeates the membrane of necrotic and late apoptotic cells.

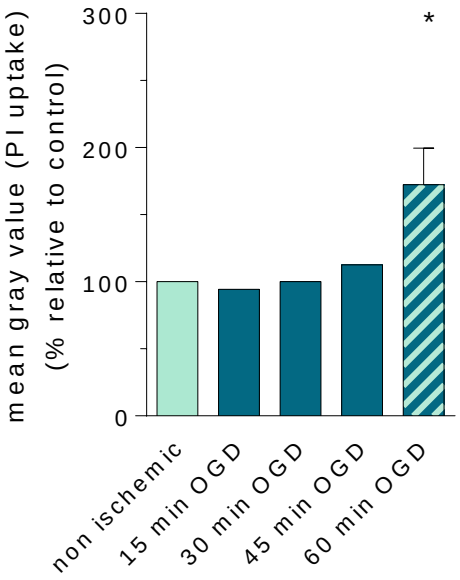

Figure 1 - Cell death quantification. Tissue was incubated with PI to assess cell death caused by OGD. Organotypic slices exposed for 60 minutes to OGD displayed significant cell death (n=1-5).
t-test: p (= 0.0305) < 0.05

The only timing that induced significant cell death was 60 minutes (**Figure 1**). Therefore, that was the time of OGD used in OHSC to evaluate other markers affected by ischaemia.

After the process of ischaemia, cerebral tissues suffer some changes in response to the injury. In this context, some biomolecules are produced in different amounts to modulate the process, which can be used as indirect markers. Some of these molecules were quantified using WB in order to understand brain response to ischaemia.

Iba-1 is a protein expressed specifically by microglia (23, 24) and consequently the amount of Iba-1 was determined in control (OHSC that were not submitted to OGD; n=2) and ischaemic OHSC (n=2). This quantification revealed that the quantity of Iba-1 on ischaemic OHSC is expressively superior to that on control (**Figure 2**). Since Iba-1 is an actin-binding protein that enhances membrane expansion, we can then hypothesize that microglia increases in number or in cell body size (activated cells indication) when ischaemia occurs.

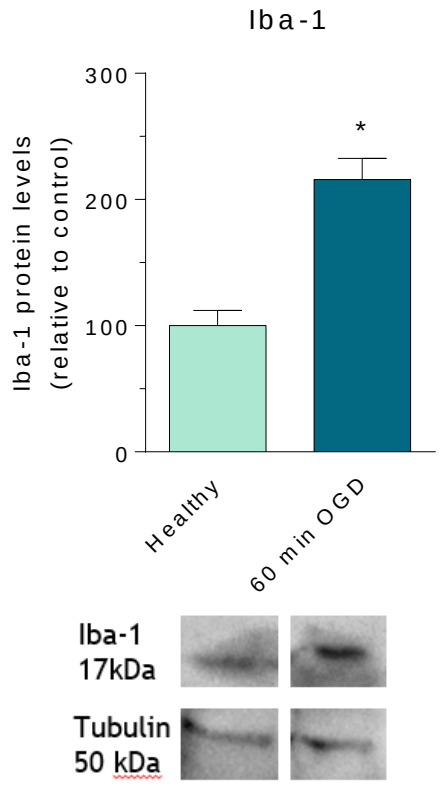

Figure 2 - Quantification of Iba-1 on control and ischemic OHSC. The expression of Iba-1 was increased in ischemic brain tissue (n=2).
t-Test: p (= 0,0305) < 0,05

Another indirect marker quantified was Arg-1, an enzyme that catabolizes arginine, which is a main substrate of nitric oxide synthase (NOS). Therefore, there is a dichotomy between Arg-1 and NOS since their affinity to arginine is determined by the inflammatory environment (mainly induced by cytokines): pro-inflammatory cytokines promote arginine metabolization by NOS and anti-inflammatory cytokines promote arginine metabolization by Arg-1 (25). Arg-1 has been referred as a marker of M2 macrophages/microglia (26, 27) which is related to neuroprotection (28, 29).

