Universidade de Lisboa

Faculdade de Farmácia

Development of new pharmacological approaches in ischemic stroke: A Systematic Review of transient MCAO model in rats

João Solas

Dissertation supervised by João Pedro Fidalgo Rocha and co-supervised by Vanessa Alexandra Pinho Mateus

Biopharmaceutical sciences

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Agradecimentos

Queria agradecer ao meu orientador Professor Doutor João Rocha, pela ajuda que sempre me deu e pela confiança que depositou em mim. Orientou-me de forma exímia e resolveu sempre os problemas que apareciam no caminho. É um profissional exemplar com o qual tive e tenho o prazer de aprender todos os dias.

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Muito obrigado.

Abstract

In vivo models of cerebral ischemia provide us with important information regarding the biochemical processes involved behind stroke, as well as playing a central role in the development of new therapeutic options. The transient middle cerebral artery occlusion model (MCAO) is one of the most widely used *in vivo* models in experimental studies and best suited to mimic cerebral ischemia since it is a robust, non-invasive and reversible method. This study aims to perform a systematic review which follows the PRISMA systematic review-protocol of the MCAO model to systemize the methods of validation of MCAO model so that there is a greater reproducibility in future studies. To perform this study a search in the Medline database was performed until March 2021 to collect all the articles. To find the articles a search expression was built using MeSH terms. The inclusion and exclusion criteria were chosen, to choose the articles to perform the systematic review. Then the rat characteristics, induction characteristics, anesthesia, infarct volume, and parameters studied data were collected from each article by two reviewers and when necessary with the help of one arbitrator. Regarding the rats characteristics the most used rats are male adult Sprague-Dawley or Wistar rats. The preferred anesthesia to perform MCAO is isoflurane and the periods of ischemia vary from 1-2h and reperfusion from 24h to 72h. It was also verified that the MCAO model causes infarct volumes between 40-50%. The parameter studied included behavior (neurological functions, sensorimotor function, and cognitive tests) and histological tests (infarct volume, brain edema, GFAP, Iba1, and NeuN) and biochemical markers, such as inflammatory factors (iNOS, COX-2, NF-κB, TNF-α, and interleukins). With the results obtained in this study, it will be possible to carry out more accurate and reproducible studies of cerebral ischemia.

Keywords: cerebral ischemia; middle cerebral artery occlusion; animal model; rats

Resumo

Os modelos *in vivo* de isquemia cerebral fornecem-nos informações importantes sobre os processos bioquímicos envolvidos nos AVCs, assim como, desempenham um papel fundamental no desenvolvimento de novas abordagens terapêuticas. O modelo de isquemia da artéria cerebral média (ACM) é um dos modelos in vivo amplamente utilizado em estudos experimentais, sendo o adequado para mimetizar a isquemia cerebral, uma vez que é um método robusto, não invasivo e reversível. Esta dissertação tem como objetivo sistematizar os vários métodos de validação do modelo animal de isquemia da ACM para haver uma maior reprodutibilidade em estudos futuros. Para a elaboração desta dissertação foi realizada uma pesquisa na base de dados Medline até março de 2021 para a recolha de todos os artigos. Foi então construída uma expressão de pesquisa recorrendo aos termos MeSH. Foram escolhidos os critérios de inclusão e exclusão, para seleção dos artigos a integrar nesta revisão sistemática da literatura. Subsequentemente, as características do rato, características de indução, anestesia, volume de enfarte e parâmetros estudados foram recolhidos de cada artigo por dois revisores e, quando necessário, com a ajuda de um moderador. Quanto às características dos ratos, as espécies mais utilizadas são os Sprague-Dawley ou Wistar adultos. A anestesia mais adequada para realizar a isquemia da ACM é o isoflurano e os períodos de isquemia variam de 1-2h e reperfusão de 24h a 72h. Foi também averiguado que o modelo de isquemia da ACM induz volumes de enfarte de 40-50%. Os parâmetros avaliados incluíram o comportamento (funções neurológicas, função sensoriomotora e testes cognitivos), testes histológicos (volume de enfarte, edema cerebral, GFAP, Iba1 e NeuN) e marcadores bioquímicos, tais como fatores inflamatórios (iNOS, COX-2, NF-κB, TNF-α, e interleucinas). Com os resultados obtidos neste estudo, será possível realizar estudos mais precisos e reprodutíveis na área da isquemia cerebral.

Palavras-chave: isquemia cerebral; oclusão da artéria média cerebral, modelo animal; ratos

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Chapter I: Cerebral ischemia

Cerebral ischemia or stroke is one of the leading causes of death and neurological disability in the world, affecting more than 15 million people annually, in which 5 million are permanently disabled and another 5 million people cannot resist producing immense health and economic burdens globally. (1–4) Several factors have been identified that increase the risk of stroke including diabetes mellitus, history of transient ischemic attacks, hypertension, atrial fibrillation, cigarette smoking, and low serum concentrations of HDL cholesterol.(5) Stroke has two pathological variants: ischemic stroke and hemorrhagic stroke. The latter is the result of the bursting of an artery in the brain (6), in turn, the ischemic stroke results from the permanent or transient disruption of cerebral blood flow or blood supply to a specific brain region especially the middle cerebral artery region by formation of a clot. This leads to lack of oxygen (hypoxia) and glucose deficits triggering a cascade of complex pathological events including abnormal recruitment of inflammatory cells, excess production of reactive oxygen species, initiation of pathological apoptosis, excessive glutamate excitotoxicity and ion dyshomeostasis, leading to necrotic death of the brain tissue (infarction). Surrounding infarction, ischemic penumbra is located, a tissue with the potential to recover if adequate treatment is provided within a tight therapeutic window. (3,6–11) Thus, it is necessary to be alert to the symptoms associated with stroke. This clinical picture presents some symptoms such as weakness, diplopia, muscle paralysis on one side of the body, loss of balance and/or coordination, aphasia, mental confusions, etc. However, these symptoms depend on the region of the brain affected. (12) After the onset of stroke symptoms, rapid intervention is very important in order to attempt the reduction of the severity of the sequelae.(13)

1. Epidemiology

Ischemic stroke is a heterogeneous multifactorial disorder with high prevalence recognized by the sudden onset of neurologic signs related directly to the sites of injury in the brain where the morbid process occurs, which leads the morbidity and mortality in industrialized countries. Throughout the years the evaluation of multiple epigenetic and genetic factors has been made to evaluate complex neurologic disorders, such as stroke, in order to diminish the individual and populational risk and help with the public health policy approaches to avert irreversible sequela and burden of stroke. (14,15)

Absolute number of people affected by stroke substantially increased across all countries despite dramatic declines in age-standardized incidence, prevalence, mortality rates, and disability. Population growth and aging have played an important role in the

observed increase in stroke. With significant geographic and regional differences and developing countries sharing the impact of stroke, the domestic and global burden of this disease may actually be carried by low- and middle-income populations making it a public health concern.(14)

The incidence of stroke rapidly increases with age, doubling for each decade after age 55. Among adults ages 35 to 44, the incidence of stroke is 30 to 120 of 100,000 per year, and for the ages 65 to 74, the incidence is 670 to 970 of 100,000 per year.(16–18) It has been noticed that age is associated with poorer outcomes, independently of stroke type. In general, older patients were more likely to be discharged to an institution other than home and were more disabled and more severely handicapped at 3 months after stroke. In-hospital mortality and case fatality also increases with age, with case fatality rate for those ≥80 years old being as high as 21%.(19,20) Stroke does occur among children, but the incidence in comparison with adults is substantially lower (i.e., approximately 1 to 2.5 of 100,000 per year), and 50 to 75% of strokes among children are as a result of haemorrhage. Sickle cell disease is the most common cause of childhood stroke, with the highest incidence between ages 2 to 5 years. Perhaps due to better control of vascular risk factors during the last decade, incidence rates of stroke in developed nations have modestly diminished or plateaued.(17)

Gender differences in heart disease are well recognized, but less is known of gender differences in stroke. Although age-adjusted stroke mortality rates for men are higher than for women overall, although due to their longevity, more women die of stroke each year.(21)

2. Pathophysiology

Cerebral metabolism and neuronal function are critically reliant on oxygen, glucose and the production of adenosine triphosphate (ATP). Maintaining an adequate cerebral blood flow (CBF) is essential for this critical supply of glucose and oxygen, since it is regulated by the metabolic necessities of the brain to adjust its functional needs (flow-metabolism coupling). Brain is the organ that requires more oxygen and glucose on a weight basis, given that it weights approximately 1400g (adult), representing only 2% of the total body weight, yet, however, it uses about 25% of total body oxygen and glucose per minute. Since the brain has such a big energy demand and it is unable to store it, any interruption of the blood supply can easily and quickly result in neuronal dysfunction and neuronal damage.(22)

Ischemic stroke occurs when the blood supply to the brain decreases or is interrupted, most of the times in the MCA region, leading to lack of oxygen and glucose in the brain, and causing the infarction. The infarction in the brain then can be classified in 2 areas: the core and the penumbra (fig.1).

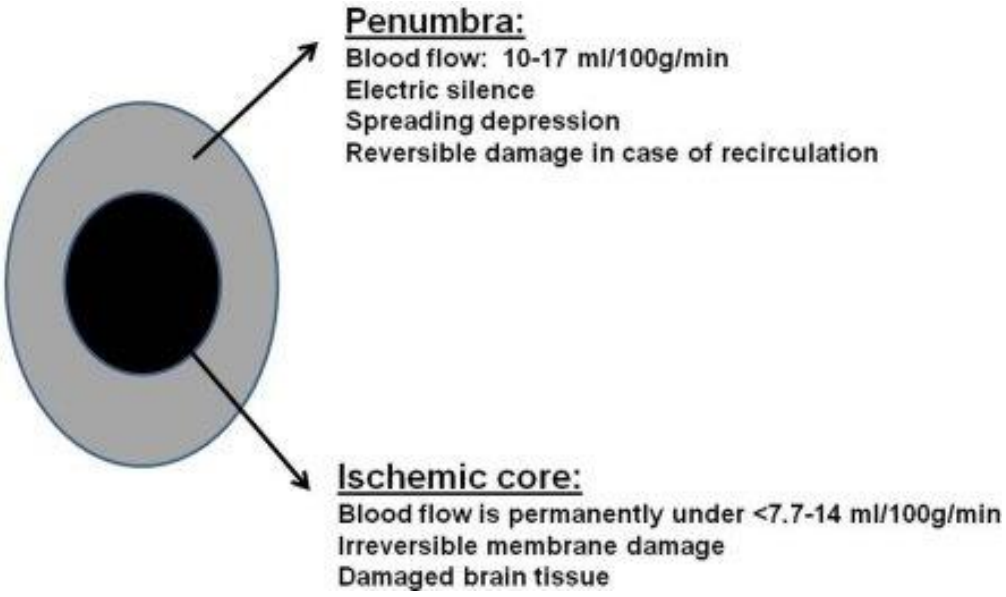

Figure 1 - Ischemic injury representation

The core is where the ischemia is most severe, resulting in a high decrease in CBF (less than 15% of baseline), leading to a reduction in the levels of ATP to 25% of the baseline. The infarction in this area occurs in minutes and the tissue is not salvageable. The penumbra is where the CBF decreases to levels between 15%-40% of the baseline and the ATP levels decrease between 50%-70%.(23–26) Additionally, the perfusion in the penumbra region is dependent on the degree of collateral circulation.

