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The effect of age, sex and strains on the performance and outcome in animal models of stroke

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ABSTRACT

Stroke is one of the leading causes of death worldwide, and the majority of cerebral stroke is caused by occlusion of cerebral circulation, which eventually leads to brain infarction. Although stroke occurs mainly in the aged population, most animal models for experimental stroke *in vivo* almost universally rely on young-adult rodents for the evaluation of neuropathological, neurological, or behavioral outcomes after stroke due to their greater availability, lower cost, and fewer health problems. However, it is well established that aged animals differ from young animals in terms of physiology, neurochemistry, and behavior. Stroke-induced changes are more pronounced with advancing age. Therefore, the overlooked role of age in animal models of stroke could have an impact on data quality and hinder the translation of rodent models to humans. In addition to aging, other factors also influence functional performance after ischemic stroke. In this article, we summarize the differences between young and aged animals, the impact of age, sex and animal strains on performance and outcome in animal models of stroke and emphasize age as a key factor in preclinical stroke studies.

1. Introduction

Stroke is one of the most common diseases in the older population. It remains the 5th leading cause of death and the leading cause of disability in the United States. Ischemic stroke accounts for about 80 percent of all stroke (Rosamond et al., 2008). Systemic thrombolysis with intravenous tissue plasminogen activator (tPA) remains the only FDA proven drug to improve clinical outcome of patients with acute ischemic stroke (Adams et al., 2007; Brott and Bogousslavsky, 2000). Due to an increased risk of hemorrhage beyond 3–5 h post stroke, only a small population of stroke patients (1–2%) can benefit from tPA. Several ischemic stroke models have been developed in a variety of species, such as rodents, canines, rabbits, cats, as well as non-human primates to understand the physiopathology and outcome of ischemic stroke in humans (Alonso de Lecinana et al., 2001; Ashwal and Pearce, 2001; Megyesi et al., 2000; Traystman, 2003). However, most animal models for experimental stroke *in vivo* are almost universally young-adult rodents used for the evaluation of neuropathological, neurological, or behavioral outcomes after stroke. Younger animals are used due to their greater availability, lower cost, and fewer health problems (Harris and Rumbaut, 2001). Ischemic stroke is a highly complex and heterogenous disorder, and its incidence, mortality, and morbidity have been increasing, especially in the aging human population. The persistent failure of human trials targeted at neuroprotective agents (Kidwell et al., 2001), which are effective in animal models of stroke, further indicates that the discrepancy between animal models and human diseases could have important clinical applications. One of the potential reasons is that ischemic stroke mainly occurs in the aged population but almost all experimental stroke research is focused on young-adult animals (Suenaga et al., 2015).

Age is a crucial factor in experimental design, which is poorly reported and overlooked in an experimental stroke study. It is well-known that age is a major risk factor of ischemic stroke, and the elderly are more inclined to suffer from stroke and have more serious outcomes (M. Knoflach et al., 2012; Sandu et al., 2017). More than half of all strokes take place in people over 75 years of age, and one-third in people over 85 years of age (Roger et al., 2012). Importantly, there are many significant neurophysiological differences between aged and young animals. It has been confirmed that neurological impairments of aged animals increase, while the recovery capacity is lower than younger animals after stroke (Buga et al., 2013). Therefore, the overlooked role of age in animal models of stroke could impact data quality and hinder

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the translation of rodent models to humans. In this article, we summarize the differences between young and aged animals, the impact of age on outcome in animal models of stroke, and emphasize age as a key factor in preclinical stroke studies. Increased scientific rigor in the use of aged rodents for stroke models may increase the translation of rodent models to humans in stroke (Herson and Hurn, 2010; Rosenzweig and Carmichael, 2013; Sommer, 2017).

2. Comparison of rodents' and human lifespan

Biomedical researchers who use adult and aged animals as experimental tools often face the question: “At what age are laboratory rats and mice considered adult or aged”? Traditionally, biomedical scientists consider sexual maturity the hallmark of adulthood. Sexual maturity in rats takes 49–60 days (Lewis et al., 2002), but social maturity is reached several months later at about 5–6 months of age (Adams and Boice, 1983). Most researchers consider 60 days for a rat to be an adult. Controversially, recent studies show that rat long bone metaphysis closed only at 7–8 months after birth (Baker et al., 1979). The newest opinion is that closure of long bone metaphysis is a more accurate sign of maturity in rats. Female rats enter menopause between 15 and 18 months of age.

Mice display a different timeline for maturity. In female mice, time to reach sexual maturity is about 30–35 days from birth (Taft, 2007). In male mice, it's about 56 days (White, 2007). Mice that are past development stages but are not yet affected by senescence are considered mature adults. This typically occurs at 3–6 months of age. Notably, mostly researchers consider mice to be adults at 3 months, which is a standard broadly used in animal experiments (Flurkey et al., 2007). In middle aged mice (10–15 months of age), some biomarkers of aging and senescent changes can be observed. Aged mice should be at least 18 months old because every aging biomarker can be detected from the 18th-month timepoint. The upper limit of aged mice is considered to be 24 months old. After 24 months, senescence may cause genomic organ failure (Flurkey et al., 2007).