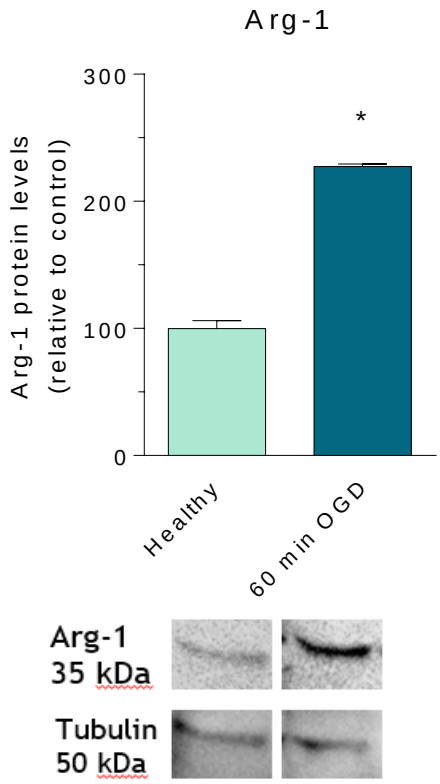

Figure 3 - Quantification of Arg-1 on control and ischemic OHSC. Ischemic brain tissue produced more Arg-1 in response to injury (n=2).
t-Test: p (= 0.0193) < 0.05.

Ischaemic OHSC (n=2) presented considerably more Arg-1 than control (n=2) (**Figure 3**). This likely illustrates an attempt to control or repair ischaemic damaged tissue by microglia M2, which activates Arg-1 via anti-inflammatory cytokines (25).

The third protein quantified was VEGFR. VEGF regulates vascular function, is essential to angiogenesis and has shown a neuroprotective effect. Nevertheless, VEGF effects on stroke are complex: a pathologic rise of VEGF promotes increased endothelial permeability and therefore also promotes permeability of the blood-brain barrier (BBB), leading to leakage and disruption (16, 17).

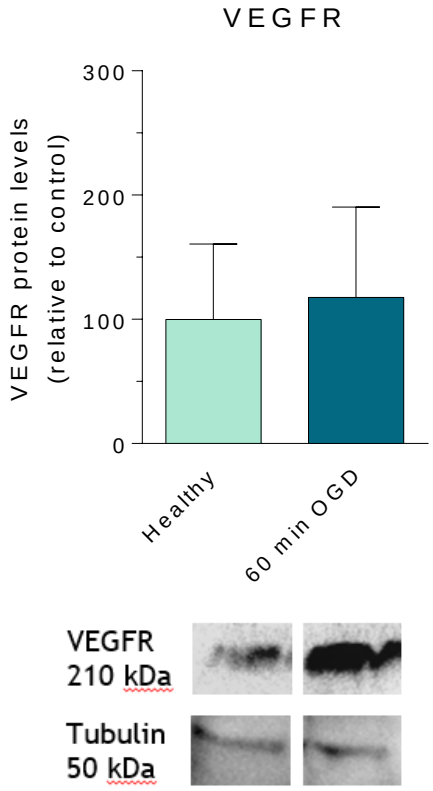

Figure 4 - Quantification of VEGFR on control and ischemic OHSC. VEGFR expression was increased in ischemic brain tissue (n=2-3).
t-Test: p (= 0,8654) > 0,05

Quantification of VEGFR in an ischaemia scenario (n=3) when compared to control had no statistically significant augmentation, due to the high variability between results. Nonetheless, VEGFR apparently tends to be increased in an ischaemia scenario (n=3), compared to control (n=2) **(Figure 4)** and this augmentation would possibly represent a response to an increase of VEGF.

Discussion

Despite perinatal ischemic stroke having a significant incidence and poor long-term outcomes (1, 5), it is still a poorly studied subject. This essay tried to elucidate some of the mechanisms that occur on cerebral tissues upon an ischemic event, considering that the ideal experimental model must be representative of the complex metabolic and pathophysiological aspects of this vascular disorder. In this way *in vitro* models can be very relatable while enabling easy assessment of cell activity, protein expression and release, and barrier properties, which is helpful in this condition context.