The normal CBF is 50mL/100g/min, however, the electrical activity in cerebral tissue only ceases at flow rates below 16-18mL/100g/min, therefore, if the CBF reaches this threshold, electrical and neuronal dysfunction will occur and the neurons become ischemic, although not necessarily infarcted. There is also a second threshold where the neuron ion homeostasis is lost below 10-12mL/100g/min, at which point the energy

production is less than the energy consumed. Furthermore, the efflux of K^+ and influx of Ca^{2+} occurs and there is rapid death or infarction (fig.2).

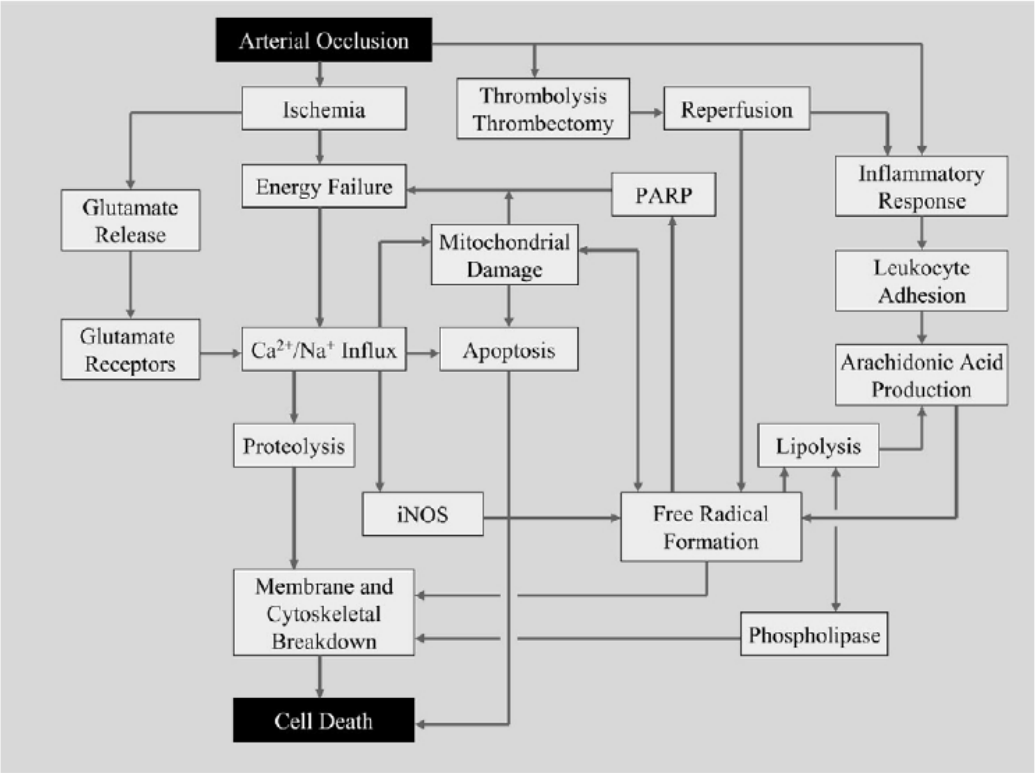

Figure 2 - Pathophysiology of cerebral ischemia (29)

These disturbances result in lactate acidosis and membrane depolarization and lead to irreversible degeneration of the neurons and glia cells in the ischemic core. In the penumbra as well as the core region, neuronal viability is time dependent, which means that the more severe the ischemia is, the less time there is before establishment of irreversible damage.(23,27,28) Inflammation is one of the most important mechanisms and plays a major role in the pathogenesis and progression of the cerebral lesion, namely penumbra region.(29) The neurons and glia release compounds such as ATP, fractalkine or other danger-associated molecular patterns, which activate microglia (resident immune cells that mediate neuroinflammation in the central nervous system) and mast cells which act as early responders (fig.3). When activated, they release proinflammatory cytokines and/or cytotoxic factors such as, nitric oxide (NO), tumour necrosis factor-α (TNF-α), interleukin 1-β (IL-1β) and reactive oxygen species (ROS), which damage the neurons and the blood brain barrier (BBB), increasing the BBB permeability. This potentiates the infiltration of immune cells, such as monocytes/macrophages, lymphocytes and neutrophils.(30,31) The reactive astrocytes which are responsible for the structural and functional modulation after ischemia will respond in a later stage of inflammation and are responsible for the formation of glial scar.(31,32)

The process by which the penumbra is destroyed is the focus of most ischemia research, as prevention of the infarct growth would be expected to salvage neuronal tissue. Given the importance of the penumbra area in the outcome of these patients, several strategies of neuroprotection are the focus of research in achieving this goal.(23)

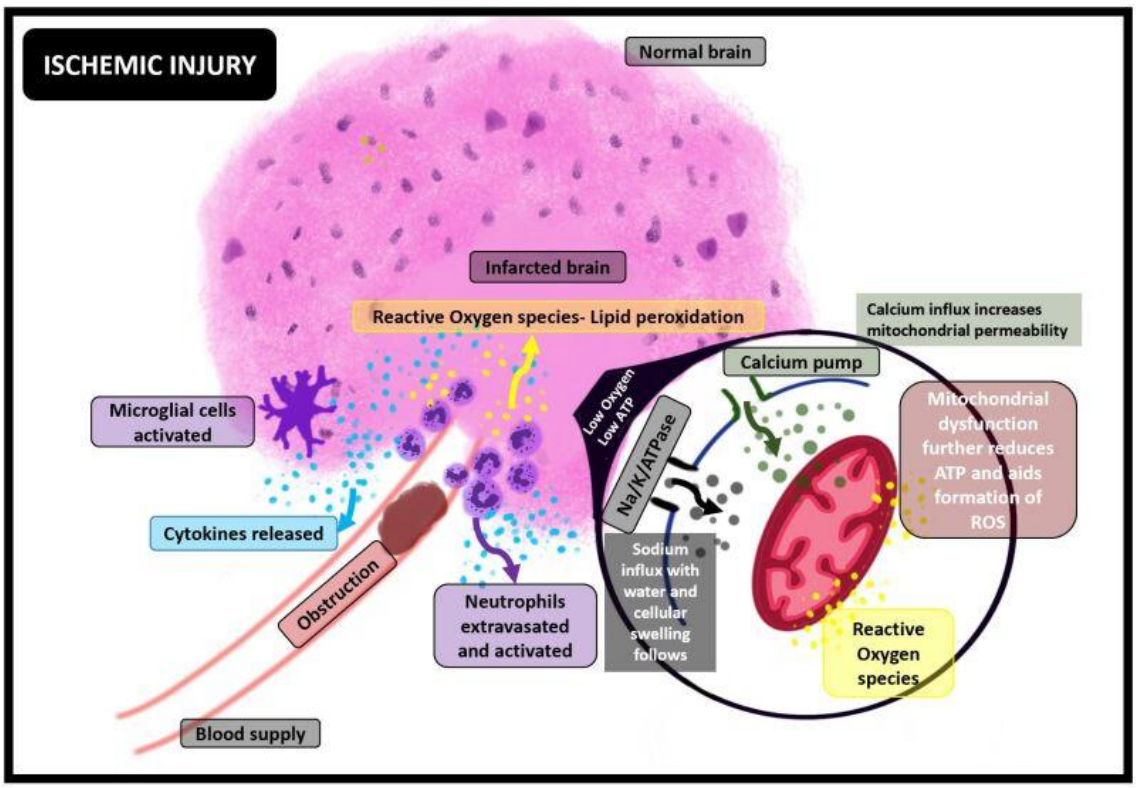

Figure 3 - Ischemia injury and its molecular consequences

Chapter II: Brain cells and blood brain barrier during cerebral ischemia

The post-stroke immune response has been the target in the treatment strategy for ischemic stroke recently. Glial cells, such as astrocytes, microglia and oligodendrocytes, are the primary components of the peri-infarct environment in the CNS and have been involved in post-stroke immune regulation. Astrocytes constitute 19-40% of glial cells, microglia constitute 10%, oligodendrocytes constitute 45-75%% of all glial cells, and the remaining cells are NG2 cells. Glial cells provide structural and nutritional support and are involved in the development of CNS under normal conditions. They also have important roles in neuropathogenesis and pathological conditions, as they participate in innate and adaptive immune responses. Studies have shown that glial cells regulate neuroinflammation after stroke. (33–35)

1. Astrocytes role during ischemia

Although astrocytes have been, in the past, considered merely a cellular layer filling of the intraneuronal space and gluing neurons together, they have been gaining recognition because of their role in maintaining central nervous system (CNS) homeostasis, providing nutrition for neuronal cells and neurotransmitter recycling. Furthermore, recently astrocytes were shown to control the number and function of neuronal synapses and blood flow in the brain. (36) During ischemia, astrocytes are well positioned to confer neuroprotection as they provide growth factors, clear glutamate, preserve BBB, interact with the immune system, impair oxidative stress and produce functional extracellular mitochondria to support neuronal viability. Additionally they are less vulnerable to ischemic injury than neurons and have a greater capacity of self-preservation, indicating that these cells may play a crucial role in post-stroke recovery. (36,37,46,38–45) However, astrocytes, in pathological cases such as stroke, also contribute to neurodegeneration by becoming reactive (fig.4).(47) They become reactive at the time that corresponds to the inflammation peak (2 days to 1 week from stroke onset) which is called the subacute phase, where the brain damage can be amplified by the inflammatory process.(40) Reactive astrocytes are characterized by high-level expression of glial fibrillary acid protein (GFAP), an intermediate filament protein, and by upregulation of intermediate filaments of cytoplasm, secretion of pro-inflammatory mediators and pro-inflammatory cytokines such as TNF- α, IL-1β and production of ROS, leading to brain injury.(36,47,48) The response of reactive astrocytes can be summarized by 2 mechanisms, through the formation of a double barrier. The first barrier is considered

physical, which is the tight astrocytic scar that is formed by polarized bundles of reactive astrocytes together with the extracellular matrix (ECM) components that surround physically the injured region.(40) This barrier is particularly important at the early stages of ischemia to surround the damaged area in order to preserve the healthy region, by keeping the immune cells inside the damaged area, although at later stages it inhibits the neurite outgrowth, contributing to the lack of regenerative capability of the brain. The second barrier is considered molecular and is composed by the signalling molecules that are produced and secreted by the astrocytes to regulate the infiltration and activation of immune cells. These molecules are not bounded to a specific physical location, but instead diffuse away from reactive astrocytes creating signalling gradients throughout the injured tissue. After the formation of these barriers, the reactive astrocytes may indirectly modulate the inflammation by altering the neuronal responses to ongoing metabolic and toxic injuries.(40,44) Therefore, astrocytes can be an important target for stroke therapy at the post-acute phase, because of their role in the post stroke inflammation and their repairing capacity. With a better understanding of the pathways involved we can augment positive aspects and supress the negative effects of reactive astrocytes, to improve the post stroke outcome.(47) Studies already revealed this role through the inhibition of the Nuclear Factor kappa B (NF-κB) signalling pathway in astrocytes which resulted in reduction of cytokine production leading to protection of neurons from ischemic damage.(48)

