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RODENT STROKE MODEL GUIDELINES FOR PRECLINICAL STROKE TRIALS (1ST EDITION)

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Abstract

Translational stroke research is a challenging task that needs long term team work of the stroke research community. Highly reproducible stroke models with excellent outcome consistence are essential for obtaining useful data from preclinical stroke trials as well as for improving inter-lab comparability. However, our review of literature shows that the infarct variation coefficient of commonly performed stroke models ranges from 5% to 200%. An overall improvement of the commonly used stroke models will further improve the quality for experimental stroke research as well as inter-lab comparability. Many factors play a significant role in causing outcome variation; however, they have not yet been adequately addressed in the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations and the Good Laboratory Practice (GLP). These critical factors include selection of anesthetics, maintenance of animal physiological environment, stroke outcome observation, and model specific factors that affect success rate and variation. The authors have reviewed these major factors that have been reported to influence stroke model outcome, herewith, provide the first edition of stroke model guidelines so to initiate active discussion on this topic. We hope to reach a general agreement among stroke researchers in the near future with its successive updated versions.

Keywords

Acute stroke; animal model; neuroprotection; middle cerebral artery occlusion; guidelines; consistency

1. INTRODUCTION

The Stroke Therapy Academic Industry Roundtable (STAIR) was established in order to address the challenges encountered in finding an effective neuroprotective therapy for acute stroke (Fisher et al. 2007; Stroke Therapy Academic Industry Roundtable 1999). Besides the

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STAIR's concern for study design, other issues relating to experimental stroke research, especially animal stroke modeling have been raised (Dirnagl 2006; Savitz 2007; van der Worp et al. 2005). This is an important issue, and it is now recommended that any stroke model that aims to achieve scientific and therapeutic value must meet certain requirements. The model must be both highly consistent in inducing injury, performed under conditions to avoid co-founding factors influencing outcomes and widely available to most investigators. With the STAIR guidelines providing an excellent framework for the design of preclinical stroke trials, a detailed guidance for conducting individual experiments using stroke models will further improve model consistency, reliability and inter-lab comparability. A review of literature shows that the infarct variation coefficient of commonly performed stroke models ranges from 5% to 200% (see following paragraphs). Many factors play a significant role in causing outcome variation; however, they are not fully defined in the STAIR guidelines. These critical factors include selection of anesthetics, maintenance of animal physiological environment, stroke outcome observation, and animal species used.

Here we provide the first edition of stroke model guidelines (SMG) so as to initiate an active discussion on this topic, with the ultimate aim of reaching a general agreement among stroke researchers in future up-dated versions. Because rodents are the mostly commonly used animals for preclinical stroke trials, the first version of SMG starts with rodent stroke models. In its successive versions, the contents may expand into stroke models of other species. The SMG ,starts with a general overview of the design and setup of a typical preclinical stroke trial, followed by more detailed guidelines on the implementation of each step.

2. GENERAL GUIDANCE FOR A PRECLINICAL STROKE TRIAL

Because investigators have used different units of measurement for the infarction volume and such data have been expressed either in mean ± SD or mean ± SEM, we adapted pSDM (percent of SD to mean,) as the coefficient for infarction volume variation for the purpose of increasing comparability between studies. The SEM, when used by many of the original investigators, has been converted to SD for the calculation of the pSDM.

Before starting an experimental preclinical stroke trial the following steps are necessary for ensuring a good quality study. Specific guidance for most steps is provided in subsequent paragraphs.

1. Revisit the latest STAIR criteria for achieving an optimized study design
2. Select the most appropriate stroke model for your study
3. Determine stroke model parameters, such as anticipated infarct size and surgical procedure
4. Determine the use of anesthetics
5. Determine the components of the inhaled gas
6. Determine the necessity and settings of intubation and ventilation
7. Set up monitoring for arterial blood pressure, blood gases, blood glucose
8. Set up temperature monitoring and maintenance
9. Set up regional cerebral blood flow monitoring
10. Determine a protocol for post-operational care
11. Determine the method and timing for infarct volume measurement

12. Determine the appropriate tests and timing for the assessment of functional deficits
13. Do a pilot study and adjust the experimental settings for further optimization
14. Assess the compliance of the trial with "Good Laboratory Practice" standards.

3. REVISITING THE STAIR CRITERIA

GUIDANCE

The design of a preclinical stroke trial should start with a revisiting of the latest version of stroke therapy academic industry roundtable (STAIR) criteria. Receiving some useful suggestions with caution from the STAIR criteria may help with improving the study design to some extent although there are debates on some issues that STAIR addressed.

SUPPORTING DISCUSSION

The STAIR criteria provides some useful recommendations for improving the design of preclinical stroke trials—To date, the STAIR group has met six times discussing and revising their recommendations for preclinical and clinical stroke trials (Fisher 2003; Fisher 2005; Fisher et al. 2009; Fisher et al. 2007; Saver et al. 2009; STAIR Group 1999; STAIR Group 2001). Recommendations provided by the STAIR consortia emphasize the design quality of both experimental and clinical stroke trials. With respect to experimental animal stroke trials, STAIR recommendations have highlighted the need for investigators to consider factors such as species and gender differences, clinical relevance of animal models, dose-response determinations, therapeutic time windows, blood-brain-barrier (BBB) permeability and tissue drug levels, treatment randomization,, physiological monitoring, and at least 2 outcome measures covering both acute and long-term endpoints.