“What is the relationship between age in mice and rats and age in humans?” is a significant question raised by many biomedical scientists. Compared to a human's lifespan, a rodent's lifespan is like a shooting star, fast and transient.

Rats in a laboratory setting live 2–3.5 years (average 3 years) (Pass and Freeth, 1993), and only 5% live beyond 3 years of age. Current life expectancy in humans is 80 years (O'Connor and Graham, 2018). Therefore, we can translate a rat's age into “human years” based on the average life expectancy of humans and rats. Such that two rat weeks (13.8 rat days) are approximately equivalent to one human year (Pass and Freeth, 1993). Like all mammals, rats also have different developmental stages in their lives, including infancy, puberty, adulthood, and old age (Sengupta, 2013). The duration of these stages is different between species, such as between rats and humans. For example, the average weaning time for laboratory rats is about 3 weeks (Baker et al., 1979), while it is about 6 months in humans. In this weaning phase, a human year is roughly equivalent to 42.4 rat days. During puberty, one human year roughly equals 3.3 rat days (Quinn, 2005). Compared to humans, 10.5 rat days equals to a human year in the adolescent phase (O'Connor and Graham, 2018; Quinn, 2005). In the adult phase, 11.8 rat days equal one human year (O'Connor and Graham, 2018; Quinn, 2005). In old age, 17.1 rat days equals a human year.

The life span of laboratory mice is nearly 24 months (Dutta and Sengupta, 2016; Wilkinson et al., 2012). Based on the average life human life expectancy of 80 years, it can be deduced that one human year is almost equivalent to 9 mouse days when correlating their entire lifespan (Flurkey et al., 2007). Similarly, one human year equals 56.77, 3.65, 2.6, 8.82 and 2.069 mice days in weaning, pre-pubertal, adult and senescence phases, respectively (Chandra et al., 2013; Dutta and Sengupta, 2016; Jackson and Abbott, 1999; Sharma and Bhattacharya, 2010; Taft et al., 2006). Table 1 shows the comparability of human, rat, and mice years.

Table 1
Comparing human and rodents' years.

Rat and mouse age in months	Rat age in human years	Mouse age in human years
1 months	9 years	14 years
3 months	15 years	23 years
6 months	18 years	34 years
9 months	24 years	46 years
12 months	30 years	58 years
24 months	60 years	70 years
30 months	75 years	85 years
36 months	90 years	100 years

3. Physiological differences between young and old animals at baseline

Aging by definition is “a functional decline that affects all living organisms in a time-dependent fashion.” Some specific cellular and molecular hallmarks include genomic instability, epigenetic modifications, loss of protein homeostasis, mitochondrial dysfunction, and cellular senescence (Lopez-Otin et al., 2013).

As we age, numerous physiological and molecular changes occur in normal functional cells and tissues (Table 2). Similar to other aging organs, the brain is also affected by these age-related loss-of-function changes. Several well-documented phenotypic changes are observed as our brain ages. The Baltimore longitudinal study on aging revealed a significant decrease in brain volume in aged individuals (Driscoll et al., 2009). Structural changes are also found in brain areas, such as the caudate nucleus, cerebellum, hippocampus, and prefrontal cortex (Raz et al., 2005; Salat et al., 2004). There is an obvious reduction in frontal areas of the brain, which establishes an anterior-posterior gradient as white matter integrity changes while we age (Head et al., 2004). This also occurs with grey matter of prefrontal, parietal, and temporal cortices (Courchesne et al., 2000; Ge et al., 2002; Good et al., 2001). Metabolic changes associated with aging also occur. For example, glucose metabolism decreases in the prefrontal, anterior cingulate, ventral and dorsolateral cortex, medial prefrontal and pre-central areas (Hsieh et al., 2012; Kalpouzos et al., 2009; Zuendorf et al., 2003). Age-related cellular changes of the brain, such as declined synaptic density and structural integrity, influences cognitive function (Gunning-Dixon and Raz, 2003; Rodrigue and Raz, 2004; Rosen et al., 2003).

The “free-radical theory of aging” (Harman, 1956) states that organisms age due to cellular accumulation of free radical damage over time. When the production of reactive oxygen species (ROS) is prolonged, the endogenous reserves of antioxidants become insufficient, leading to cellular damage. This suggests that antioxidants play a critical role in an organism's lifespan. For example, a decreased level of antioxidant moieties in plasma results in the damage of age-dependent memory (Berr et al., 2000; Perkins et al., 1999; Perrig et al., 1997). Glutathione, which is the most abundant endogenous antioxidant, is also reduced in several regions of aged rodent brains, including the hippocampus (Balu et al., 2005; Calabrese et al., 2004; Zhu et al., 2006), compared with the young-adult brain. On the other hand, antioxidant enzyme activity such as glutathione peroxidase (Rodrigues Siqueira et al., 2005), catalase (Tian et al., 1998) and Mn- and CuZn-superoxide dismutase (SOD) are reduced and oxidative stress is increased in aged brains (Gupta et al., 1991; Navarro et al., 2004). Oxidative stress has an effect on many organ functions, especially the brain. ROS are main products of oxidative phosphorylation in the mitochondrial inner membrane (Balaban et al., 2005). Mitochondrial integrity declines as we age resulting from inefficiencies in the electron transport chain, reducing generation of energy rich molecules (ATP) and favoring the formation of reactive oxidants (Shigenaga et al.,

Table 2
Physiological differences between the young and aged brain at baseline.