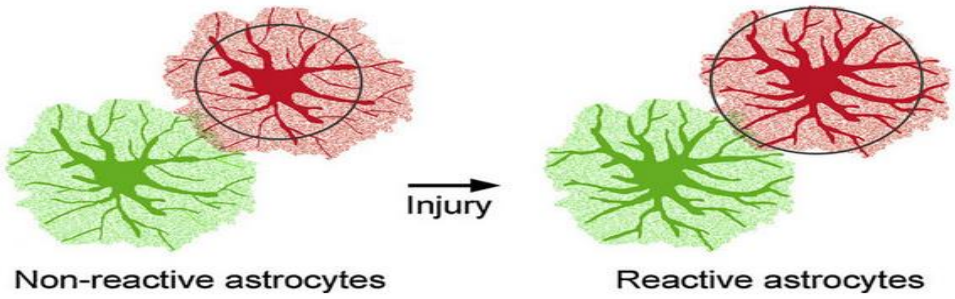

Figure 4 - Astrocytes during ischemia

2. Microglia role during ischemia

Microglia are the resident macrophages of the CNS and are the major immune-competent cell type in the brain.(49–52) Microglia has the major role of surveying the CNS environment and serve as the first line defenders, being considered the main mediators of neuroinflammation. Basically their role is to protect the CNS from infections, tissue damage and metabolic imbalances, however, if acute inflammation persists for a prolonged time this can be detrimental and damage the neurons.(50,53,54) Once cerebral ischemia occurs, the microglia is activated within minutes by NF-κB triggering:

phenotypic changes such as morphological (amoeboid shape, like systemic macrophages), proliferation, migration, phagocytosis, and secretion of inflammatory mediators. Activated microglia act like a double edge sword, since in one hand they can phagocyte pathogens and necrotic cells, supress inflammation and aid in brain repair. However, in the other hand they produce excessive inflammatory cytokines (TNF-α, IL-1β, IL-6), chemokines, and oxygen (ROS)/nitrogen (NOS) reactive species such as hydroperoxyl radical (HO_2) and nitric oxide (NO) respectively. Matrix metallopeptidases (MMPs) are also produced and disrupt the BBB which will initiate an influx of monocytes/macrophages, exacerbating the neuroinflammation and brain damage.(8,50,53,55–57) This double edge sword can be explained by functional dichotomy, which are the classical (M1, pro-inflammatory) and alternative (M2, anti-inflammatory) activation processes (fig.5).(58,59) M1 activation is induced by lipopolysaccharide/interferon-c (LPS/IFN-c) or Th1 cytokines associated with the production of pro-inflammatory molecules such as TNF-α, NO, IL-6, ROS and TNF-α, this activation process responsible is for neural damage. M2 activation is induced by Th2 cytokines such as IL-4, IL-13 or IL-10, as these M2 microglia express different molecules associated with the expression of scavenger receptors and proangiogenic factors such as mannose receptor, dectin-1, and arginase, which are involved in neuroprotective effects.(58,60) Hence, a potential approach to brain ischemia could be the modulation of microglia activation, by promoting the M2 activation and inhibiting the M1 activation, in order to enhance the neuroprotective effects and supress the damaging effects, promoting a better outcome.(51)

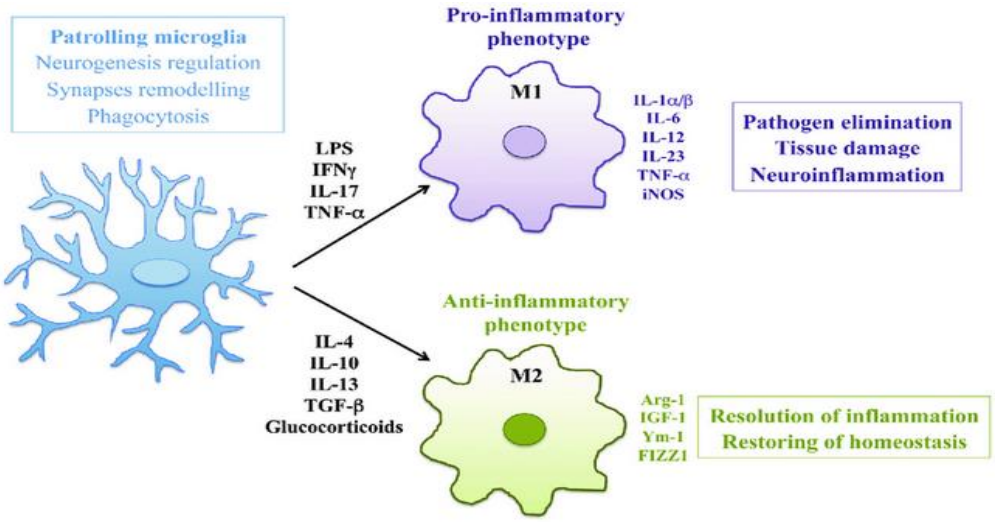

Figure 5 - Microglia differentiation during an ischemic event

3. Blood Brain Barrier disruption during cerebral ischemia

The BBB is an essential physical and biochemical barrier that separates the CNS from the systemic circulation, which constitutes an interface between the vasculature system and the neural tissues. It regulates the transport of substances in a bidirectional way and protects the central nervous system from unwanted compounds, playing a crucial role in maintaining its homeostasis by regulating the paracellular permeability ion balance, nutrient transport, brain hemodynamics (cerebral blood flow and angiogenesis) neuronal development and synaptic activity.(5,61,62)

The BBB is composed of endothelial cells (ECs), basement membrane, astrocytic foot processes and pericytes.(63) Anatomically, the BBB endothelial cell is distinguished from other capillary counterparts by higher mitochondria amount, less cellular penetrations and lower pinocytic activities. The limited permeability of the BBB is mainly due to the existence of tight junction (TJ), which connects the two adjacent ECs.(64–67) During cerebral ischemia, one of the major events occurring is the disruption of BBB, which is an early event that occurs prior to the onset of neuronal injury. It develops soon after the onset of artery occlusion and continues for several days to weeks after stroke. BBB disruption can be associated with reperfusion and is consequently attributed to dysfunctional TJs causing increased paracellular solute leak and endothelial damage. This leads to increased permeability of the affected vessels (remarkable pathological feature of stroke), modulation of transport proteins and endocytotic transport mechanisms (leading to changes in transcellular transport for some substances), and inflammatory damage, causing cognitive and motor impairment.(5,62,68) Therefore, BBB disruption following ischemic stroke can have severe pathological consequences (edema and inflammation) that can exacerbate brain injury and contribute to cognitive impairment. It is critical to note that BBB breakdown is a precursor to serious clinical consequences of ischemic stroke such as hemorrhagic transformation.(69) One of the most significant contributors to BBB breakdown in stroke is activation of proteinases such as matrix metalloproteinases (MMPs)(5) They include MMPs that are activated by hypoxia-inducible factor-1 (HIF-1 α)-dependent mechanisms (i.e., MMP-2) and MMPs whose activation is triggered by cytokines (i.e., TNF- α, IL-1α) such as MMP-3 and MMP-9.(70,71) Current knowledge in the BBB field has uncovered a multiplicity of molecular pathways that can lead to significant changes at the level of the cerebral microvasculature. Indeed, identification and characterization of these molecular pathways has provided opportunities to develop novel therapeutic approaches to protect against BBB dysfunction in the context of ischemic stroke.(5)

Chapter III: Pharmacological therapies for ischemia injury

Currently, therapy for ischemic stroke consists of two potentially complementary strategies of thrombolysis and neuroprotection. Thrombolysis can be accomplished using either thrombolytic drugs which include drugs that act as plasminogen activators to dissolve the thrombus or mechanical devices to disrupt and remove the offending thrombus, seeking to re-establish blood flow and salvage brain ischemic tissue. Neuroprotective drugs aim is to limit the injury the results from ischemia and subsequent reperfusion of brain tissue.(72)

1. Plasminogen Activators

Since the late 1950s, many studies have used pharmacologic thrombolysis in the patients which suffered ischemic stroke.(73) These agents work by converting the inert plasminogen into plasmin, an active protease that both dissolves fibrin and solubilizes its degradation products, resulting in clot breakdown.(74) Initial studies revealed that the benefits of re-establishing circulation to an ischemic area must be weighed against an increased risk of haemorrhagic complications, due to this the plasminogen activators were only approved in 1995, when a phase III clinical trial described a population who benefited from thrombolysis.(75) They have limited use which depends on the time of stroke onset and the patients age. The European stroke organisation (ESO) recommends using plasminogen activators until 4.5h after stroke onset. This therapeutic window can be extended to 9h in some cases for patients with CT or MRI core/perfusion mismatch, and for whom mechanical thrombectomy is either not indicated or not planned.(76) Thus, research has been focused on expanding the therapeutic time window in which the patients can receive the plasminogen activator as well as the setting in which the patient can receive treatment.(77,78) Currently, the plasminogen activators used for ischemic stroke treatment that are approved in Portugal consist of alteplase, reteplase and tenecteplase.(79) Reteplase is more convenient to administer and produces a faster thrombolytic effect when compared to alteplase. Tenecteplase has a lower risk of bleeding complications but has a similar mortality rate after one-year treatment to that of alteplase.

With the advances in neurointerventional techniques, an emerging technique for the treatment of ischemic stroke is the intra-arterial delivery of plasminogen activators. This technique involves the delivery of plasminogen activators directly into the thrombus

through a microcatheter, this technology also allows for mechanical disruption and clot retrieval. Despite the good results of this new technique, the gold standard still is the early intravenous administration of plasminogen activators.(72)

2. Neuroprotective agents

Regarding the neuroprotective agents, their aim is to alleviate excitotoxicity, calcium dysregulation, mitochondrial dysfunction, oxidative and nitrosative stress and inflammation. In addition, they act to relieve tissue acidosis, blood-brain barrier disruption, and neuronal apoptosis, necrosis and autophagy.(80) Ion channels are membrane proteins that mediate ionic homeostasis in neurovascular units during ischemic stroke. An excess of sodium influx to neurons induces cell swelling and leads to cytotoxic edema. Intracellular calcium overload can trigger a series of pathological events that ultimately result in neuronal apoptosis as well as necrotic death, while an unchecked outflow of potassium changes neuronal polarization and excitability. Therefore, modulation of ion channel function can be a novel approach in the treatment of stroke. (81) Free radicals are high reactive molecules capable of chain reactivity. They can react with and damage proteins, lipids and nucleic acids. They are present in low levels, although during ischemia their levels increase and contribute to cerebral edema. Hence, free radical scavenging can be a new therapeutic approach to grant neuroprotection.(82,83) The excitotoxicity is a type of neurotoxicity centred in glutamate. Glutamate concentration during ischemia has a quickly extracellular increase and stimulates *N*-methyl-D-aspartate receptor (NMDAR), that acts via calcium permeability. Excitotoxicity cell death is triggered through PTEN, cdk5 and DAPK1, among other downstream targets. All these targets might be potential new pharmacological targets to excitotoxicity blockage therapies.(84) Nootropics, also called cognitive enhancers or smart drugs, are supplements or endogenous substances which improve behavioural outcome and cognitive function in healthy individuals and patients, probably by promoting neurogenesis or decreasing neuronal death. Supplementing endogenous substances, such as neurotrophic factors, intermediates of phosphatidylcholine and urinary kallidinogenase, may constitute an effective approach for alleviating stroke symptoms.(81) Neuroprotective agents are only experimental therapies that are in clinical trials. They still require a deep comprehension of its mechanisms and until now none has presented significant therapeutic results. Consequently, further research is required to develop new therapies to improve the outcomes in patients that have suffered an ischemic event.