The STAIR criteria should be used with caution because there may be conflicts between its suggested late treatment and therapeutic windows—In the STAIR I, the ideal neuroprotective drug trial was described thus: "should demonstrate efficacy in at least 2 species, in at least 2 laboratories that use different models, is effective in both permanent and transient focal ischemia, and improves short-term and long-term histological and functional outcomes, even when administered several hours after the onset of ischemia" (STAIR Group 1999). Keeping the therapeutic window in mind, it may be simply impossible to demonstrate robust neuroprotection when the treatment is delivered too late. The therapeutic window is roughly a few hours in rodent MCA occlusion (MCAO) models. For example, in a 300 g rat, a 2-hour duration of transient MCAO produces a large infarct volume of 400-450 mm^3, which is similar in size to the infarct caused by permanent MCAO after 24 hours (Greco et al. 2007; Masada et al. 2001). Hence, it is likely that a preclinical stroke trial using a 2 hour transient MCAO model and a late treatment time point (e.g., 6h post-MCAO) (Simard et al. 2009; Yin and Zhang 2005) would have missed the therapeutic window and the opportunity to observe a treatment effect. In this instance, a baseline injury quantification study, performed at different treatment time points (e.g.,, 2, 4, 6 hours post-MCAO) would improve study design and increase the chance of obtaining a positive neuroprotective effect.. Some histopathological methods and diffusion-weighted imaging techniques described in the infarct volume measurement section of this guideline can be used for the detection and quantification of baseline injury starting several hours after ischemia.

The STAIR criteria should be used with caution because there may be conflicts between its suggested observational time and the natural history of stroke evolution—As discussed in more detail below, both the infarction evolution and changes in functional deficits have their own natural histories. Measuring infarct volume

evolution is time sensitive and methodology dependent (see section on Infarction volume measurement). Assessment of functional recovery is even more complicated, is model dependent,and is time sensitive in relation to the recovery pattern and functional test being used (see section of Functional evaluation). Therefore, it may sound arbitrary to recommend all outcome measures be performed in 1-3 days and in 7-30 days.

Would the STAIR criteria help with increasing the chance of a true discovery (no, but this was not really its aim)?—As stated in the STAIR I, the purpose of STAIR is "to propose recommendations for ways to optimally preclinically assess neuroprotective and restorative drugs for acute ischemic stroke". However, these recommendations were condensed into a few designing principles even with the latest update (Fisher et al. 2009), which has been functioning like an elevated threshold limiting experimental discovery entering into clinical trials. These STAIR criteria may help with improving the design quality of preclinical stroke trials, reducing bias and false positive conclusions (Fisher et al. 2009), but has little to do with increasing the chance of scientific discoveries. It is the research direction that holds the chance of scientific breaking through while the optimized methodologies increase the sensitivity for positive findings. The research directions that hold the continued promise for neuroprotection for ischemic stroke has been discussed in our previous review paper (Liu and Levine 2008). Optimization of preclinical stroke trials is more complicated than that the STAIR has recommended, which is what we need to address in this stroke model guidelines

4. STROKE MODEL SELECTION

Various stroke models have been developed to mimic different stroke subtypes or pathological mechanisms and can be generally classified into two categories: focal cerebral ischemia models and global cerebral ischemia models. Global ischemia models mimic the clinical conditions of brain ischemia following cardiac arrest or profound systemic hypotension, focal models represent ischemic stroke, the most common clinical stroke subtype. The most commonly used focal ischemia models are the intraluminal filament model (Koizumi et al. 1986) and the Tamura model (Tamura et al. 1981a). Some additional stroke models involve special mechanisms to induce artery occlusion/ischaemia, such as the thromboembolic, endothelin and photochemical models.

GUIDANCE

There are mainly two factors that influence the selection of in vivo stroke models for preclinical trials. These are the potential protection mechanism of the neuroprotective candidate and the highest achievable model quality with a particular lab setting.

For examples, if the candidate is predicted to reduce ischemic injury by attenuating cerebral edema after thrombolytic therapy, the thromboembolic model should be used; if the predicted neuroprotection is associated with a particular brain cortex region, the photochemical model will be preferable because this model is able to produce ischemic injury in an arbitrary geometric shape at any location on the brain surface. If the predicted protection mechanism of a drug candidate is shared by several stroke models, the selection of a preferred model could be determined by the achievable model quality, as judged by success rate and outcome consistency. In most cases, the choice is between the intraluminal model and the Tamura model.

SUPPORTING DISCUSSION

About the photochemical model—Implementation of the photochemical model involves injection of a photosensitive dye that penetrates the BBB. The photochemical

reaction produces singlet oxygen and free radicals, which causes endothelial injury and formation of microthromboses. The light used for inducing this reaction can be laser or filtered non-laser light, and can be shone onto a section of artery wall or any location of the skull. Therefore, this model is useful for neuroprotection that is associated with a particular brain cortex region and involves free radical scavenging as a protective mechanism (Chen et al. 2004; De Ryck et al. 1996; De Ryck et al. 1989; De Ryck et al. 2000; Eichenbaum et al. 2002; Futrell et al. 1988; Lozano et al. 2007; Ostrovskaya et al. 1999).

About the autologous clot model—Although the autologous clot model that mimics thromoboembolic stroke has been developed (Kudo et al. 1982), and efforts have been made to improve its outcome consistency, (Wang et al. 2001; Zhang et al. 1997b) this model is still not suitable for validating neuroprotective effects because of its uncontrollable reperfusion and unacceptable variation of infarct area (Wang et al. 2001; Zhang et al. 1997a; Zhang et al. 1997b). Therefore, this model is reserved for clot-related protection mechanisms which other stroke models cannot address.

About the endothelin-1 model—Endothelin-1 (ET-1) is a potent vasoconstrictor. It reduces regional cerebral blood flow and produces ischemic injury when being injected directly into brain tissue (Windle et al. 2006) or adjacent to the MCA (Biernaskie et al. 2001; Nikolova et al. 2009). The magnitude and duration of reduction of cerebral blood flow is variable, dose dependent (Nikolova et al. 2009), and strain dependent (Horie et al. 2008), persistent up to 7-16 hours (Biernaskie et al. 2001). ET-1 has a much less potent effect for producing an infarct in mice than in rats (Horie et al. 2008).

Intraluminal model versus Tamura model—In experienced hands, the intraluminal model and the Tamura model can achieve similar success rates and outcome consistency (Table 2). However, some types of the Tamura model may cause just cortical injury with small infarction volume, which does not produce consistent functional deficits (Chen et al. 1986;Roof et al. 2001).