Aspect	Difference in the aged brain *vs.* the young	References
Brian volume	Decrease	(Driscoll et al., 2009)
Structural changes such as cortical areas	Decrease	(Courchesne et al., 2000; Ge et al., 2002; Good et al., 2001; Head et al., 2004; Raz et al., 2005; Salat et al., 2004)
Metabolic changes e.g., glucose metabolism	Decrease	(Hsieh et al., 2012; Kalpouzos et al., 2009; Zuendorf et al., 2003)
Cellular changes such as synaptic density and structural integrity	Decrease	(Gunning-Dixon and Raz, 2003; Rodrigue and Raz, 2004; Rosen et al., 2003)
Level of antioxidant moieties such as glutathione	Decrease	(Balu et al., 2005; Berr et al., 2000; Calabrese et al., 2004; Perkins et al., 1999; Perrig et al., 1997; Zhu et al., 2006)
Antioxidant enzyme activity	Decrease	(Gupta et al., 1991; Navarro et al., 2004; Rodrigues Siqueira et al., 2005; Tian et al., 1998)
Oxidative stress	Increase	(Balaban et al., 2005)
Mitochondrial integrity and ATP	Decrease	(Shigenaga et al., 1994)
Mutation rate of mtDNA	Increase	(Richter et al., 1988)
Gene expression related to antioxidation functions	Decrease	(Erraji-Benchekroun et al., 2005; Fraser et al., 2005; Lu et al., 2004)
Single base modifications of DNA	Increase	(Lu et al., 2004)
Global methylation related to changes in DNA methylase activity	Decrease	(Gravina and Vijg, 2010; Vanyushin et al., 1973)
Level of inflammatory cytokines and activation of inflammation	Increase	(de Magalhaes et al., 2009; Helenius et al., 1996; Lee et al., 2000; Singh and Newman, 2011)

ATP: Adenosine tri-phosphate; mtDNA: mitochondrial DNA.

1994). The mitochondrial DNA (mtDNA) is usually the first to be damaged by oxidative stress due to the proximity to ROS that are being generated and also because it lacks histones for protection. Therefore, mtDNA has a higher mutation rate than nuclear DNA (Richter et al., 1988).

There are significant changes in genome-wide gene expression in the brain at a molecular level as we age. Genes involved in learning and memory, calcium signaling, vesicle-mediated protein transport, and mitochondrial function are downregulated, while genes associated with antioxidant functions, such as DNA repair, and the inflammatory response are upregulated in the aged brain compared with the young brain (Erraji-Benchekroun et al., 2005; Fraser et al., 2005; Lu et al., 2004).

Mutations in DNA damage or DNA repair genes may be responsible for some of the changes in gene expression. Single base modifications of DNA are a major part of oxidative DNA damage observed in the aged brain, which are located at the promoter region of most downregulated genes during aging (Lu et al., 2004). In mice and humans, some mutations in DNA repair genes that induce accelerated aging phenotypes are featured with biological degeneration and neurodegeneration, often known as progeria syndrome (Lombard et al., 2005).

There is increasing evidence that epigenetic effects, such as histone modification and DNA methylation, are connected to age-related changes in gene expression (Gravina and Vijg, 2010). It is well-known that aging tissues, including the brain, heart, and spleen, show a reduction in global methylation related to changes in DNA methylase activity (Vanyushin et al., 1973). These changes are the basis of a new epigenetic clock that measures the age of tissue from DNA methylation (Horvath, 2013). Moreover, alterations in normal chromatin structure result from changes in DNA methylation and posttranslational histone modifications and induce global heterochromatin loss with aging, which has been suggested as the main reason of the deleterious processes observed in aged tissues (Tsurumi and Li, 2012).

Another main change as brain the ages is the increasing inflammatory environment, which has been referred to as “inflamm-aging”. Inflamm-aging is a mild chronic inflammation on the aging organism, which would reduce neuronal dendritic and axonal branching, synapse density, dendritic spines, and presynaptic markers and reduces the aging organism's efficient response against stressor stimuli (Yankner et al., 2008). Such a phenomenon is encountered throughout the whole process of life by living organisms experiencing constant pressure (Franceschi et al., 2000). Chronic mild inflammation can also cause age-related immune changes, namely immune aging (Larbi et al., 2008). Studies have shown that age-related gene expression changes associated with inflammation and immune response (de Magalhaes et al., 2009; Lee et al., 2000) can increase the plasma levels of inflammatory cytokines (Singh and Newman, 2011) and augment the activation of inflammatory pathways (Helenius et al., 1996).

Age-related physiological changes may have a more significant impact on experimental variables and the quality of data obtained from rodent models, including animal models of stroke (Jackson et al., 2017).

4. Animals used for stroke models

Rodents, such as mice, rats and gerbils, are the most commonly used animals in stroke studies. Larger animals, such as cats, dogs, and non-human primates, have also been frequently used in the stroke research.