Therefore, we used a novel model of OHSC obtained from P2-3 mice. The usage of OHSC is innovative itself. OHSC provide unique characteristics and several advantages over cell models, since they preserve the whole hippocampi structure and maintain neuronal activities and synapse circuitry (30). Moreover, this approach offers several benefits such as the possibility of using younger animals if needed (e.g. P1-3 mice), the refinement of experimental doses/conditions and the reduction of the number of animals for *in vivo* models. To the best of our knowledge only one group has reported to use it for the study of perinatal stroke, and once more they used P8-10 rats (31). Since oxygen levels should be kept preferably under 2% to be representative of the ischemic core and around 7% if studying the ischemic penumbra (20), we have exposed OHSC to significantly lower O_2 rates (0.1%), in opposition to the majority of models that use much higher O_2 rates (5 to 12%). A considerable drawback from this model is the absence of blood flow and infiltrating immune cells once tissue slices are maintained in culture. Nevertheless, they provide a snapshot on how the neurovascular unit is modulated up to the time of brain isolation if a lesion and/or treatment is applied *a priori*. Subsequently, therapeutic agents and stimuli can be further administered over tissue to evaluate their impact on neuronal and glial activity.

By using OHSC from P2-3 mice, the physiopathology of the cerebral tissues response to an ischemic episode is comparable to a preterm infant between 23 and 32 weeks of gestation (19). This development correlation was preferred because prematurity can be associated with perinatal stroke (1, 14, 32) and most of the studies available use older mice, corresponding to a correlation with term infants (2, 19). The relevance of age pertains to the fact that the different gestational periods represent different development stages regarding the status of the BBB and the immune system: in preterm infants, the vascular, glial and immune systems are still in development (5, 16, 19). Since interfering with these systems greatly affects stroke outcome, experimental models should be able to evaluate the immediate and initial ischaemic events affecting them. Inclusively, perinatal stroke in term and preterm newborns implicate different clinical outcomes. In term newborns grey matter

is preferentially damaged, in a focal pattern (2, 5), whereas stroke in preterm infants leads mainly to PVL, which is the leading cause of brain injury in preterm infants (2, 5, 9).

Briefly, development of vascular systems during brain development occurs through two distinct processes (i) vasculogenesis, in which angioblasts differentiate into endothelial cells forming the perineural vascular plexus; this plexus will function as a substrate for (ii) angiogenesis, the process of generating new vessels from pre-existing ones (33-35). Furthermore, BBB is formed by inter-endothelial cells junctions, such as tight and adherens junctions, thus functioning as a physical barrier that promotes transcellular transport instead of paracellular (33, 34). It is noteworthy that, after a stroke, integral membrane proteins that form these junctions (e.g. occludin, claudins and zonula occluden proteins) are altered, affecting BBB permeability (5).

The major cell types responsible for development, regulation and maintenance of central nervous system angiogenesis and BBB are pericytes, astrocytes and neurons (34). These cells control the several identified factors that regulate the development of vascular systems and BBB, such as VEGF, angiopoietin and Wnt ligands (33, 34, 36). Inclusively, diminished quantities of pericyte and astrocyte along blood vessels are one of the reasons preterm brain is more susceptible to ischaemic injury (2, 5). Microglial cells migrate to the brain even before the development of brain vessels and therefore it has also been proposed a role for microglia in brain vasculature development (36, 37).

Regarding this pathophysiology, we considered important the study of variation in quantity of Iba-1, Arg-1 and VEGFR, used as indirect markers to approach the role of activated microglia, BBB permeability and angiogenesis stimulation, immediately after a perinatal stroke.

The elevation of Iba-1 on an ischemic context suggested an augmentation of the quantity of microglia, since it is one of the most used markers of activated microglia (27, 29). This can be interpreted as a defensive mechanism (28). In response to a *stress* event, microglia is able to control its own proliferation, migration and production of inflammatory cytokines, in order to modulate the changing environment conditions (38). However, activated microglia can express two different phenotypes: a damaging M1 or a repairing/neuroprotective M2 profile (28, 29).

M1 microglia produce pro-inflammatory mediators, which will have a neurotoxic role (29). M2 microglia are activated by apoptotic cells and are responsible for an anti-inflammatory response by several mechanisms (29, 39), including the expression of Arg-1 (25, 40), which was found to be more prevalent on ischaemic OHSC than on control. Arg-1 will likely modulate the inflammation (41), supressing immune system and promoting tissue regeneration (25, 41). Some studies have reported that Arg-1 influences the prognosis of

ischaemic stroke through improved cerebral tissue remodelling and behavioural recovery (41, 42). Although Arg-1 is an M2 marker (27), it is also expressed by astrocytes (42). Hence, we cannot exclude the participation of these cells in the repairing process. Additionally, we cannot conclude why the increased levels of Arg-1 are not able to protect from cell death/tissue damage; more studies are needed in this sense.