5. THE INTRALUMINAL MODEL

For the intraluminal model, the key factors that affect outcome consistency are the physical properties of the occluder, the MCAO surgical procedure and the strain of animal. Critical physical properties of the occluder that affect stroke outcome include its tip diameter, tip length, tip shape, and flexibility. Some specific surgical procedures have also been developed for different purposes, such as for confirming a successful occlusion, for supplemental occlusion of proximal arteries, and for prevention of premature reperfusion. Animal strain is not the focus of the first version of SMG.

A. THE SELECTION OF MCAO OCCLUDERS

GUIDANCE—It has been shown that silicone-rubber coated filaments are superior to flame blunted and PLL coated monofilaments for producing consistent ischemic injury (Spratt et al. 2006). There are insufficient data for an accurate comparison between silicon rubber coated monofilaments and glue-coated, resin-coated or nail polish-coated monofilaments. Flame/heat-blunted and PLL coated monofilaments are generally considered unacceptable for neuroprotection studies because of their low success rate, high subarachnoid hemorrhage (SAH) rate, and large variability in infarction volume.

SUPPORTING DISCUSSION—The intraluminal MCAO models can be induced using different filaments. In the Koizumi model, a silicone-rubber coated monofilament is used, while in the Longa model a flame-blunted monofilament is used. Other occluders include

the poly-L-Lysine (PLL) coated monofilament (Spratt *et al* 2006), methyl methacrylate glue coated monofilament (Shah *et al* 2006), silicon resin coated monofilament (Yamauchi *et al* 2005), and nail polish coated monofilament (Matsushima and Hakim 1995). The physical characteristics of the occluder influence outcome variation by causing insufficient occlusion, premature reperfusion, and/or filament dislodgement. The following paragraphs review the MCAO model quality obtained using the most common occulders and their optimizations.

The PLL coated occluders: The PLL coated monofilament has the lowest success rate, the highest SAH rate and highest mortality rate among all monofilaments in rat models. MCAO models using PLL coated occluders have been reported to have a success rate as low as 13-14% in rats, with model mortality of around 21-31% (Spratt *et al* 2006). High mortality (50-60%) and infarct size variation have also been reported when using PLL coated sutures in mouse models (Huang *et al* 1998). While most authors reported low success rates, high SAH rates, and high mortality rates when using PLL coated sutures for both rat models and mouse models, Belayev et al reported increased infarct volume and experimental consistency as compared to uncoated sutures, although in some instances brain infarction did not occur (Belayev *et al* 1996).

Flame/heat-blunted occluders: Tsuchiya et al. (Tsuchiya *et al* 2003) showed that using flame blunted monofilaments to induce MCAO caused a 40% rate of subarachnoid hemorrhage, and pSDM was greater than 100%. In another study (Schmid-Elsaesser *et al* 1998), models using heat-blunted 3-0 filaments had a success rate of 46% (without further repositioning of the occluder according to laser Doppler flowmetry (LDF) monitoring), with 44% occurrence of SAH. Premature reperfusion occurred very frequently with a rate of 24% when using the heat-blunted filament group as shown through LDF monitoring (Schmid-Elsaesser *et al* 1998). The authors' own experience confirmed a less than 40% success rate when using flame blunted monofilaments. Personal communication with other MCAO model performers who have experience with the Longa model confirm that a flame blunted occluder is not an acceptable choice for neuroprotection studies. In the mouse intraluminal model, SAH rates can reach as high as 40% if uncoated heat-blunted filaments are being used. In such cases, the pSDM can be more than 50% (Tsuchiya *et al* 2003).

Silicone-rubber coated occluders: Studies using silicone rubber coated monofilaments have reported success rates ranging from 66% (Schmid-Elsaesser *et al* 1998) to 100% (Liu *et al* 2006), and SAH rates from 0% (Chen *et al* 2008) to 8% (Schmid-Elsaesser *et al* 1998). Premature reperfusion rates have been reported to be 26%; readjusting filament location for correcting premature reperfusion could increase the success rate of MCAO(Schmid-Elsaesser *et al* 1998). The pSDM when using a silicone-rubber coated filament ranges from 30% (Schmid-Elsaesser *et al* 1998) to around 5% (Maysami *et al* 2008). It also seems that bilateral laser Doppler flowmetry can be a useful tool for detecting premature reperfusion (Hungerhuber *et al* 2006).

B. OPTIMIZATION OF MCAO OCCLUDERS

GUIDANCE—The physical properties of the occluder tip play a critical role in causing infarct variation and SAH occurrence. For a certain range of animal body weights, an optimal occluder diameter can be found through a series of pilot experiments. It has been reported that the optimal occluder diameter for rats weighing 275-320g is around 0.38 mm for silicon rubber coated monofilaments (Spratt et al 2006). The silicone rubber coating length is another important factor that influences the occluder's ability to block the back-flow from communicating arteries (Chen et al 2008). Therefore, an optimal coating length may also exist for animals within a certain body weight range, so matching the occluder size with animal size would theoretically improve model consistency. Note too that a shorter

coating can preserve blood supply to the hypothalamus, minimizing post-surgical thermoregulatory dysfunction, particularly the occurrence of spontaneous hyperthermia. In order to match the wide range of rodent animal body weights, a large number of different occluders in standard size would be needed. To this end, our recommendation is to obtain commercially made occluders, which are available in different diameters and silicone rubber coating lengths (www.doccol.com).

SUPPORTING DISCUSSION

When the occluder is matched to animal size, improved success rates and reduced SAH rates can be achieved: In rat models of 60-min transient MCAO to 24-h permanent MCAO using correct-sized occluders (Candelario-Jalil et al. 2008; Khan et al. 2006; Liu et al. 2006; Shimamura et al. 2006a; Shimamura et al. 2006b; Solaroglu et al. 2006; Tsubokawa et al. 2007; Tsubokawa et al. 2006a; Tsubokawa et al. 2006b), the success rate was found to be 88-100%, and the SAH rate to be 4%. In mouse models of 60-min transient MCAO(Chen et al. 2008; Kleinschnitz et al. 2007; Maysami et al. 2008; Pignataro et al. 2007b; Pignataro et al. 2007c), the success rate was found to be 96% and the SAH rate 0%.