Chapter IV: In Vivo pre-clinical models of ischemic stroke

1. Animal Models

The use of *in vivo* research, and specifically the use of animal models, is an essential part of the development process of new drugs. The importance of animal models in biomedical sciences is not a novel concept and it is noteworthy that since the beginning of the 20th Century, 94 out of 106 Nobel Prizes awarded for Physiology or Medicine were dependent on research using experimental animals.(85–87) They are a key component of the strategy to develop optimal experimental conditions facilitating analysis of the different factors leading to disease enabling scientists to explore the complex dynamic which exists between health and disease(85). Furthermore, the preclinical pharmacological, toxicological and pharmacokinetic data required for the conduct of the first phase of clinical trials in humans is often collected with the use of animal models.(86,87) When animal models are employed in the study of human disease, they are frequently selected because of their similarity to humans in terms of genetics, anatomy, and physiology.(88) Rodents are the most common type of mammal employed in experimental studies, because they share the majority of their genome with humans, making rodents particularly applicable to human disease, also they are a cost-effective and efficient tool to speed research and the development of drug therapies. (88)

2. Animal models of ischemic stroke

Ischemic strokes are the most common and results from a thromboembolic occlusion of a major cerebral artery or its branches. It results in oxygen and energy deprivation, ROS formation, glutamate secretion, intracellular accumulation of calcium and triggers inflammation processes. This events will ultimately lead to irreversible tissue injury associated to infarction.(89) During the last four decades, a variety of ischemic stroke animal models were developed, with the objective of identifying the mechanisms that underlie cerebral ischemia and develop new agents for stroke therapy. The animal models of ischemic stroke are an indispensable tool for many reasons such as (9):

- Human ischemic stroke is highly variant in its manifestations (causes, anatomic localization), whereas an experimental model is highly reproducible, well controllable and standardized, allowing a better analysis of stroke pathophysiology and pharmacological effects;

- Molecular, genetic, biochemical and physiological research require most of the time invasive direct access to the brain tissue;
- Pathophysiological events occurring during the first minutes of an ischemic stroke are not detectable by imaging techniques used in human stroke and thus can only be studied in an animal model;
- Perfusion and vasculature are essential in pathophysiology of stroke and cannot be modelled in *in vitro* models.

The majority of stroke experiments are executed in small animals (mice, rats, rabbits), with the rat being the most popular, because of its body size that allows an easy monitoring of physiologic parameters, the brain size is well suited for fixation procedures, the homogeneity of results between strains and its high study reproducibility.(90–92) The most common models utilized are the middle cerebral artery occlusion (MCAO), craniotomy model, photothrombosis model, endothelin-I model and embolic stroke model (table 1).(9)

Table 1 - Advantages and disadvantages of the most common ischemic stroke animal models (9)

	Advantages	**Disadvantages**
Intraluminal suture MCAo model	Mimics human ischemic stroke Exhibits a penumbra Highly reproducible Reperfusion highly controllable No craniectomy	Hyper-/hypothermia Increased hemorrhage with certain suture types Not suitable for thrombolysis studies
Craniotomy model	High long-term survival rates Visual confirmation of successful MCAo	High invasiveness and consecutive complications Requires a high degree of surgical skill
Photothrombosis model	Enables well-defined localization of an ischemic lesion Highly reproducible Low invasiveness	Causes early vasogenic edema that is uncharacteristic for human stroke Not suitable for investigating neuroprotective agents
Endothelin-1 model	Low invasiveness Induction of ischemic lesion in cortical or subcortical regions Low mortality	Duration of ischemia not controllable Induction of astrocytosis and axonal sprounting, which may complicate the interpretation of results
Embolic stroke model	Mimics most closely the pathogenesis of human stroke Appropriate for studies of thrombolytic agents	Low reproducibility of infarcts Spontaneous recanalization High variability of lesion size

Abbreviation: MCAo, middle cerebral artery occlusion.

3. MCAO model

The most common model of ischemic stroke utilized in rodents is the MCAO model. MCAO is less invasive and does not require craniectomy, avoiding damage to cranial structures. This technique temporarily occludes the common carotid artery (CCA), introducing an adapted suture directly into the internal carotid artery (ICA) and advancing

the suture until it interrupts the blood supply of the middle cerebral artery (MCA)(9) although there is a modified version in which the suture is inserted into the external carotid artery (ECA), using the ECA trunk to advance through ICA (fig.6). The laser doppler flowmetry can be useful to ensure that the MCA was occluded. This version has the advantage of maintaining the anatomic integrity that his required for reperfusion, being the better choice for transient ischemia with reperfusion.(93) This model has several advantages, with one of the most relevant ones being one of the anatomically closest to human stroke, since the MCA and its branches are the cerebral vessels most affected in humans ischemic stroke (approximately 70%).(9,94) Therefore, the use of this model also allows for the achievement of a penumbra region similar to that exhibited in human strokes, a large infarct volume and high reproducibility. The reperfusion and thus the duration of ischemia is precisely controllable. (9) Depending on the duration of MCAO, the area of brain that is affected may increase or decrease. The most used durations are 60 minutes, 90 minutes, 120 minutes and permanent. The model can be utilized to study the ischemic stroke and respective neuronal death, cerebral inflammation and BBB damage.

However, this technique sometimes may lead to vessel rupture and subsequent subarachnoid haemorrhage. To avoid this problem, a silicon-coated suture is utilized as well as a laser doppler-guided placement of the suture.(9,95) Therefore, as this model allows the induction of a controlled and consistent ischemic insult in experimental animals, it enables the study of cellular and molecular mechanisms of poststroke brain damage as well as new therapeutic approaches.(96)

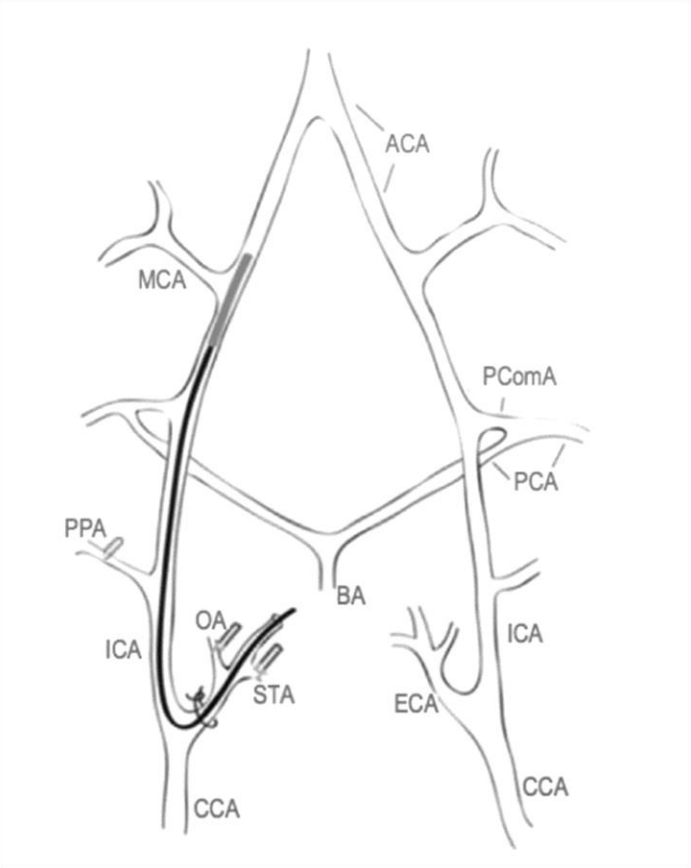

Figure 6 - Scheme of the MCAO model through intraluminal suture. MCA - Middle Cerebral Artery; ACA - Anterior carotid artery; PPA - Pterygopalatine artery; ECA - External carotid artery; CCA - Common carotid artery.

Chapter V: Aim

The aim of this study is to perform a systematic review which follows the PRISMA systematic-review protocol, with the objective to define the best way to implement the MCAO model. The findings of this study will be posteriorly experimentally applied to obtain preliminary results of the cell behaviour during an ischemic event. This will allow us to better guide experimental research in terms of a more rational choice of pharmacodynamic targets and cellular pathways in order to obtain a pharmacological approach with a higher potential for clinical translation success.

This study will allow us to understand the best way to standardize the MCAO model and implement this preclinical model (including surgical parameters) with more consistent results. Additionally, a thorough analysis of the experimental parameters will allow us to perform a following study to better study the influence of the several CNS cells and guide the choice on pharmacological approaches. An evaluation of the experimental variables of this preclinical model will also enable an independent approach on the 2 phases of injury induction of ischemic stroke: ischemia phase and reperfusion phase. Understanding the mechanisms involved in both phases will allow us to better decide the experimental parameters to be used.

Afterwards, it is our intent to continue the study at a PhD degree, using the established animal model and continue with the evaluation of new pharmacological approaches. Given that ischemic stroke is one of the leading causes of death and that the therapeutic options available today are not only very limited but even then still lead in some cases to neurological disabilities, there is a big opportunity for an added value of preclinical translational research.

Chapter VI: Material and methods

1. Research question

A well formulated research question is required to perform a systematic review. Consequently, PICO (P – Population or participants, I – intervention, C – comparison, O – outcome) worksheet was used to define the type of population and interventions areas in order to help with the decision of which articles will be included in this review, by creating a specific, clear and answerable research question. Population was defined as rats, intervention was established as development of MCAO model, comparison different methods to implement the MCAO model, outcome was defined as the best way to perform MCAO model in rats. Resulting in the research question of: What is the better way to perform a MCAO model in rats?

2. Search strategy

Following the establishment of a review protocol based on PRISMA methodology, a computerized literature search was conducted to find publications that study the ischemic stroke in rats with middle cerebral artery occlusion. This research covered the electronic database MEDLINE via the PubMed platform, in which all studies with the middle cerebral artery occlusion model in rats were searched from the beginning of the database until March 2021.

The search strategy included the insertion of keywords, and consequently, combinations of them were carried out until forming an appropriate search expression. The search expression for MEDLINE is shown below:

"Rats"[Mesh] OR Rats[tiab] OR Rat* AND "Animal Experimentation"[Mesh] OR "Animal Experimentation"[tiab] OR "Animal Experimentation" OR "Drug Evaluation, Preclinical"[Mesh] OR "Preclinical studies"[tiab] OR "Preclinical studies" OR "Non-clinical studies"[tiab] OR "Non-clinical studies" OR “animal model”[tiab] OR “animal model” AND "Brain Ischemia"[Mesh] OR "Brain Ischemia"[tiab] OR "Brain Ischemia" OR "Ischemic Stroke"[Mesh] OR "Ischemic Stroke"[tiab] OR "Ischemic Stroke" OR “ischemic brain injury”[tiab] OR “ischemic brain injury” OR "cerebral ischemic injury"[tiab] OR "cerebral ischemic injury" OR neuroinflammation[tiab] OR neuroinflammation AND "Infarction, Middle Cerebral Artery"[Mesh] OR "Middle Cerebral Artery"[tiab] OR "Middle Cerebral Artery" OR MCAO[tiab] OR MCAO OR "middle cerebral artery occlusion"[tiab] OR "middle cerebral artery occlusion".

The results from this search expression are shown below in table 3.

3. Selection of studies

The articles resulting from our research expression were selected according to the following inclusion criteria: 1) only original articles; 2) studies where the transient middle cerebral artery occlusion (tMCAO) model is described; 3) *in vivo* preclinical studies using rats; 4) articles that were published in english. The exclusion criteria are as follows: 1) preclinical studies in mice; 2) studies with other associated pathologies; 3) experiments performed in cell cultures (*in vitro*); 4) all other models than tMCAO; 5) articles before 2016. The selection of studies was performed by two independent reviewers and disagreements between the two reviewers were resolved by mutual consensus and when required with the arbitrator. To perform the selection of studies, Rayyan platform (https://rayyan.ai/) was utilized.