Concerning VEGFR, the rise on ischemic OHSC compared to control was not significant (13, 43). It is controversial if VEGF offers beneficial effects in a stroke context or if it is injurious (16). VEGF is responsible for several processes upon an ischaemic stroke, including disruption of endothelial cells junctions and endothelial cells endocytosis, followed by an augmentation in the BBB permeability, with disruption and leakage, and consequently to brain oedema, intracranial haemorrhage and intracranial hypertension (16, 17). On the other hand, VEGF plays an essential role on angiogenesis, stimulating it by promoting proliferation and migration of endothelial cells (16, 17, 19), which is crucial for recovery after stroke (17). Furthermore, it enhances the perfusion, diminishing infarct volume/preservation of penumbra (16, 19), and has shown to have a neuroprotective function (16), including in neonatal stroke (19, 43). Due to these characteristics, VEGF could be a potential therapeutic target. In this way, it could be useful to treat OHSC with VEGFR agonist and observe whether cell death could be reduced.

There is a particularity that is worth discussing. Hypothermia proved, so far, to be the only effective therapeutic measure in perinatal stroke (2, 5, 14). However, small mice with only a few days of life, have a natural propensity for hypothermia (44). Consequently, there is the possibility this fact could compromise results if using an *in vivo* model, with mice natural low body temperature to be a good prognostic factor in ischaemic stroke. In our model, ischaemia was only induced on P2-3 brain tissue that was previously maintained at 37ºC.

Therefore, our research supports a model that can be related to early perinatal stroke in humans. Inflammation modelling is a main mechanism in perinatal stroke (11, 13), and our results were in accordance, suggesting the validity of proposed methodology. Furthermore, microglia seem to be widely implicated in the protective mechanisms occurring after a perinatal stroke.

Conclusion and Future Perspectives

In conclusion, the proposed OHSC model using P2-3 animals appears to enable the possibility of detailing neuronal damage and the respective mechanisms of repair that are specific of perinatal stroke using a more representative model of preterm infants. However, more studies need to be developed to more accurately portray the extent of damage occurring specifically in perinatal stroke in order to allow the study of repair mechanisms and the discovery of new therapeutic targets.

As future perspectives, there are some issues that could be better explored in order to better evaluate some items and interesting facts. Firstly, it would be important to increase the number of samples and understand if the raise of VEGFR is statistically significant. Secondly, in order to clarify the participation of astrocytes in the repairing process, we could use other markers, such as glial fibrillary acidic protein, an astrocytes marker which is augmented in case of brain damage and/or degeneration of CNS (45). Moreover, brain response to ischaemia could be further assessed in our proposed model by immunohistochemistry, which would enable to characterize the circumstances inflammatory response occurs, inclusively allowing to address the specific localization of specific cells, such as microglia and astrocytes. By exploring the complex intercellular connections occurring after a stroke, this could facilitate the discovery of new therapeutic targets.

In order to assess Arg-1 role on cell death/tissue damage, we could quantify Arg-1 and Iba-1 expression in the OHSC exposed to 15 to 45 minutes of OGD (reduced cell death), and then compare the Arg-1/Iba-1 ratio: a higher ratio suggests a neuroprotective response, with higher activation of M2 microglia; whether a lower ratio suggests a higher activation of M1 microglia. These would allow to clarify if there is a proportional relation between cell death and microglia response.

Another important study would be to understand if perinatal stroke really is gender dependent, and what is influencing that outcome. An interesting but poorly studied fact found in literature, is that perinatal stroke appears to be gender-dependent both on incidence, symptoms and therapy response, with males being more commonly affected and with poorer outcomes (2, 4). The protection provided by female sex hormones is one of the possibly explanations supported in literature for this fact (2). To address this issue, we would divide pups by a physical trait: male mice have a visible pigment spot on the scrotum (females do not have this spot in the anogenital region) (46).

Considering the outcomes of perinatal stroke, further investigation in this topic would be a contribution for a better future.

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