When the occluder is matched to animal size, impressive improvements in infarct consistency have been reported both in rats and in mice: A technical paper by Shimamura showed consistent infarction and a tight error bar in rat models even with inexperienced surgeons (Shimamura et al. 2006a). For a 60-min to permanent occlusion in rats, the pSDM is around 10% to 20% depending on experimental design and selection of right-sized filaments. (Candelario-Jalil et al. 2008; Khan et al. 2006; Liu et al. 2006; Shimamura et al. 2006b; Solaroglu et al. 2006; Tsubokawa et al. 2007; Tsubokawa et al. 2006a; Tsubokawa et al. 2006b). A 15-min occlusion could also produce a consistent caudate infarction with little variation in mice (Pignataro et al. 2007a). For a 30-min occlusion, the pSDM was reported to be around 20% in mouse models (Cho et al. 2007; Kim et al. 2008). Even better consistency has been reported in 60-min MCAO models in mice, in which the pSDM was around 5-10% (Kleinschnitz et al. 2007; Maysami et al. 2008; Pignataro et al. 2007b; Pignataro et al. 2007c).

Standard-sized occluders are available for matching with animal size: Varying sized occluders can be conveniently obtained commercially (www.doccol.com) with desired tip diameter and silicone rubber coating length. Tip diameter can be selected within a range from 0.17 mm to 0.49 mm and the coating length in a range from 2 mm to 10 mm. This makes it possible to match animal body weight with occluder diameter so as to achieve better results. Although there is not enough available data to make a detailed match chart between occluder size and animal size, a preliminary matching chart is provided by the vendor to guide investigators' selection of occluders, covering animal body weights from 15 to 400 grams.

C. OPTIMIZATION OF SURGICAL PROCEDURES

GENERAL GUIDANCE—The surgical procedure of inducing MCAO models plays an important role in the stroke outcome variation; and it can be optimized to achieve better success rates and reduce outcome variation. Modifications and optimizations have also been reported concerning the inserted distance of the MCA occluder, CAA approach versus ECA approach, and supplemental occlusion of proximal arteries.

SPECIFIC GUIDANCE ON KEY SURGICAL STEPS

The inserted distance of the MCA occluder

The inserted distance of the MCA occluder: ***GUIDANCE:*** The inserted distance of the occluder is critical to a model's success. For the rat model, the distance from the common carotid artery (CCA) bifurcation is 18-20 mm for a 300 g (Belayev et al. 1996; Lee et al. 2004), and 20-22 mm for a 400 g rat (Lindner et al. 2003). For the mouse model, a distance of 9-11 mm (Dimitrijevic et al. 2007; Yamashita et al. 2006) rostral to the CCA bifurcation needs to be reached.

SUPPORTING DISCUSSION: It has been reported that different insertion distances produce significant differences in infarct size (Zarow et al. 1997). Over-insertion may cause a rupture of the anterior cerebral artery (ACA) and subsequent SAH whilst insufficient insertion may not be able to block the back-flow from the anterior communicating artery, leading to incomplete occlusion of the MCA.

In vivo confirmation of MCA occlusion

In vivo confirmation of MCA occlusion: ***GUIDANCE:*** A reduction in regional cerebral blood flow (rCBF) of at least 75% from baseline is generally accepted as an indicator of successful MCAO (Schmid-Elsaesser et al. 1998).

SUPPORTING DISCUSSION: Because of the anatomic variation of carotid arteries between individuals and between strains (Dittmar et al. 2006; Oliff et al. 1995a; Oliff et al. 1995b), and inaccuracies in measuring the inserted distance, investigators often use LDF to instantly confirm successful occlusion of the MCA. Due to neck movement or artery wall retraction, the occluder may dislodge from its original location if it is not properly affixed against the arterial wall. Occluder dislodgement can result in premature reperfusion and SAH. For reducing occluder dislodgement, investigators have used various techniques. The smooth nylon surface of the occluder at the affixation position can be made serrated to increase traction. A microclip with proper biting force or a tight knot applied onto the artery and the serrated occluder section may help reducing dislodgement.

CAA approach versus ECA approach

CAA approach versus ECA approach: ***GUIDANCE:*** The ECA approach is a better choice for transient MCAO because it maintains the anatomic integrity required for reperfusion. The CCA approach may, on the other hand, be a simpler surgical procedure for permanent MCA occlusion.

SUPPORTING DISCUSSION: When inducing MCA occlusion, the occluder may be introduced into the internal carotid artery (ICA) via a cut in the CCA (the CCA approach) or a cut in the external carotid artery (ECA). Most intraluminal models that appear in the literature were induced through the ECA approach. Some stroke investigators introduced the occluder through an arteriotomy of the common carotid artery (Wetzel *et al* 2008; Xi *et al* 2004). The CCA approach changes the dynamics of cerebral blood flow when the occluder is withdrawn for reperfusion because blood flow will enter only from the contralateral side through the Circle of Willis. Changes in proximal blood supply may also affect infarct volume and model consistency. Studies have shown that occluding additional proximal arteries along with MCA can achieve a larger and more consistent infarct (Woitzik *et al* 2006).

Supplemental occlusion of proximal arteries

Supplemental occlusion of proximal arteries: ***GUIDANCE:*** Supplemental occlusion of proximal arteries (PTA and/or CCA) decreases infarct volume variation.