4.1. Rodents

Rodents, such as mice and rats, are the most widely used animals in stroke research. The advantages of rodent stroke models includes: 1) similar cerebral vasculature and physiology to humans (Yamori et al., 1976); 2) low cost, abundant source, and easy transport and operation (Mhairi Macrae, 1992; Yan et al., 2015); 3) moderate size, which is easy to monitor physiologic parameters and continue subsequent examination of brain specimens after stroke operation (Takizawa et al., 1991); 4) the application of transgenic technology, which allows researchers to control genes to create relatively homogenous strains (Gob et al., 2015; Kraft et al., 2013); 5) a variety of neurosensory and motor behavior tests that have been standardized for rodents, which facilitate the assessment of functional outcomes after experimental stroke; 6) compared to larger animals, there are fewer animal welfare concerns regarding rodents in research (Kleindorfer et al., 2004).

Most stroke researchers use Sprague-Dawley (SD) rats to generate a range of stroke models. However, extreme variability in cerebral infarction volume is encountered when SD rats are used. The major reason is their middle cerebral arterial (MCA) anatomy varies widely from one animal to the other (Fox et al., 1993; Spratt et al., 2006). Wistar rats and F344 rats are also widely used strains for stroke study. Morphological analysis of the major cerebral arteries showed a significantly higher number of proximal side branches of the long proximal MCA segment in Wistar rats than in F344 rats (Fox et al., 1993;

Spratt et al., 2006), which may cause extreme variability in cerebral infarction volume in Wistar rats compared with F344 rats. Owing to a lower occurrence of vascular variability, many experts recommend that the Wistar-Kyoto rat tend to be the better choice for rat ischemic stroke models (Howells et al., 2010).

Since the strain differences in arterial collaterals and sensitivity to excitotoxic cell death, mice exhibit profound inter-strain differences in infarct volumes after MCAO (Carmichael, 2005), which is common focal stroke model of intra-arterial suture occlusion of the MCA. Several studies confirmed that C57BL/6 mice showed significantly larger infarct volumes than the Sv129 strain (Connolly et al., 1996; Maeda et al., 1999), and in global ischemia, C57BL/6 is more sensitive than BALB/c and other strains (Yang et al., 1997). However, in distal MCAO (see below), cortical infarcts in BALB/c mice were 3-fold larger than those in 129X1/SvJ and C57BL/6J mice (Majid et al., 2000).

Distal occlusion of the MCA (dMCAO) is another model of MCAO that involves occlusion of the MCA on the surface of the brain together with bilateral common carotid artery (CCA) occlusion also commonly known as the three-vessel occlusion model (Carmichael, 2005). The dMCAO model produces more restricted damage to the cerebral cortex, avoiding large area damage seen in suture occlusion of MCA of more than 60 min (Carmichael, 2005). There are some differences between rats and mice in terms of procedure time to produce consistent and comparable infarction. In rats, there needs to be a period of at least 60 min of occlusion of both MCA on the surface of the brain and bilateral CCA (Chen et al., 1986). However, in the mouse, permanent distal MCA and bilateral CCA occlusion limited to 15 min produces infarction restricted to the cortex, but bilateral CCA occlusion for 30 min or more produces global infarction bilaterally and affect the striatum and globous pallidus (C57BL/6 strain, Ohab, J. and S. T. Carmichel, personal observations). Additionally, permanent occlusion of MCA and ipsilateral CCA in the mouse produces a similar degree of cortical infarction as the bilateral CCA occlusion model (Sugimori et al., 2004).

However, there are limitations to the use of rodents as models of stroke. Rodents have been recognized to have a higher degree of brain plasticity, which makes comparative aspects of rodents and humans less relevant (Durukan and Tatlisumak, 2007). The subsequent inflammatory pathways between rodents and humans are also notably different. Several studies have showed that genomic responses in mouse models poorly mimic human inflammatory disease (Seok et al., 2013).

4.2. Non-human primates

Non-human primates (NHPs) resemble humans in terms of cerebrovascular system, sensorimotor integration, rich behavioral repertoire, grey-to-white matter ratio, and have closer evolutionary ties to human beings (Fan et al., 2017). The brains of NHPs are generally made up of multiple gyri. Similar to humans, NHPs have a complete cerebral arterial ring. The distributions of the internal carotid and vertebral arteries in NHPs are also very similar to those in humans (Fukuda and del Zoppo, 2003). According to statistics, there are five species of NHPs, including Papio Anubis (Baboon), *Callithrix jacchus* (Common marmoset), *Saimiri sciureus* (Squirrel Monkey), *Macaca mulatta* (Rhesus macaque), and *Macaca fascicularis* (Cynomolgus macaque), that are most often used for mimicking stroke (Cook and Tymianski, 2012).