4. Data Extraction

All data were extracted independently by two reviewers in a Microsoft Excel spreadsheet (Windows 10 edition; Microsoft Corporation, Lisbon, Portugal), with disagreements resolved by consensus and when required with the arbitrator. The following information was extracted from each study: characteristics of the rats (strain, weight, gender and age), characteristics of anesthesia, characteristics of induction (ischemia and reperfusion), therapeutic approach (treatment, dose, duration of administration, volume of cerebral infarction), parameters studied, authors and year of publication.

5. Quality assessment

The quality of eligible studies will also be assessed by two independent reviewers and the disagreements will be resolved by consensus and when required with the arbitrator. The methodological quality of these preclinical studies will be assessed based on the recommendations of Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guideline. This guideline is divided in two lists of items, the ARRIVE essential 10 which contains the basic minimums required in a manuscript and the recommended set which complement the essential 10 and add important context to the study. The essential 10 consist of ten items which are the study design, sample size, inclusion and exclusion criteria, randomisation, blinding, outcome measures, statistical methods, experimental animals, experimental procedures, results. The recommended set has eleven more items that complement the essential 10 and consist of, abstract, background, objectives, ethical statement, housing and husbandry, animal care and monitoring, interpretation/scientific implications, generalisability/translation, protocol registration data access and declaration of interests. All these items were assessed in each eligible

study and scored from 0 to 3: 0 – not mentioned; 1 – incomplete; 2 – complete; 3 – too complete. The scores of each item were summed and an overall grade was given. Low quality from 0 to 19, moderate quality from 20 to 39; high quality scores higher than 39. These results are shown below in table 2.

Chapter VII: Results and discussion

After applying the set of keywords in the electronic databases, a total of 452 publications were found and identified which, screening the titles and abstracts, 395 articles were excluded, as they weren't within the scope of this work. Therefore, we obtained a total of 57 references which were assessed for eligibility and, after assessing these articles, 22 of them were also excluded as they did not comprehend the inclusion criteria. The remaining 35 articles were included in the qualitative analysis, since these studies described the occlusion of the middle cerebral artery in rats and were in accordance with the inclusion criteria (fig.7). The agreement between the authors of the review on exclusion was 100%.

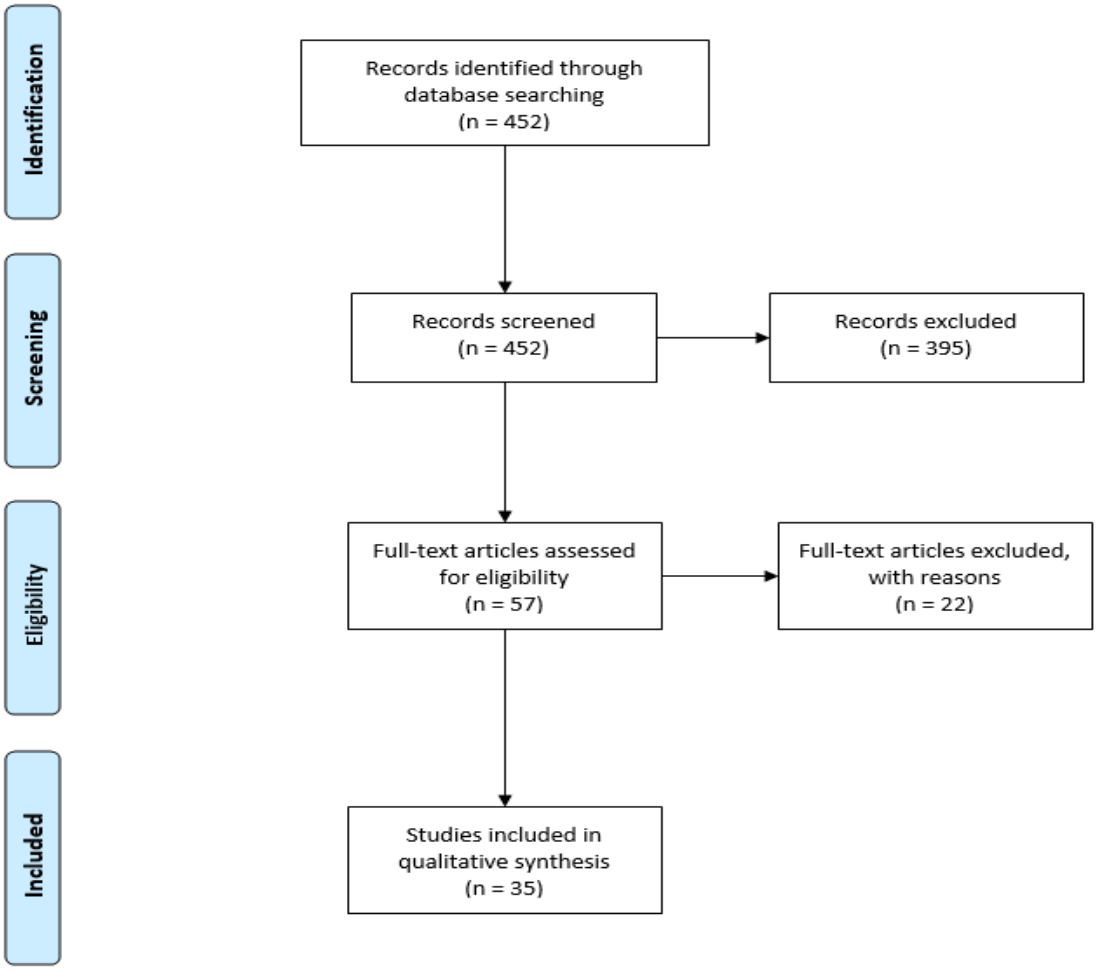

Figure 7 - PRISMA flow diagram

The quality assessment regarding the articles reviewed in this study is shown below in table 2. After summing the scores regarding quality, 26 articles had moderate quality and 9 articles had high quality. Also, no article had a bad quality score.

Table 2 - Quality assessment of the articles reviewed

Study design	Sample size	Inclusion and exclusion criteria	Randomisation	Blinding	Outcome measures	Statistical methods	Experimental animals	Experimental procedures	Abstract	Background	Objectives	Ethical statement	Housing and husbandry	Animal care and monitoring	Interpretation/ scientific implications	Generalisability/ translation	Protocol registration	Data access	Declaration of interests	total	Quality	Refences
3	2	2	1	1	3	3	2	2	1	3	3	1	0	1	3	3	0	0	0	34	Moderate	(97)
3	2	2	1	0	3	3	3	3	2	1	2	3	3	1	2	2	0	2	2	40	High	(98)
3	2	0	1	0	3	3	3	3	2	2	2	3	0	0	2	0	0	0	2	31	Moderate	(99)
3	2	2	3	3	3	2	3	3	2	2	2	0	3	3	3	2	0	3	3	47	High	(100)
3	0	0	2	2	3	2	3	3	1	2	2	3	2	1	2	2	0	0	0	33	Moderate	(101)
3	0	0	1	2	3	3	3	3	1	1	0	3	3	2	2	1	0	2	3	36	Moderate	(102)
3	2	0	1	2	3	2	3	3	2	3	2	0	2	1	3	2	0	0	2	36	Moderate	(103)
3	1	0	0	0	2	1	3	3	1	2	2	3	2	0	2	0	0	0	0	25	Moderate	(104)
3	1	2	3	2	3	2	3	3	2	2	2	3	3	0	3	0	0	3	3	43	High	(105)
3	2	2	1	2	3	2	3	3	2	3	3	2	2	0	2	1	0	0	0	35	Moderate	(106)
3	2	2	3	2	3	3	3	3	1	2	2	3	0	2	3	3	0	0	3	43	High	(107)
3	2	2	0	0	3	3	2	3	2	2	2	3	3	0	3	2	0	0	2	37	Moderate	(108)
3	1	2	0	1	3	3	3	3	2	2	2	3	2	2	2	2	0	0	2	38	Moderate	(53)
3	2	2	1	2	3	2	3	3	2	3	3	2	3	1	2	2	0	0	3	42	High	(109)
3	2	0	1	2	3	3	3	3	3	3	2	2	2	0	2	1	0	0	3	38	Moderate	(110)
3	1	0	1	1	3	3	2	3	3	2	3	3	3	2	2	2	0	2	3	42	High	(111)
3	2	0	1	2	3	2	2	3	2	2	2	3	2	0	2	2	0	0	2	35	Moderate	(112)
3	2	0	0	2	3	3	2	3	1	3	2	2	2	2	3	1	0	0	2	36	Moderate	(113)
3	3	2	2	2	3	3	3	3	2	2	2	2	2	2	3	2	0	0	1	42	High	(114)
3	3	0	1	1	3	2	1	3	1	1	2	3	2	1	2	1	0	0	2	32	Moderate	(115)
3	0	0	0	0	2	2	2	3	2	2	2	2	0	1	2	2	0	0	3	28	Moderate	(116)
3	2	0	0	0	3	3	3	3	3	3	2	3	3	1	3	2	0	1	0	38	Moderate	(117)
3	2	0	0	0	3	2	1	2	1	2	1	2	0	0	2	1	0	0	0	21	Moderate	(118)
3	3	0	0	0	2	2	1	2	1	3	2	2	0	1	2	1	0	0	0	25	Moderate	(119)
3	2	0	0	0	3	2	2	2	1	2	2	3	2	2	2	2	0	0	2	32	Moderate	(120)
3	0	2	2	2	3	3	2	2	3	3	2	2	2	0	2	0	0	3	3	39	Moderate	(121)
3	3	0	1	0	3	2	2	3	1	2	3	2	2	1	3	2	0	0	3	36	Moderate	(122)
3	1	0	1	1	3	1	1	3	1	2	2	3	1	0	2	1	0	0	2	28	Moderate	(123)
3	3	3	2	1	3	3	3	3	2	2	2	3	3	2	3	2	0	3	3	49	High	(124)
3	2	0	0	0	3	1	1	3	2	3	2	3	3	0	2	1	0	0	3	32	Moderate	(125)
3	1	0	2	1	3	3	3	3	2	2	2	2	3	1	3	2	0	0	2	38	Moderate	(126)
3	2	0	1	2	3	3	1	3	3	2	2	2	3	1	2	1	0	0	0	34	Moderate	(127)
3	3	0	1	0	3	3	3	3	2	2	3	3	2	1	2	1	0	0	2	37	Moderate	(128)
3	3	0	2	2	3	2	3	3	2	2	1	3	2	2	3	2	0	0	2	40	High	(129)
3	1	0	1	2	3	2	1	3	1	2	2	2	0	0	3	2	0	3	2	33	Moderate	(130)

Like mentioned before, there are two types of stroke: ischemic and haemorrhagic. Ischemic stroke results in the obstruction of an artery that impairs blood flow in the brain (10). Hemorrhagic stroke results in the rupture of a weakened cerebral blood vessel and, consequently, blood leakage (hemorrhage) into the brain space (6). This study focuses only on ischemic stroke and all articles involving hemorrhagic stroke were excluded. The information taken from each article regarding cerebral ischemia in rats is shown in table 3.