SUPPORTING DISCUSSION: When the MCA is being occluded, there may be residual blood flow to the MCA territory, which causes insufficient occlusion of the MCA. Residual blood flow could come from the anterior and posterior communicating arteries (AComA and PComA) of the Circle of Willis, the ICA itself, or from leptomenigeal anastomoses on the cortical surface (i.e., collateral supply). Blood flow can also reach the MCA cortex indirectly by external carotid collateral flow though the pterygopalatine artery. Applying a vessel clip on the CCA can increase infarction volume by reducing the residual blood flow through the ICA, especially when smaller filaments are being used (Tsuchiya *et al* 2003). A more recent study has shown that blocking pterygopalatine artery (PTA) blood flow decreases infarct volume variation (Chen *et al* 2008). However, from an anatomical perspective, these surgical modifications will not be effective in reducing the residual flow originating from AComA, PComA, or leptomeningeal anastomoses. A more practical option is to use optimized, silicone rubber-coated, standard-sized monofilaments, which match animal body weight, to induce MCA occlusion.

6. THE TAMURA MODEL

In 1981, Tamura described a rat model of middle cerebral artery occlusion (Tamura *et al* 1981a; Tamura *et al* 1981b) which can induce either permanent or temporary occlusion of the MCA. The former could be achieved by direct electrocoagulation of a section of the MCA whereas the latter by either microclip application or artery ligation/retraction by a nylon suture or a rigid wire. In recent years, infarct variation with the intraluminal models has been noticed (Chen *et al* 2008; Shimamura *et al* 2006a), and has become a concern in preclinical neuroprotective trials, especially with suboptimal models (Savitz 2007). Using just one rodent model may not be sufficient for screening neuroprotective candidates in preclinical stroke trials. Therefore, the Tamura model may serve as a supplemental or alternative approach for validating neuroprotective efficacy in rodents.

A. ABOUT THE MCA OCCLUDER

GUIDANCE—The MCA occluder and occlusion mechanism play no role in the Tamura model. A careful selection of microclips will be necessary for reducing artery wall mechanical injury.

SUPPORTING DISCUSSION—Similar success rates and stroke outcome consistency can be achieved with different occluders and occlusion techniques, such as electrocoagulation, applying a microclip, or suture ligation. Model success rate, mortality rate, and infarct variation will differ in response to changes of occlusion site, occlusion extensiveness, ischemia duration, CCA occlusion, and the animal's blood pressure.

Microclips with a biting force not greater than 15 g are usually used. The size, weight, and biting force of the microclip are important for a successful Tamura model. Not all microclips are suitable for rodent MCA occlusion. Microclips that have been used in this model include Codman Sundt AVM microclip #1-3, Zentype microclip, and Scoville Lewis clip.

B. SURGICAL OPTIMIZATION

GUIDANCE—Ensuring a complete occlusion of the MCA is a necessary step for this model. In addition, the occlusion site and extensiveness must remain consistent. In the transient MCAO model, simultaneous occlusion of either common carotid artery increased

the model success rate (Coert et al 1999). On the other hand, MCAO combined with bilateral CCAO should be used with caution because of a higher incidence of mortality. Moreover, transient MCAO by the Tamura model is often suboptimal and should be used with caution.

SUPPORTING DISCUSSION—Visual inspection under a stereo microscope is commonly used for this purpose. Even with an ensured occlusion through transection of the MCA, infarct consistency still largely depends on the length of coagulated MCA (Bederson *et al* 1986b).

Bederson et al first reported (Bederson *et al* 1986b) the correlation of success rate with the anatomic location where the MCA is occluded. In their experiments, a 100% success rate was achieved with 3 or 6 mm occlusion of the MCA beginning proximal to the olfactory tract. 1-2 mm occlusion of the MCA from its origin, at the olfactory tract, or lateral to the inferior cerebral vein, however, only produced infarction in 13%, 67%, and 0% of rats, respectively.

When assessed at 3 days post surgery, one hour MCAO only caused cerebral infarction in 40% of rats; with simultaneous occlusion of ipsilateral or bilateral CCAs, the success rate reached 60% and 75%, respectively. A higher success rate was also observed when the duration of MCA occlusion increased. One hundred percent success rate was observed with permanent MCAO plus bilateral CCAO.

Chen et al reported (Chen *et al* 1986) that the mortality rate could reach 60% when the CCAs were permanently ligated bilaterally; the high mortality could be reduced to 7% if the contralateral CCA was released after 60-min of occlusion.

Although transient direct MCA occlusion with subsequent reperfusion is possible by applying a microclip on, or suture tying, the MCA, the implementation of these techniques demands extremely delicate surgical skills, especially in mice. Consequently, this model has gradually become less popular since the emergence of the intraluminal model of transient MCAO (Koizumi *et al* 1986), which is relatively easier to perform and does not require a craniotomy.

C. PURE CORTICAL INFARCTION VERSUS COMBINED INFARCTION

GUIDANCE—Pure cortical infarcts in this model produce very mild and inconsistent functional deficits and are therefore not suitable for functional recovery evaluation. Permanent occlusion at a site proximal to the lenticulostriate branches (pMCAO) produces a larger infarct with more persistent functional deficits (Roof et al 2001), and may be preferable for assessing a robust protective effect of therapeutic agents for stroke.

SUPPORTING DISCUSSION—The site of MCA occlusion has also been shown to influence the severity and consistency of histologically-revealed damage as well as functional deficits. Occlusion of the MCA lateral to the olfactory tract produces a pure cortex infarction because the basal ganglia blood supply from the lenticulostriate branches is spared; occlusion of the MCA at its origin produces a combined infarction including both cortex and basal ganglia. Microclips and retraction/release methods are used for transient MCAO/reperfusion and produce a pure cortical infarct due to operational limitation. Electrocoagulation necessarily produces permanent occlusion; it can be applied at various sites along the MCA to produce a variety of lesions from a pure cortical infarct to combined infarcts of both basal ganglia and cortex.