Compared with rodents, NHPs are much expensive to purchase and feed (Murphy et al., 2008). The ethics and operation requirements for NHPs are also much stricter than those for rodents (Cook and Tymianski, 2012). However, there are several distinct advantages for using NHPs for stroke models: 1) the anatomical, cognitive and behavioral complexities of NHPs are similar to humans, which make NHPs more suitable to mimic human neurological disease (Bakken et al., 2016); 2) NHPs and humans have a greatly expanded neocortex and functional partitioning, especially the primary visual cortex (Bakken et al., 2016); 3) rodents have rich collateral anastomoses and some rodents do not have a complete circle of Willis, A study (McColl et al., 2004) found that only 10% of C57BL/6 mice have a complete Circle of Willis, which decreases the translational significance of rodent models. There were no significant differences in anterior and middle segments of the circle of Willis between the SV-129 and C57BL/6 strains. Gerbils lack posterior communicating arteries (PComA) necessary to complete the circle of Willis. The PComA in Wistar rats were significantly thinner than that in SD rats. Of note, a few features, such as prolonged myelination, synapse production, and pruning are unique in primates, which limit their application in some human neurological diseases (Fan et al., 2017).

4.3. Canines

Canines possess a series of characteristics that make them one of the ideal animals for use once small-animal studies are done, right before human clinical trials. Unlike other small animals with lissencephalic brains, canines have gyrencephalic brains with a percentage of grey/white matter that is more similar to humans (Ferrer et al., 1986; Wynshaw-Boris et al., 2010). The neurovascular anatomy of canines enables their arteries to be catheterized in a similar manner to humans under angiographic guidance, allowing for minimally invasive endovascular stroke induction (Rink et al., 2008). Since the surgical procedure can be visualized in real-time by angiography for canines, endovascular procedure evaluation is thus reliable.

However, ischemic stroke model of canines has its own set of disadvantages. First, it is hard to obtain a consistent infarct size as the blood supply in the canine brain is provided by a cerebral arterial circle system consisting of multiple cerebral arteries wherein the canine MCA receives leptomeningeal circulation, which makes redirection of blood supply of occluded vessels from other arterial branches possible (Atchaneeyasakul et al., 2016; Symon, 1960). Second, like primates, using canine models as a stroke model also include high purchase and accommodation fees. Viewed as “man's best friend”, the use of canines carries with it many ethical issues and considerations in our present age (Hasiwa et al., 2011).

As for the selection of which canine to use as a stroke model, it is recommended to choose larger-sized canines compared to smaller ones. Many factors should be considered when choosing canines, like canine breed, strain, and the increased difficulty in performing surgical procedures in smaller animals (Atchaneeyasakul et al., 2016).

5. Factors influencing stroke model performance

5.1. Brain structure

It has been demonstrated that there are differences between humans and other species in terms of brain anatomy and function, which are relevant with respect to the infarct location and size after stroke induction. However, infarct size only mimicks human conditions to a limited extent, whereas the location of the ischemic lesion within specific connections may be more relevant for the clinical syndrome and long-term outcome (Sommer, 2017). Ischemic damage of white matter is closely relevant to the prognosis of stroke outcome. The percentage of white matter in humans is about 60%, compared to 35% in dogs, 20% in rabbits, 15% in rats and 10% in mice (Krafft et al., 2012). Therefore, the pattern of injury after stroke is different in various species, and even in same species. For example, rats subjected to right MCAO showed transient hyperactivity, while this effect was not seen after occlusion of the left MCA (Robinson, 1979).

5.2. Vascular structure

The vascular system in the brain is comprised of many vessels that play an integral role in blood transfer throughout the circulatory system. However, the blood vessels in the brain have undergone many

structural evolutionary changes to protect the brain from fluctuations in blood supply (Sommer, 2017). Many cerebral perfusions provided and protected by three collateral systems in the cerebral vasculature: shunts between the branches of extra-and intra-cerebral arteries, the circle of Willis at the base of the brain allow redirection of the blood flow when one artery is occluded and a highly interconnected network of vessels in the surface of cortex (Blinder et al., 2013; Eftekhar et al., 2006). In addition, at a functional level, blood supply is regulated by auto-regulation of cerebral resistance arteries and endothelial regulation of the vascular tone by releasing vasoconstrictive or vasorelaxation molecules. Second, the neurovascular network unit is closely involved in neuronal activity (Moskowitz et al., 2010). However, the impact of aging on blood vessels is profound in terms of structure and function (Xu et al., 2017). Age-related structural changes of arteries mainly include elongation and dilation, stenosis as well as distortion. The primary function of large blood vessels in the brain is also reduced with aging (Franklin et al., 1997). It is more difficult to perform stroke in aged animals as opposed to young animals. In addition, there is a wide variation in the Circle of Willis (Eftekhar et al., 2006), which partly explains the high variance of infarct volumes even in the same species or strains after stroke. While differences in the gross anatomy of arteries and collaterals may cause variations in brain injury patterns, functional differences between young and aged animals may have deeper implications in the pathophysiology of the ischemic cascade (Sommer, 2017). Differences in blood systems between species will result in different patterns of ischemic damage. For example, variability in distal MCA branch patterns may contribute to variability in infarct size, as was shown in Sprague Dawley rats after distal occlusion of the MCA. These alterations and even variations will influence survival of brain tissue after stroke.