Table 3 - Outcomes with tMCAO model

Rats Characteristics				**Anesthesia Characteristics**	**Induction Characteristics**		**Infarct volume**	**Parameters studied**	**References**
Strain	**Weight**	**Gender**	**Age**	**Anesthesia**	**Ischemia**	**Reperfusion**			
Sprague-Dawley	…	male	7-9 weeks	isofluorane	90 min	24h	…	BE, BM, IV, NF	(97)
	250-280g		…			3,7,28,33 days	28.84±6.86%	A, BM, CV, HA, IV, NF	(98)
	300-320g		adult			15 days	66.7 ± 2.50%	BM, HA, IV, NF	(99)
	240-270g		6-7 weeks			24h	…	BM, HA, IV, NF	(100)
	250-300g		adult			1, 3, 7 or 14 days	20%	BM, CV, IV, NF	(101)
	275-320g		adult			48h	300-400 mm^3	BE, IV, NF	(102)
	150-200g		…	chloral hydrate		3 days	~70%	A, BM, IV, NF	(103)
	250-330g		8 weeks			24h	23.8±1.4%	A, BM, CI, HA, IV, NF	(104)
	200-230g		3 months			3 days	40%	BM, BW, HA, IV	(105)
	200-220g		adult			24h	30%	A, BM, IV, NF	(106)
	240-300g		adult		120 min	9 or 16 days	50,50%	BM, HA, IV, NF	(107)
	200-240g		…			14 days	40-50%	BM, HA, IV, NF	(108)
	250-300g		adult			3 days	…	A, BM,CV, HA, IV, NF	(53)

	220-240g		adult			14 days	40%	BM, HA, IV, NF	(109)
	280-310g		…			22h	~30%	A, BM, CV, HA, IV, NF	(110)
	250-300g		…			1,3, 7 or 14 days	30%	BM, HA, IV, NF, NI	(111)
	250-280g		…	…		24h	40%	BM, BW, IV, NF	(112)
	280 ± 10g		…	…		24h	24.59 ± 7.17%	HA, IV, NF	(113)
	300-340g		adult	enflurane		1 or 7 days	48.5% ± 2.4%	A, BM, CV, HA, IV, NF	(114)
	…		…	chloral hydrate		1, 3 or 7 days	28.3 ± 4	A, BM, IV, NF	(115)
	250-300g		adult			5 days	145.5 + 10.6 mm^3	BM, IV, HA, NF	(116)
	320-350g		10-12 weeks	isofluorane	60 min	24h	25-30%	A, BM, HA, IV, NF	(117)
	280-300g		…			24h	40-50%	A, IV	(118)
	280-300g		…			24h	~40%	A, BM, IV	(119)
	300-350g		…	inactin		5,5h	15-20 mm^3	BP, HA, HR, IV, NF	(120)
	260-330g		…	isofluorane	120 min or 180 min	1, 35, 60 or 90 days	23.4 ± 1.6 %, 19.6 ± 1.8 %,16.1 ± 1.3 % and 15.7 ± 1.6 %	IV, NF	(121)
Wistar	270-290g	…	8 weeks		90 min	3 or 21 days	20-25%	A, BBB, CV, HA, HR, IV, NF	(122)
	…	male	…		120 min	1 days	100-150 mm^3	IV	(123)
	240-260g		9-10 weeks	chloral hydrate		24h	250-300 mm^3	A, BM, CV, HA, IV, NF	(124)
	…		8 weeks	halothane	60 min	28 days	60%	HA, IV, NF	(125)
	230-280g		adult	chloral hydrate		6h, 12h, 24h	~50%	BM, IV, NF	(126)

	200-300g		3-4 months	isofluorane		24h	40mm^3	BM, IV, HA, NI	(127)
	250-350g		adult	…		24h	212.13±20.2 mm^3	BBB, BE, BM, HA, IV, NF, NI	(128)
	220-270g		8 weeks	chloral hydrate	30 min	24h	40-60 mm^3	A, BM, HA, IV, NF	(129)
…	240-280g	…	…	isofluorane	120 min	24h	26.82%	A, BBB, BM, BW, CV, HA, IV, NF	(130)

Legend: A - Apoptosis; BBB – Blood brain barrier; BE – Brain edema; BM - Biochemical markers (e.g. TNF-α; GFAP; NF-κB; Iba-1; IL-1B; IL-6; IL-10; MCP-1; COX-2; iNOS; NeuN); BP – Blood pressure; CV – cell viability; HA – Histological assay (e.g. inflammation); HR- Heart rate; IV – Infarct volume; NF – Neurological function (sensorimotor, cognitive and/or behavioral); NI – Neuronal injury

Due to the severity of stroke in humans, several ischemic stroke models in rodents have been developed for the disease to be studied, such as, for example, permanent or transient occlusion of the middle cerebral artery and thromboembolic models. (131) In fact, these models are very versatile as they have many associated advantages. (9,132,133) The transient middle cerebral artery occlusion model in rats is the most widely used to induce cerebral ischemia. These MCAO models are appropriate for the development and testing of new therapeutic strategies for the treatment of cerebral ischemia. This is a very versatile technique, since it is not invasive and it is not necessary to perform a craniectomy. (9) It consists in the insertion of a filament into the ICA and its advance it to the origin of the MCA so that it heavily decreases or interrupts the blood flow. Thus, oxygen supply will be reduced in brain tissue, as it occurs in humans, and cerebral ischemia will occur. This concept is associated with several events that irreversibly damage the brain. (9,134)

1. Rats Characteristics

1.1. Strain

This article focused only on studies in which tMCAO was developed in rats. According to the table 3, the strain of rats most often used in preclinical studies to induce tMCAO are Sprague-Dawley (n=25) and Wistar strains (n=9) (one additional study with unspecified strain).

In the experimental practices of cerebral ischemia, a wide range of animal models have been used, including dogs, cats, non-human primates, rabbits, mice and rats. However, the species most used for these tests are rats and mice due to several reasons: 1) their small size, which allows for easy monitoring of physiological parameters; 2) several anatomical and physiological similarities in comparison with humans; 3) its use is relatively cheap compared to larger animals; 4) ethical issues. (9,135)

Wistar and Sprague-Dawley rats have some differences between them that influence their choice in studies of cerebral ischemia. On one hand, Wistar rats have thinner arteries and larger and more concise volumes of cerebral infarction. On the other hand, Sprague-Dawley rats have an atypical MCA branch, which causes smaller infarction volumes and more considerable results variations. (9,133,136)

tMCAO was initially developed by Koizumi, who interestingly described the model in Wistar rats (137), which is the strain with the highest reproducibility of results. The Wistar strain was found to not be the most common one in this review, since Sprague-Dawley rats were used in the most studies and also having showed the best results in terms of

brain neuroprotection (9). Even so, these findings seem to suggest that the authors are probably not using the most appropriate strain to induce cerebral ischemia, given the high dispersion of results regarding brain damage due to the presence of an atypical branch. (133) Thus, the Wistar strain is the most adequate to perform the tMCAO model, since is the one that has the higher reproducibility and the better MCA branch morphology when compared with humans.

1.2. Age and weight

With regard to the age of the animals, the articles analyzed used mostly adult rats (n=21). However, some studies (n=14) haven't referred the age of the animals, despite that, the articles that don't mention the age of the animals, usually refer to the animals' body weight, an aspect that tells us approximately their age / stage of development. Of the 14 articles that didn't mention their ages, 12 described the animals' weight, indicating that the tested rats were all in adulthood. Thus, of the total number of articles under study, 33 of the studies used adult rats, results that match the age group that is at greatest risk of having a cerebral ischemic stroke.

Regarding the body weight of the animals used, through the table 1 it is possible to verify that 31 of the 35 articles reported the weight of the animals used. The body weight of rats in the articles under study varied between 150-350g, which is in line with the weight of an adult rat. It's also worth noting that none of the rats has overweight.

1.3. Gender

The gender is an important factor in stroke since the statistics show differences between men and women in mortality and incidence. Age-adjusted stroke mortality rates for men have a higher mortality rate than women overall, although due to their longevity, more women die of stroke each year than men. Also studies have shown that women have more severe strokes than men, and stroke outcomes of women are less favourable than men. (15)

When analysing the articles, we found that almost all studies (n= 33) were performed in male rats, the other 2 articles didn't mention the gender of the animals utilised. It might look strange to only use male rats when there is such a difference between the male and female gender, even more when the female gender has the poorest stroke outcomes, although this happens because the female gender has a hormonal variability that male gender does not have, which makes standardization and the translation into human more difficult. Also, oestrogen has been shown to have neuroprotective effects, which leads to misunderstanding of results. To avoid this problem, the female animals must be

ovariectomised, making it more difficult and expensive since one more intervention is required.(136) Thus making the male gender preferred to study stroke.

2. Anesthesia characteristics

In this review, the use of different types of anesthetics was verified in animals subjected to MCAO. The most used anesthetics are isoflurane (n = 14) and chloral hydrate (n = 15) accounting to more than 80% of studies, halothane (n = 1), enflurane (n = 1), inactin (n = 1) were the other used anesthetics, although all of them were used only once. In addition, some articles did not mention what anesthesia they provided to the rats (n = 3). The predominance of the use of these anesthetics is in line with what is described in the literature, since these are the agents most commonly used during MCAO.(138)

The use of anesthesia in animals to induce stroke can cause some changes in experimental results, influencing the metabolism, blood pressure and cerebral blood flow, as well as inducing neuroprotective effects, modulating some aspects of the processes after cerebral ischemia.(133)

Some anesthetics such as pentobarbital induce neuroprotective effects against cerebral ischemia, reducing infarct volume and cell death.(139) This type of anesthetics are not suited to study ischemic stroke, since they affect the results of studies.

The choice of anesthesia is very relative, since its choice depends on what is intended with the study, the budget and available conditions. But considering the results obtained in this review article, isoflurane and chloral hydrate are the most used anesthetic.

An anesthetic alternative that is not mentioned in this study is the ketamine + xylazine cocktail which is widely used in rodents surgical procedures. It is a safe anesthetic and is easily administered and does not require specialized equipment to be administered.(140) Also, ketamine does not have neuroprotective effects, and has absence of cardiac and respiratory depression.(141,142)

Recently an ethical issue has been raised regarding the use of chloral hydrate, due to its mutagenic and carcinogenic effects in animals, so the attention has turned to finding out an alternative agent able to meet not only potency, safety, and analgesic efficacy, but also reduced neuroprotective effect for stroke research. Isoflurane is a volatile halogenated gas that is currently used in stroke and has presented itself as the alternative to chloral hydrate with additional advantages, such as: it is easy to administer and to titrate, has a rapid onset and recovery, produces adequate and reproducible anesthesia depth, and causes minimal cardiac depression. Furthermore, this anesthetic agent displays a slight analgesic effect, fulfilling an important ethical criterion for animal

experimentation as referred to in the guidelines of ethics committees. Also, MCAO in animals asleep with either chloral hydrate or isoflurane did not present significant difference in terms of infarct volume and mortality.(143) Due to the factors mentioned above and the fact that this agent has a rapid effect in anesthetizing animals, isoflurane is the most preferred viable option.(144)

3. Induction characteristics

3.1. Ischemia

In this study the time which animals were submitted to ischemia in the MCAO model were assessed. The most common time of ischemia performed in MCAO was 120 min