D. INFARCT VOLUME VARIATION AND OPTIMAL ISCHEMIA DURATION

GUIDANCE—Transient MCA occlusion of 30-min by the Tamura model is too mild to produce a brain infarct, but selective neuronal damage in the striatum and subcortex areas in the ipsilateral side could be observed (Yang et al 2001). A 3-h occlusion time is preferable for transient cortical ischemia because of increased consistency. Infarct volume consistency could be improved with supplemental CCA occlusion. Similar results have been reported with different occlusion methods.

SUPPORTING DISCUSSION—Improved infarct consistency has been reported with simultaneous occlusion of the common carotid arteries (Coert *et al* 1999). For example, the pSDM for 1-h MCAO was 200%, 1-h MCAO plus ipsilateral CCAO was 133.5%, and 1-h MCAO plus bilateral CCAO 100.9%. The pSDM for 3-h MCAO plus bilateral CCAO was 59%. The best consistency was observed with permanent MCAO plus bilateral CCAO with the pSDM being 43.75%.

A pSDM range of 10% to 160% has been reported when using a Zen-type microclip for direct MCA occlusion in rats. Margaill et al achieved excellent consistency in rats, with a pSDM of 10% for striatum infarcts and 15-16% for cortex infarcts in transient MCAO of 60-90 min (Margaill *et al* 1996). However, in a 1-h MCAO model, David et al reported significant variation with the pSDM being 68% (David *et al* 1996). Morikawa et al reported an even higher pSDM of 160% for cortex infarcts and 83% for striatum infarcts with a 2-h transient MCAO(Morikawa *et al* 1992). Using a Zen-type microclip for MCA occlusion in mice seems to result in less variation than in rat models. Kitagawa et al achieved a pSDM of 10% for permanent MCAO, and of 45.65% for 60-min transient MCAO (Kitagawa *et al* 2004).

When proximal arteries were occluded in addition to micro-clip occlusion of the MCA, a pSDM range of 16% to 119% could be achieved. Using a Sundt microclip for direct MCAO plus bilateral CCAO, Buchan et al (Buchan *et al* 1992) achieved pSDMs of 119%, 86% and 16% for 1-h, 2-h, and 3-h transient MCAO respectively, when combined with the same duration of contralateral CCAO and permanent ipsilateral CCAO, in normotensive rats. A pSDM of 32% could be reached for 24-h permanent MCAO combined with permanent bilateral CCAO. In a 3-vo transient MCAO model with all three vessels released at the same time after a period of occlusion, the pSDM was 38% (Schielke *et al* 1999) for 3-h transient MCAO, and 32% (Coert *et al* 2003) for 2-h transient MCAO. In hypertensive rats, a 90-min transient MCAO combined with permanent occlusion of the ipsilateral CCA can produce a consistent infarct volume with a pSDM of 25% (Colbourne *et al* 2000).

Using suture tying methods in rats, the pSDM range has been reported as being from 13% to 99%. Selman et al reported the pSDM in a 1-h transient MCAO model to be 99% (Selman *et al* 1994). Better consistency could be achieved by increasing the duration of MCA occlusion. Permanent MCA ligation combined with permanent ipsilateral CCA ligation and transient 60-min occlusion of the contralateral CCA (Chen *et al* 1986) produced a pSDM of 19%. Infarct variation coefficients of 13% have been reported (Drummond *et al* 1995) with 3-h transient MCAO in hypertensive rats.

For MCA cauterization and permanent cut methods, a pSDM range of 6.6% to 149% has been reported. Morikawa reported a pSDM of 53% for cortex infarcts and 34% for striatum infarcts (Morikawa *et al* 1992). In a permanent distal MCAO model with ipsilateral CCA occlusion, Brint et al also reported (Brint *et al* 1988) a pSDM of 16-149% in Wistar rats and 6.6-35% in spontaneously hypertensive rats, which was associated with a more severe infarct in Wistar rats.

A pSDM range of 12.5% to 50% could be reached in a 3-vo Tamura model. Yanamoto et al evaluated a 3-vo model both in normotensive rats and mice, in which the MCA was cauterized and cut permanently, along with temporary bilateral CCAO (Yanamoto *et al* 2003). When the CCAs were released after 60-min, the pSDM reached 50 % in rats and 24% in mice. When the CCAs were released at 2-h post occlusion, the pSDM in rats was reduced to 12%; this is an improvement over their earlier work which described (Yanamoto *et al* 1998) a pSDM of 22% in a 3-vo transient MCAO of 2-h in normotensive rats (all three vessels were released after 2-h occlusion).

7. INFARCTION VOLUME MEASUREMENT

A. METHODS FOR INFARCTION VISUALIZATION

Measuring infarct volume evolution is time sensitive and methodology dependent. Tissue processing for histopathological staining may produce significant volume variation. Definitive determination of cerebral infarct is made by microscopic examination of hematoxylin and eosin (H&E) stained brain sections. Infarcted brain tissue appears as a sharply delineated pan-necrotic area on H&E stained brain sections (Garcia *et al* 1993). On H&E stained brain sections ischemia-induced neuronal morphological changes can be detected within a few hours after MCA occlusion while it usually needs 24-h for these ischemic changes to mature into a well-developed infarct. There are other more sensitive staining methods that can detect ischemic injury as early as 15-min post MCA occlusion. These staining methods include the arginophilic III staining (Czurko and Nishino 1993; Liu and Guo 2000a) and the immunohistochemical staining of microtubule-associated protein 2 (MAP2) (Pettigrew *et al* 1996). The early infarct area revealed using the above-mentioned pathological methods does not usually have enough contrast when compared with adjacent non-ischemic tissue. This makes it difficult for direct macrometric measurement of infarct volume. Alternatively, the macrometric measurement of infarct volume can still be achieved after microscopic delineation of the infarct area (Liu and Guo 2000b). The above mentioned methods also require tissue fixation followed by a complex staining process, which may produce 7-12% variation of hemisphere volume (Overgaard and Meden 2000). Therefore, a standard tissue processing protocol for these methods is needed for reducing variation. Currently, direct macrometric measurement of brain infarction is most often conducted by using 2,3,5-triphenyltetrazolium chloride (TTC) to stain fresh brain sections. The TTC staining method is able to offer a reasonably sharp contrast between infarcted and normal areas as early as 3-h in rats, and 12-h in mice. It is relatively simple to conduct and is widely accepted by most stroke investigators.