5.3. Strain

Different species and different strains of the same species may have different outcomes even if using the same protocol for ischemic model preparation. Thus, it is important to select the appropriate animal strain according to the purpose of the study. For example, Sugimori et al. found that BALB/c mice showed significantly larger and more reproducible infarctions ($44.1 \pm 5.2\,mm^3$) than C57BL/6 mice ($25.2 \pm 13.7\,mm^3$) using krypton (Kr) laser-induced photothrombosis to occlude the MCA (Sugimori et al., 2004). Maeda et al. demonstrated that the infarct area induced by the MCA is larger in C57BL/6 than in SV129 mice (Maeda et al., 1998). The infarct volume after permanent or transient focal ischemia by the suture occlusion technique is larger in C57BL/6 than in SV129 mice (Connolly et al., 1996). In addition, C57BL/6 mice are more susceptible to global ischemia by bilateral carotid artery occlusion *vs.* SV129 mice (Fujii et al., 1997). Similarly, BALB/c show much bigger infarct volumes than C57BL/6 and SV129 mice following MCAO (Barone et al., 1993; Majid et al., 2000), which may be due to the relatively undeveloped circulation of the posterior communicating artery in BALB/c mice. In terms of a more accurate situation of clinical reality, the spontaneously hypertensive rat (SHR) and the stroke-prone spontaneously hypertensive rat (spSHR) have often been used, as hypertension is the single most important risk factor for stroke in humans (Alberts and Atkinson, 2004; Sommer, 2017). Other spontaneous stroke models, such as the male-inducible hypertensive rat or (R+/A+) mice, which are double transgenic for the human renin and human angiotensinogen genes, also develop spontaneous ischemic and hemorrhagic cerebral lesions after induction with specific diets (Hainsworth and Markus, 2008). Indeed, the different species and strains affect the outcomes of stroke.

5.4. Sex

Sex may be another factor affecting stroke outcome. According to preclinical and basic studies, female hormones like estrogen and progesterone have the ability to reduce the incidence of stroke, cardiovascular disease and the tissue damage after ischemia (Paganini-Hill, 2001; Simpkins et al., 2005). For example, E2 has neurotrophic, anti-apoptotic, vasodilatory, anti-inflammatory, and antioxidant effects, each of which could contribute to improved outcomes in ischemic male and female brains (McCullough and Hurn, 2003). Many studies have shown the neuroprotective role of estrogens in young-adult animals (Hoffman et al., 2006; Lebesgue et al., 2009; Simpkins et al., 1997). Consistently, it is increasingly recognized that the epidemiology of ischemic stroke shows a distinct sex difference (Sudlow and Warlow, 1997) and therapeutic agents also have different effects in terms of improving the functional recovery after stroke in male and female subjects. This may partly explain why some neuroprotective agents failed in clinical trials as many animal studies exclusively use male animals. In human studies, pre-menopausal women have a lower risk of stroke relative to age-matched men but have a higher risk after menopause (Sacco et al., 1997; Wenger et al., 1993). Therefore, stroke risk has been attributed to the presence of protective female sex hormones. In addition, many different inbred and outbred strains of female rats and mice have smaller brain tissue damages after a similar insult of ischemia (Simpkins et al., 2005).

Even for female animals, the outcome after ischemic stroke is different with age. Leon et al. confirmed that chronic estradiol (E2) administration in aged female rats led to worsened ischemic brain injury and increased mortality after an experimental stroke (Leon et al., 2012). Clinical trials conducted by the Women's Health Initiative (WHI) and other small organizations showed that women who are in hormone replacement therapy (HRT) or taking estrogen have higher a risk of stroke or greater stroke severity (Bath and Gray, 2005; Sare et al., 2008; Wassertheil-Smoller et al., 2003). All these findings suggest a potential age-related difference in the effects of E2 on stroke. A recent study in mice demonstrated that prolonged loss of E2 prior to replacement (10 weeks) ameliorated the neuroprotective and anti-inflammatory effects of E2 after MCAO-induced stroke (Suzuki et al., 2007). One possible explanation of these findings could be the unexpected pro-inflammatory effects of estrogen administration after a long period of “hypoestrogenemia” (Liu and McCullough, 2011). Therefore, it appears that the timing of estrogen replacement after menopause is critical for functional recovery in both animal models and clinical trials.

In addition to age and time, other factors, such as the dosing of replacement, may also affect the effects of E2 in MCAO-induced brain injury. A recent study confirmed that slow-release commercially purchased pellets of 17β-estradiol, which produce an early supraphysiological peak followed by a substantial decrease in serum levels of E2, are detrimental and exacerbate cerebral brain injury. Silastic capsules, however, yielding 17β-estradiol concentrations within the physiological range for at least 4 weeks was beneficial in reducing MCAO-induced brain damage (Strom et al., 2010).