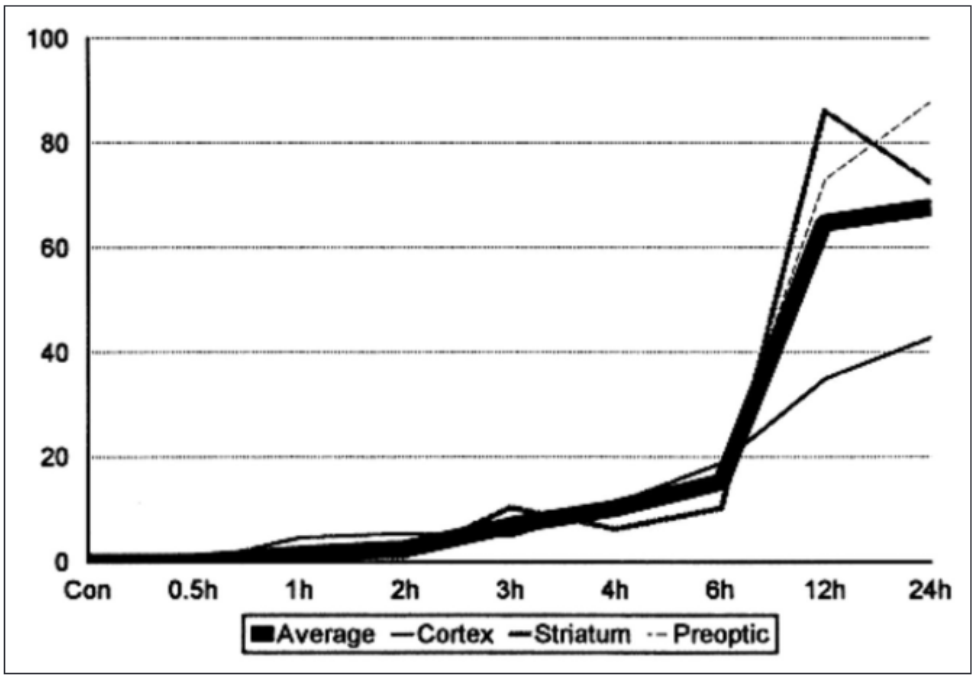

Figure 8 - Effects of MCAO on cell death in function of time and location (145)

(n=11) and 90 minutes (n=11) followed by 60 minutes (n=10). Also, one article used 2 timepoints, 120 min and 180 min and other used 30 min. The predominance of these times (60 minutes, 90 minutes, and 120 minutes) of ischemia is in accordance with the literature description. (9) Depending of the time of ischemia performed, the brain area affected (fig.8), which involves the striatum and the overlaying frontoparietal and temporal cortices, some of the occipital cortex and the thalamus and hypothalamus, will vary, increasing the area affected with the extension of ischemic time (fig.9).(145)

The infarction after MCAO follows a kind of stereotypical progression from early ischemia in the striatum to delayed infarction in the cortex overlaying the striatum.(146) Thus, there is no preferable time for ischemia, although what needs to be considered is the region of the brain that is the subject of the study, since the timepoint used to ischemia affects the area damaged and consequently the brain regions.

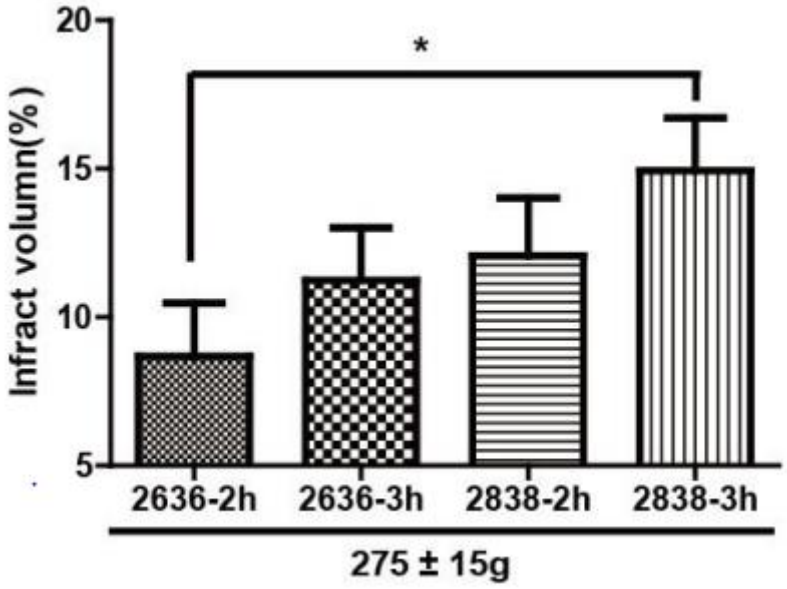

Figure 9 - Example of Infarct volume increasing with time and the variation of infarct volume depending on the filament utilized. 2636 and 2838 correspond to filaments. *$P < 0.05$ (120)

3.2. Reperfusion

In this study the time of reperfusion before the sacrifice of the animals was assessed. As expected, all articles under analysis (n=35) induced reperfusion in animals and a wide range of times was used in the articles, oscillating from 6 hours to 90 days. In fact, the study of reperfusion is very variable among the selected studies, because many authors chose to study only one timepoint (n=26), however, the remaining 9 studies tested reperfusion between 2 and 4 timepoints. Furthermore, since reperfusion is a consequence of the thrombolytic therapy, its study is of great importance and better mimics the human strokes. (94) Reperfusion processes contribute to exacerbation of the ischemic injury with an significant increase on infarct volume within 6-24h following the start of reperfusion when compared to permanent occlusion, confirming that reperfusion contributes to exacerbation of brain damage.(147) Spontaneous reperfusion also occurs commonly after stroke, in about 50-70% of stroke patients. (148,149) Hence, reperfusion

has a significant role on ischemia/reperfusion injury (fig.10), so it is extremely important when studying stroke to choose a model that allows to perform and study reperfusion.

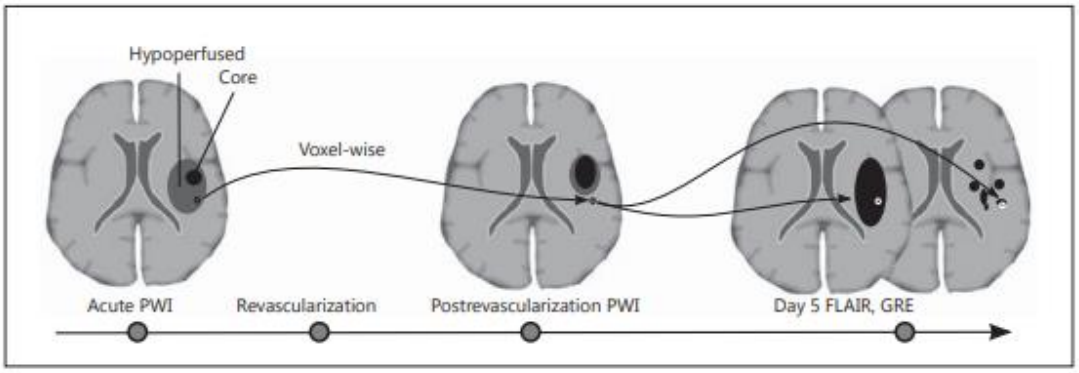

Figure 10 - Ischemia/reperfusion injury progress after re-establishing blood flow (147)

4. Infarct volume

Infarct volume was also assessed in this study with all studies evaluated (n=35) evaluating results of the cerebral infarction volume. The results oscillated between 20-66% of infarct volume, being 40-50% the most common infarct volume. Some studies presented the infarct volume in mm^3, where the results fluctuated between 15-400 mm^3. The infarct volume, unlike of what is stated in the literature, did not increase with the extension of ischemic time.(145) This might be due to the characteristics of the filament, crucial to have a consistent MCAO model, with a slight variation of the filament capable of affecting the results obtained (Figure 9).(150) For instance, the suture insertion needs to be taken into account, because a slight alteration in the distance of the insertion, such as 4 mm, alters the infarct volume.(151) The method utilized to measure the infarct volume is also important, since each method has an implied variation. The methods identified in the articles reviewed were the TTC staining method (n=29), MRI (n=4) and cresyl violet staining (n=2). The TTC method was the most utilized and this is in accordance with what is stated in the literature. This method can be used in fresh brain sections and a sharp contrast between infarcted and non-infarcted area can be observed as early as 3 hours in rat brains. This method is widely accepted in the literature, being relatively simple to conduct, with low cost and high reproducibility, making this method attractive and reliable.(152,153) The cresyl violet method is also a viable method to measure the infarct volume and has a high degree of correlation of infarct volume with TTC method. Although this method requires tissue fixation before the application of the stain and it is not specific for neuron cells, it instead stains the nissl substance that is present in high quantities in the neurons, giving the stained appearance.(153) The MRI

method is non-invasive, and rats are anesthetized before the procedures. This method can be applied multiple times to evaluate the evolution of ischemic lesion, although it requires complex equipment with associated high costs. Summing everything it seems that depending on the study objective the method to measure the infarct volume may vary between MRI and histopathological methods. Also, between histopathological methods, the TTC stain is the gold standard, as it as a lot of advantages when compared to the others. The MRI method is more suited to a long-term study as it does not require the rat to be sacrificed in order to measure the infarct area, and consequently allows to make multiple measurements in a single rat, enabling measurements at several timepoints within the same experiment.

5. Parameters studied

5.1. Biochemical markers

The biochemical markers concentration is measured in blood or tissue samples, reflecting the severity or presence of pathology.(154) They are a characteristic which is objectively measured and evaluated as an indicator of biological, pathogenic, or pharmacologic responses to a therapeutic intervention.(155) All the articles reviewed (n=35) evaluated biomarkers to determine the severity of stroke.

The biomarkers evaluated trough the articles analysed were interleukins, iNOS, COX-2, TNF-α, NF-Kb, MPO, ROS, etc. The inflammatory factors have a major role in the pathogenesis and progression of cerebral lesion of stroke. During a stroke event, microglia, in response to neurons and glia stimulation, releases pro-inflammatory cytokines and/or cytotoxic factors such as, NO, TNF- α, IL-1β and ROS, which are associated to neurons and the blood brain barrier (BBB) damage, increasing the BBB permeability. (30,31) These biomarkers should be measured in the brain because their augmentation is associated to stroke pathophysiology and severity. On the other side, IL-4, IL-10 or IL-13 are associated with anti-inflammatory and neuroprotective effects. (58,60) These biomarkers are many times overlooked, although they also play a major role in ischemia, since they allow the enlightenment of the pathophysiology of ischemia and the so called double edge sword of microglia with 2 phenotypes (inflammatory and anti-inflammatory).

According to our data, most of the biomarkers used to study tMCAO are inflammatory and have the objective of proving the reliability of the tMCAO model and to study the inflammation that results from ischemia and reperfusion. This is also explained by the mechanism that brain damage leads to post-ischemia, where the core is the region damaged during ischemia and the penumbra region being salvageable with a damage

that is mainly characterized by inflammatory processes. This region is the one that scientists have been mainly focusing their attention, sepcifically on how to prevent it by decreasing the extension of the lesion, as it is believed that, by inhibiting the inflammatory processes and modulating brain cells response such as microglia, that will stop the damage that occurs post ischemia, the so called reperfusion injury. Thus, the knowledge and understanding of which players and how they act during ischemia, will lead us to new therapeutic approaches and better outcomes to patients suffering an ischemic event.