B. DIRECT VISUALIZATION OF INFARCT ON TTC STAINED FRESH BRAIN SECTIONS

GUIDANCE—TTC staining is the most widely used technique to identify infarcted versus viable tissue. It is not selective for brain tissue or cell types. A brain matrix or vibratome is necessary for providing clean cut sections. The extent of brain infarction is optimally seen between 24-36 h post ischemia by the staining of fresh brain sections. Species differences in mitochondrial dehydrogenases may account for differences in the times at which infarction can become apparent (see below). In vivo TTC staining should be used only for transient ischemic models in its reperfusion stage after excellent reperfusion has been ensured. Better contrast and infarct boundary delineation may be obtained with use of lower TTC concentration.

SUPPORTING DISCUSSION—TTC serves as a proton acceptor for many pyridine nucleotide-linked dehydrogenases (such as succinate dehydrogenase); it is reduced by these enzymes in viable brain tissue into a red, lipid-soluble formazan, while infarcted or non viable tissue remains unstained (Bederson *et al* 1986a; Liszczak *et al* 1984). The TTC

method requires the brain to be sectioned into several thin parallel sections with even surfaces for infarct volume calculation. It has been reported that there is good correlation between TTC, H&E (Bederson *et al* 1986a; Lundy *et al* 1986), and cresyl violet staining (Tureyen *et al* 2004). Although TTC staining is widely used for infarct volume measurement, there are some issues that need to be considered regarding its use.

Macrophage/glia infiltration may confound the staining results after 36-h post-ischemia. Infiltrating cells may cause staining in infarcted tissue. For example, 36-h after stroke, macrophages and glial cells infiltrate infarcted areas, and result in tissue TTC staining, which would not have been evident at an earlier time point (Liszczak *et al* 1984). Another issue that needs to be considered is the species difference of mitochondrial enzymes (Stewart *et al* 1998). For example, ischemic injury can be visualized as early as 3 hours after stroke in rats (Bederson *et al* 1986a; Liu *et al* 2004), but may require at least 12 hours in mice.

TTC is able to pass the blood-brain barrier, which allows *in vivo* staining (Isayama *et al* 1991). However, such *in vivo* staining relies on the regional cerebral blood flow (Dettmers *et al* 1994), and may only be suitable for transient cerebral ischemia at the reperfusion stage; it cannot be used in permanent cerebral ischemia (Benedek *et al* 2006).

The TTC staining process is affected by several factors, such as TTC concentration, staining duration, and incubation temperature. A methodology paper (Joshi *et al* 2004) demonstrated that staining with lower TTC concentration (0.05-0.1% versus 0.6%) at 37°C for 30-min could reduce non-specific staining and improve contrast between infarcted and normal tissue, and hence provide better delineation of infarct boundaries.

C. DIGITAL METHODS FOR DEFINING THE INFARCTED AREA

GUIDANCE WITH SUPPORTING DISCUSSION—The traditional way of acquiring a digital image of brain infarct is to digitalize the brain section through a stereoscope equipped with a macro lens. TTC-stained brain sections can also be scanned into digital files for automated infarct recognition (Goldlust *et al* 1996). Manual delineation of the infarct area may be needed if the contrast is insufficient for an automatic infarct selection. Due to field limitation of the regular objective lens, additional optical modification may be required in order to be able to view the entire brain section with a regular microscope. For volume calculation, the infarct area must have enough contrast against the non-infarcted area that it can be distinguished from its surrounding areas. Infarct area can be measured using imaging analyzing software such as Image Pro Plus(Liu *et al* 2006), Adobe Photoshop (Horita *et al* 2006), NIH image J (Tureyen *et al* 2004), or other appropriate image processing programs. If the contrast is excellent, as it usually appears on TTC stained sections, the infarct area can be automatically selected and calculated based on color differentiation. With spatial calibration the infarct area can be expressed in real measurement units (e.g., mm^3).

D. CALCULATION OF INFARCT VOLUME

GUIDANCE—When comparing infarction volumes at different time points, cerebral edema and infarct shrinkage should be corrected for.

SUPPORTING DISCUSSION—Ischemic infarction evolution involves different temporal-spatial pathological processes that may influence infarction volume measurement. Studies on the natural progress of infarct evolution show significant differences in infarction volume between early and late time points (Gaudinski *et al* 2008; van der Worp *et al* 2005). Cerebral edema is more severe 2-3 days after acute stroke. Edema may significantly increase the brain tissue volume as well as the directly measured infarct volume. On the other hand,

when an infarct has been evolving for one week, it will begin to shrink because of attenuated edema, tissue loss, and scar contraction. When comparing infarction volumes at different time points, cerebral edema and infarct shrinkage should be adjusted. In this situation, a corrected infarction volume against edema or shrinkage (Leach *et al* 1993; Lin *et al* 1993; Swanson *et al* 1990) will be more suitable.

E. CALCULATION FORMULAE FOR INFARCTION VOLUME

The following formulae can be used for calculating the corrected infarction volume for both edema and shrinkage.

Corrected infarct area = measured infarct area + area of contralateral corresponding structure − area of ipsilateral corresponding structure (Swanson *et al* 1990)

Corrected infarct area = measured infarct area × area of contralateral corresponding structure / area of ipsilateral corresponding structure (Leach *et al* 1993)

Infarction volume for continuous macrosections can be calculated as Σ(thickness × ½ (corrected infarct area of a section's rostral surface + corrected infarct area of a section's caudal surface)

If the infarct volume calculation is based on thin microsections (5-10 μm thickness) at fixed interval, the formula will look like this: Infarct volume = (interval + slide thickness) × (Σ (corrected infarct area) − ½ corrected infarct area of the first slide − ½ corrected infarct area of the last slide)

F. MEASUREMENT OF INFARCTION VOLUME VARIABILITY

GUIDANCE—Use relative volume for comparison between studies and indicate infarct volume variation.