6. The necessity of aged models for stroke research

Aging is the principal independent risk factor for stroke. Along with aging, the incidence of stroke remarkably increases in humans, with half of all strokes occurring in people over 75, and one-third in the population over 85 (Roger et al., 2012). People over the age of 85 are have been associated with worse functional reserves, indicating an impaired response to stressors and illnesses (Sandu et al., 2017). Compared with younger patients, older patients have higher in-hospital mortality and poorer functional outcomes after an ischemic stroke (Rojas et al., 2007). Age is thus considered as one of the most important prognostic markers for poor outcome (Rasmussen et al., 1992). A number of neurochemical and physiological changes occur during aging (Anyanwu, 2007). Studies have documented that age affects the incidence, functional recovery and mortality in both human stroke patients and animal stroke models (Bergerat et al., 2011). After experimental stroke, old animals have less edema formation (Liu et al., 2009)

and lower level of NKCC, a Na-K-Cl cotransporter, compared to young mice (Liu et al., 2010). Consistently, more robust edema formation was found in the young brain after stroke in clinical postmortem studies (Jaramillo et al., 2006). Many studies reported that some promising neuroprotective agents, like Compound C, an adenosine monophosphate-activated protein kinase (AMPK) inhibitor, have been proven to be effective in young animals but fail to show the same results in aged animals (Li et al., 2007; McCullough et al., 2005). One possible reason for the ineffectiveness is that stroke research is mostly carried out in young animals and thus neglect the co-morbidities and risk factors in elderly subjects, such as arterial hypertension, diabetes and atherosclerosis (Bacigaluppi et al., 2010). Fortunately, appropriate stroke models have been established in aged rats over the past two decades (Bacigaluppi et al., 2009).

Aged animals are rarely used for stroke models for several reasons: 1) it is much more difficult to perform the required surgeries to induce stroke; 2) aged rats or mice are less tolerant to anesthesia; 3) high cost of purchasing and accommodation; 4) higher mortality after stroke due to frailty, peripheral immunosuppression and other comorbid diseases (Liu et al., 2009). Although age is an important factor in experimental design, it is often overlooked in stroke research, as only a few studies in the literature exist that use middle-aged and aged animals in stroke models.

7. Effect of age on the ischemic stroke model

7.1. Mortality and aging

Age is one of the pivotal risk factors in ischemic stroke. Aging leads to the progressive deterioration of multiple body systems. Studies have shown that with increasing age, the tolerance of vasculature to blood pressure and the capacity of brain's self-healing are notably in decline, contributing to increase the susceptibility of brain ischemia (Kim and Vemuganti, 2015; Yang and Paschen, 2017). Aged animals appear to have significantly higher mortality rates and more severe neurological impairments. Wang et al. examined a group of 3–4 month-old rats and another group of 22–24-month-old male rats, and results demonstrated a mortality rate of 43.5% in aged rats *vs.* 10% in young rats (Wang et al., 2003). This can be attributed to decreased neurogenesis with aging, which may affect infarct size, inflammatory response and cell mortality. However, the distal MCAO model caused smaller infarct size and had lower mortality both in young and aged animals *vs.* the MCAO model. Therefore, the distal MCAO model is more appropriate for long-term research of ischemic stroke, particularly in the aged population.

7.2. Injury size and aging

In acute ischemic stroke, the progress of the stroke is mainly surrounded by the penumbra, where the brain tissue is in dormant or semi-dormant condition (Astrup et al., 1981). The tissue in the penumbra is able to be revived again when the ischemic and anoxic state is improved within a limited time window. It needs to be pointed out that the potential of salvageable penumbra to amelioration infarct size is decreased with age, suggesting that the infarct size after ischemic stroke is related to age. Studies have documented that the infarct size in aged animals (20- and 27-month-old) is larger than that in young animals (4-months-old) post-stroke. Shapira et al. reported that the histological infarct damage in young (3-months-old) rats was significantly higher compared to old (24–26-months-old) male rats (Shapira et al., 2002). In contrast, another study shows that the aged animal infarct size in the neocortex and striatum was shown to be 3% larger than the young (Sutherland et al., 1996). Kharlamov et al. measured the infarct size of male Fisher rats of three different age groups (4-, 20-, and 27-months-old), and reported no significant difference (Kharlamov et al., 2000). However, another laboratory has also showed aging decreased infarct volumes in male mice but with worse functional outcomes (Manwani et al., 2013). This consequence may result from the different types of ischemic stroke models being utilized, such as the proximal MCAO and the distal MCAO models. Another study demonstrated that the infarct size was the largest when rats were sacrificed after 7 days, but its diminished after 10 days (Persson et al., 1989). However, there is no evident variance of infarct size with age in humans (Engelter et al., 2003).

7.3. Neurobehavior and aging

The neurological outcome depends on a great variety of factors, such as sex, comorbidities, heredity and age in particular. Clinically, older patients have worse outcomes than their younger counterparts after stroke, suggesting that the severity of neurological deficit after stroke has a positive correlation with age (Kim and Vemuganti, 2015). A recent report showed that 6- and 20-months-old rats after ischemic stroke appeared to have worse neurological outcomes *vs.* 2-months-old rats (Tu et al., 2017). For instance, aged rats have worse performance on complex tests like neurological score (measuring synthetical functions), the rotarod test or adhesive removal test (measuring somatosensory dysfunction) and the water maze test (measuring spatial memory). However, aged rats recovered just as well on simple tests such as the corner test and foot-fault test (measuring motor asymmetries) as the young rats did (Aurel et al., 2007). Since the aged brain displays a higher susceptibility to hypixia (Popa-Wagner et al., 2007), it was demonstrated that aged rats have more severe impairment of behavioral outcomes and poorer neurological functional recovery after ischemia *vs.* younger animals, although the gradual improvement in neurobehavior would continue beyond 2 months (Brown et al., 2003). Interestingly, the recovery process in humans lasts about 3 months up to several years. The mechanism underlying this difference remains unclear.