5.2. Histological evaluation

Histological evaluation of the brain samples, removed at the end of the experiment, play an important role in the study of stroke in these experimental animal models. Depending on the methods used, the brains may or may not be fixed.(152) The histology in the MCAO models can be used to perform multiple assays, such as the evaluation of the brain infarct area/volume, the brain edema, brain water and the brain cells. In this study all articles presented histological evaluation (n = 35). The infarct volume presented itself as the most important parameter as it was the most studied (n = 35), and provided the possibility for comparison among studies. Brain edema (n = 3) and brain water were also evaluated, although by only 3 studies. NeuN, GFAP and iba1 were biomarkers evaluated through the studies, being related to neurons, reactive astrocytes and microglia respectively and were evaluated through immunohistochemistry methods. NeuN is associated to pathological changes in existing neuronal populations, namely during stroke, since in this process the NeuN immunoreactivity is usually diminished or disappears in ischemic area due to neuronal damage or death.(156) This makes NeuN an important biomarker to assess the integrity and the severity of the damage in the ischemic area. GFAP is a marker of astroglial injury that is an attractive candidate to mark brain injury screening due to his upregulation during traumatic brain injuries.(157) The astrocytes during stroke become reactive which corresponds to the inflammatory peak and they are mainly characterized by the high-level expression of GFAP, making it an excellent biomarker to assess the inflammation and its severity during and post stroke.(36,47,48) The iba1 is a protein expressed in all microglia, which is involved in the membrane ruffling of microglia. The membrane ruffling is essential to microglia morphological changes from quiescent ramified microglia to activated amoeboid microglia, causing the iba1 to be overexpressed during this process, making it a viable biomarker to detect the alterations during an ischemic event, where the microglia is activated within minutes by NF-κB triggering: phenotypic changes such as morphological (amoeboid shape, like systemic macrophages), proliferation, migration, phagocytosis,

and secretion of inflammatory mediators.(8,50,53,55,56,158) These parameters allow the understanding of the injury severity caused by ischemic stroke and the cell behaviour during this event.

5.3. Behaviour evaluation

Usually, the parameters evaluated in this animal model are behaviour tests. The behaviour tests aim to evaluate the sensorimotor impairment and recovery, cognition and mood in stroke animals.(159) These parameters were evaluated in almost all studies analysed (n = 30) as a reflection of its importance. These studies are indispensable as they allow to comprehend the brain plasticity and help the developing of therapies. The sensorimotor function is scored with the help of tests such as rotarod, cylinder test, corner test. It's expected that stroke rats are impaired and have superior scores of impairment when compared to sham groups.(160,161) Neurological functions are characterized by a modified neurological severity score that measures the reflexes to multiple stimuli, balance and simple motor functions. In these tests it is also important to evaluate the sensorimotor impairment in stroke animals when compared to the expected regular response in sham animals.(159) Cognitive tests can also be applied with the Morris water-maze being one of the most popular ones where the cognitive function is evaluated. In these tests the latency time to reach the platform of the maze is measured, and multiple trials are performed in different regions of the maze. It is expected that the time spent in maze test to be higher in stroke animals compared to sham animals.(159,162) All these expected results are consistent with the damage post-stroke of tMCAO model.

Chapter VIII: Conclusion

Stroke animal models have been evolving over the last four decades with the objective of identifying the mechanisms that underlie cerebral ischemia and therefore develop new therapeutic approaches. The animal models are an indispensable tool in stroke research, since *in vitro* models are not fitted to mimic some structures/processes such as the vasculature and reperfusion which are crucial to study ischemic stroke, physical, molecular, genetic and biochemical investigations require invasive access to brain tissue, the evaluation of pharmacokinetic and pharmacodynamic parameters of drugs cannot be accessed on i*n vitro* models, and toxicological studies also require animal models. Despite the evolution in the stroke animal models, the search for new therapeutic agents has not been successful, and so we still only have plasminogen activators as the only pharmacological therapy approved by EMA to treat stroke.(9,76) With the evolution of neurological surgeries some techniques that have been developed such as the intra-arterial procedures were adapted to treat the ischemic stroke, this technique allows to administrate the plasminogen activators directly at the blockage or mechanically disrupt the clot and retrieve it, although the better outcomes after an ischemic stroke still are achieved with the early IV administration of plasminogen activators.(72) The failure on translation to clinical trials has been justified in many cases by the lack of standardization of the ischemia-inducing method and to the parameters chosen to evaluate the results. To validate and make the translation into clinical practice, the clinical trials design will need to be robust. The stroke translational failures comprehend neuroprotective agents, stem cells and immunological treatments. Clinical trials for ischemic stroke have failed because they are frequently based on weak preclinical evidence or inappropriate models.(163) Thus, it is really important to standardize the preclinical models of stroke and choose the most adequate to the intended study in order to properly mimic the human stroke and acquire strong evidences. This will potentiate the chances of successful translation to clinical trials.

The clinical trials have also missed due to the lack of crosstalk between the preclinical side of stroke and the clinical side. What happens is that the investigator is rarely familiarized with clinical reality and clinical needs of stroke and rarely translates clinical advances to his benchside.(164) To avoid this problem, both sides should stay permanently in contact during the preclinical and the clinical trials. These would help the investigators to take into account the clinical reality.

The heterogeneity of types of clinical stroke, in terms of size, location, time-windows, degrees of reperfusion or collaterals, comorbidities, and the presence or not of salvageable penumbra, its one of the main reasons for translational failure.(165) Also,

the time points that are tested in rats are frequently a lot different from what is done in the clinical practice. The rats have their reperfusion at an exact timepoint while human's reperfusion has a huge heterogeneity, which causes differences to the severity of stroke and the timepoints on the administration of treatment, while rats receive it at the beginning of ischemia, or at early stages, patients receive it at much later stages (time to call ambulance, diagnosis, exclude bleeding). At this point the core damage is bigger in comparison with rats. This means that the patients in clinical trials that receive the treatment are out of the effective time window that was teste in the preclinical studies.(166) Improvements in the preclinical trials should be performed in order to match the reality of clinical trials. The timepoints at which the therapies are administered should be similar what is seen in the clinical trials, in order to simulate the conditions that occur during clinical trials. By doing this, possibly the translation to clinical trials would have less disparity and the results would be more promising.

The differences between rats and humans must also be taken into account to the failure of clinical trials, since the rats have different pharmacokinetics and pharmacodynamic profiles which lead to different responses to the same pharmacological therapy.(167) The brains of rodents and humans are also different in the rate of white matter to grey matter. The brains of rodents such as mouses have 90% of grey matter while human brains have 50% of grey matter, this means that the portion of grey matter and white matter affected by ischemia are different. Furthermore, despite their similarities, histologically the astrocytes, neurons and microglia have anatomical, functional, morphological and genetic differences.(168–170)

These differences must be considered when translating the studies to clinical trials, in order to prevent the bad outcomes of clinical trials. New methods to make a comparison between these differences should be developed.

The translation to the clinical trials have also failed because of methodological problems. The internal validity, low internal validity means low chances for the results to represent the truth and it tends to positively bias the results and increases the odds of failure in the translation to humans.(163) So internal validity should be robust and not biased, in order to dimmish the odds of results not representing the truth and consequently diminish the chance of failing the translation to humans. The pre-testing subjects, where the results are biased because the subjects of the study are adapted to the tests.(171) Detection bias where the investigators are non-blinded leading to positively biased results.(172) Selection bias, where the randomization is not adequate and is influenced.(173) Performance bias where each group receives different animal care, group sizes per cage, housing conditions, diet and cage location.(172) All of this will influence the animals and the results of the study, so every animal must have the same conditions,

the randomization should be performed correctly and by a blindsided investigator and the tests should also be performed by a blindsided investigator, to reduce the variability of the study and improve the impact of its outcomes.

Statistical methods such as the regression to the mean which bias to false-positives results since the extreme results are removed in favor of positive results. Underpowered studies, where the groups are small and cannot lead to statistically significant results, leading to overestimation of the results. The use of inadequate statistical tests, leading to false-positive results. (174–176)

Lack of validation, replication and confirmatory studies, after obtaining results they should be replicated and validated by an independent well design study.(172) If this does not happen, the study validation will be low and the chances of getting a success translation are minor. Looking to our study, ischemic stroke induced by MCAO model is usually performed in rats and a microfilament is utilized to achieve the ischemia. This model has avoided craniectomy and is probably the closest to human stroke. The rats are the favourite animal due to his characteristics (DNA similarity, cost, easy handling, ethical perspective, and the cerebral vasculature and physiology similarity to humans) which allow to conduct reproducible studies. The intraluminal filament coated with silicone is the optimal method as it is the one which lowers the infarct size variability and avoids the risk of hemorrhage.(136)

In this study we conclude that the MCAO model can be achieved by using adult male Wistar rats or adult male Sprague-Dawley rats with weight between 150-350g and with the insertion of an intraluminal filament. The anesthesia that should be used is isoflurane, the ketamine + xylazine cocktail can also be used as an alternative.

The ischemia time should be between 60 to 120 minutes with a reperfusion time between 24h and 72h. The most used parameters to characterize this model were the inflammatory factors (iNOS, COX-2, NF-κB, TNF-α, and interleukins), histological evaluation (infarct volume, brain edema, GFAP, Iba1, and NeuN) and the behavioural tests (neurological functions, sensorimotor function and cognitive tests). The infarct volume expected to be achieved is 40-50%. Our group will use this data to implement a MCAO model which is validated and reproducible with the objective of comprehend the pathophysiological mechanisms of ischemic stroke and test new therapeutic targets.

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Annex 1 – Construction of search string

"Rats"[Mesh] OR Rats[tiab] OR Rat*
Results: 1.800.000 articles

AND

"Animal Experimentation"[Mesh] OR "Animal Experimentation"[tiab] OR "Animal Experimentation" OR "Drug Evaluation, Preclinical"[Mesh] OR "Preclinical studies"[tiab] OR "Preclinical studies" OR "Non-clinical studies"[tiab] OR "Non-clinical studies" OR “animal model”[tiab] OR “animal model”

Result: 365.000 articles

AND

"Brain Ischemia"[Mesh] OR "Brain Ischemia"[tiab] OR "Brain Ischemia" OR "Ischemic Stroke"[Mesh] OR "Ischemic Stroke"[tiab] OR "Ischemic Stroke" OR “ischemic brain injury”[tiab] OR “ischemic brain injury” OR "cerebral ischemic injury"[tiab] OR "cerebral ischemic injury" OR neuroinflammation[tiab] OR neuroinflammation

Result: 163.000 articles

AND

"Infarction, Middle Cerebral Artery"[Mesh] OR "Middle Cerebral Artery"[tiab] OR "Middle Cerebral Artery" OR MCAO[tiab] OR MCAO OR "middle cerebral artery occlusion"[tiab] OR "middle cerebral artery occlusion"

Result: 35.000 articles

((("Rats"[Mesh] OR Rats[tiab] OR Rat*) AND ("Animal Experimentation"[Mesh] OR "Animal Experimentation"[tiab] OR "Animal Experimentation" OR "Drug Evaluation, Preclinical"[Mesh] OR "Preclinical studies"[tiab] OR "Preclinical studies" OR "Non-clinical studies"[tiab] OR "Non-clinical studies" OR "animal model"[tiab] OR "animal model")) AND ("Brain Ischemia"[Mesh] OR "Brain Ischemia"[tiab] OR "Brain Ischemia" OR "Ischemic Stroke"[Mesh] OR "Ischemic Stroke"[tiab] OR "Ischemic Stroke" OR "ischemic brain injury"[tiab] OR "ischemic brain injury" OR "cerebral ischemic injury"[tiab] OR "cerebral ischemic injury" OR neuroinflammation[tiab] OR neuroinflammation)) AND ("Infarction, Middle Cerebral Artery"[Mesh] OR "Middle Cerebral Artery"[tiab] OR "Middle Cerebral Artery" OR MCAO[tiab] OR MCAO OR "middle cerebral artery occlusion"[tiab] OR "middle cerebral artery occlusion")

Result: 452 articles