7.4. Neurobiological effects of age after stroke

It is well known that regeneration potential is at a low level in senescent animals, diminishing the ability of aged animals to recover from stroke relative to young animals. One evident explanation accounting for this phenomenon is that activated microglia and astrocytes are prematurely accumulated in the lesioned areas, which increases the susceptibility of the cells to apoptosis on account of the decline of plasticity of the cerebral vessels and increased inflammatory reaction to stroke (Aurel et al., 2007). Another study showed that stroke-induced neurogenesis is reduced with aging by inhibiting the proliferation and migration of neural progenitor cells (Moraga et al., 2015). Aged individuals delay the activation of growth-promoting genes such as GAP43, CAP23 and c-Jun, which is a growth-promoting transcription factor (Li and Carmichael, 2006). In addition, the neurotoxic carboxyl terminal modulator protein (CTMP) of the β-amyloid precursor protein (βAPP) is significantly increased in the aged group. It is proposed that increased CTMP may deteriorate the outcome of stroke with aging through the Akt signaling pathway (Popa-Wagner et al., 1998).

8. Age as a key factor in preclinical stroke studies

Aging can change the efficacy of many neuroprotective drugs through influencing pharmacokinetics and pharmacodynamics (Mangoni and Jackson, 2015). Firstly, aging is associated with a reduction of nephrons, the hyalinization of renal arterioles, and the reduction in both renal plasma flow and glomerular filtration rate (Mclachlan, 1978). These changes in renal function in the elderly affect the clearance of many drugs, such as water-soluble antibiotics (Triggs et al., 1980), lithium (Hewick et al., 2012), and nonsteroidal anti-inflammatory drugs (Oberbauer et al., 1993). Secondly, aging is accompanied by the gradual reduction of liver volume and liver blood flow (Koff et al., 1973). Drug clearance in the liver depends on the ability to

extract drugs from blood passing through the liver and on hepatic blood flow, and the extraction rate depends on the metabolizing capacity of the liver. The decrease of hepatic blood flow with aging will mainly affect the clearance of drugs with high extraction rate, such as chlormethiazole, glyceryl nitrate and propranolol. Thirdly, age-related changes in body composition, including a progressive reduction in body water and lean body mass, result in a relative increase in body fat (Fülöp et al., 1985). Therefore, the volumes of distribution of lipid-soluble drugs increase with aging thus extending the half-life of drugs. It has been observed that the volumes of distribution and the half-life of some lipid-soluble drugs, such as diazepam (Reidenberg et al., 1978), thiopentone (Christensen et al., 1981), and chlormethiazole (Roberts et al., 1978) increased with aging. Finally, age-related pharmacodynamic changes include increasing the sensitivity to several kinds of drugs, including anticoagulants (Shepherd et al., 2012) and psychotropic drugs. The elderly were more likely to have adverse drug reactions (ADRs) to these several types of drugs compared with the young (Maixner et al., 1999). In fact, drugs with lower doses and lower plasma concentrations can have the same effect in elderly subjects.

Furthermore, drug metabolism may change with aging through the following factors (Waring et al., 2017); the first of these is inflammation. The elderly population is usually associated with many diseases, such as Alzheimer's disease, type 2 diabetes, and chronic obstructive pulmonary disease. Elevated levels of inflammatory activity has been observed in these diseases. Thus, inflammation is common in elderly people (Shin et al., 2011). The inflammatory state of the elderly will affect drug metabolism, reduce enzyme activity, increase the release of cytokines, and increase adverse drug reactions (ADRs) (Waring et al., 2013). The second factor that changes with aging is circadian rhythms. Drug metabolism of lipid-soluble drugs is known to be subjected to one's circadian rhythm. Drug absorption is faster in the morning, and drug metabolism is regulated by the biological clock (Baraldo, 2008). The elderly population is often associated with depression, anxiety, neurological disorders, and cancer, all of which can interfere with circadian rhythms and affect drug metabolism. Thirdly, our gut microbiota plays an important role in drug metabolism. When the composition of gut microbiota changes with age, drug metabolism will be altered (O'Toole and Jeffery, 2015). The last factor is epigenetics. DNA methylation patterns at specific CpG islands are thought to be associated with aging. In addition, high methylation levels are linked to the diagnosis of cancer (Gautrey et al., 2014). The human CYP2C9 gene is involved in the metabolism of many drugs and its activity is regulated by epigenetic mechanisms (Ingelman-Sundberg et al., 2013).

9. Conclusion

In summary, several ischemic stroke models have been invented, which provides reliable stroke data for researchers to translate to the bedside. However, despite overwhelming experimental literature demonstrating substantial therapeutic success on rodent stroke models, the translation of findings in animal stroke models to the clinical setting remain unsuccessful. Therefore, it is timely to ask whether animal stroke models that are widely used for stroke research truly mimic the human stroke condition. We believe that the role of age in animal models of stroke is overlooked and reiterate that age is a key factor, which could have an impact on data quality and hinder the translation of rodent models to humans.

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