SUPPORTING DISCUSSION—Experimental stroke models are performed with one or more of a variety of possible subject animals according to the needs of the study design. In addition, researchers measure infarct volume using different units and at various time points post ischemia. This situation generally precludes direct comparison of infarction volumes between studies. Standard deviation (SD) reflects the variability in a set of data while standard error of mean (SEM) reflects the accuracy of a mean value. Therefore the SD of infarct volume reflects the stroke model outcome variation. SD can be normalized to its corresponding mean as a Percentage of SD to Mean (**pSDM**). Some authors in the cited papers expressed data as mean ± SEM; in these cases we have converted SEM to SD according to the formula SD = SEM × SQRT N.

8. FUNCTIONAL EVALUATION

A. THE TIMING OF FUNCTIONAL EVALUATION

GUIDANCE—The natural process of functional recovery should be considered in functional evaluation after experimental focal stroke. Evaluation should be conducted at the same post-occlusion time point in all groups but not during the 6-12 hours post-occlusion because at this time accelerated functional recovery occurs. Maximal sensitivity for functional evaluation can be achieved between 2-6 hours post-occlusion.

SUPPORTING DISCUSSION—The extent of functional recovery after stroke is dependent on time, age and environmental factors (Buchhold *et al* 2007). Some functions recover faster and better than others. The most severe sensorimotor deficits can be observed

at 2-6 hours post stroke with a fast recovering speed being observed between 6-12 h post-MCAO (Reglodi *et al* 2003). For validating neuroprotective efficacy, functional tests with a slow or absent natural recovery process may be most appropriate, such as forelimb flexion, gait disturbance, and lateral resistance (Reglodi *et al* 2003). The well-known "circling phenomenon" can be observed as soon as the animal is fully recovered from anesthesia, but may not be apparent when evaluated at 24 hours in some stroke models (Erdo *et al* 2006) despite significant infarct volume maturation at this time. In a permanent MCAO model resulting in cortical infarction, it has been reported that most young rats (3-4 mo) do not show "circling" when evaluated on day 2 post ischemia (Buchhold *et al* 2007). Hence, such a highly time-dependent motor deficit may not be suitable for preclinical neuroprotection stroke studies. In order to achieve a better sensitivity in detecting neuroprotective efficacy, MCAO models of moderate severity should be used and appropriate functional tests should be conducted between 2-6 hours post ischemia because functional deficits usually reach maximum severity at this time. For confirming robust neuroprotection, functional tests that have a slow recovery pattern may be more appropriate.

B. EVALUATION SYSTEMS FOR NEUROLOGICAL FUNCTIONAL DEFICITS

GUIDANCE—Select an appropriate evaluation system to cover important functional deficits. It is reasonable to include more tests to cover different functional deficits and subsequently analyze both the total score and individual scores because each function has a different recovery pattern. Functional tests that have a slow recovery pattern may be most suitable to confirm a neuroprotective effect.

SUPPORTING DISCUSSION—Behavioral changes after ischemic stroke can be evaluated using specially designed scales. Many scales are available for the detection of ischemic injury, but not all scales can be used for the validation of an intervention's neuroprotective capability. To qualify for neuroprotection studies, the neurological scale must be able to detect the major ischemia-induced behavioral changes, including motor, sensory, motion coordination, spontaneous activity, reflexes, consciousness, and alertness changes. Bederson' 4-point scale (Bederson *et al* 1986b), modified Bederson's scale (Becker *et al* 2001; Zausinger *et al* 2000), and Rogers' 8-point scale (Rogers *et al* 1997), although frequently used, are primitive measurements of motor deficits. It may be more appropriate if these scales are merely used for confirming a successful occlusion of middle cerebral artery after completion of surgery. For a more informative functional assessment, more complex evaluation systems like the 18 and 42 point scales should be considered (Chen *et al* 2001; Reglodi *et al* 2003). Although several functional tests have been developed to provide an effective neurological evaluation scale for preclinical neuroprotection studies (Buchhold *et al* 2007; Reglodi *et al* 2003; Schallert 2006), there are no guidelines regarding their use.

C. ANALYSES FOR NEUROLOGICAL FUNCTIONAL DEFICITS

GUIDANCE—Ensure a blind method is adhered to for conducting the evaluation process. Analyze both the total score and individual scores. When a complex battery of tests is being used, stratified analysis of functional deficits will be preferable because the changing pattern will be different in functional deficits. When indicated, use non-parametric statistical methods for data analysis.

SUPPORTING DISCUSSION—During the evaluation process, behavioral scores are given by the examiner based on the examiner's observation and understanding of the tests; therefore, these scores are subject to the examiner's bias. Adapting a blind method for functional evaluation will be necessary for reducing bias in preclinical neuroprotective trials. Moreover, the data set obtained from scaled neurological evaluation may not always conform to a normal distribution, especially when the sample size is small. Non-parametric

statistical analyses should be used if the data can't pass a normality test. For example, one should use a Mann-Whitney U test for two group comparison and a Kruskal-Wallis analysis of ranks for multiple group comparison.

9. ANESTHETICS

GUIDANCE

When designing a preclinical study for neuroprotection, the protection provided by anesthetics should be taken into account. When neurotransmitters or neuroplasticity are the main foci of a study, anesthetics such as urethane, which do not disturb the action of neurotransmitters should be used. Fasting animals should be utilized in the experimental design of neuroprotection studies though caution should be used to reduce hypoglycemia-related mortality when fasting small rodents (mice, gerbils).

SUPPORTING DISCUSSION

Many commonly used anesthetics have neuroprotective effects against cerebral ischemic injury. These anesthetics include isoflurane (Kawaguchi *et al* 2004; Xiong *et al* 2003), sevoflurane (Nakajima *et al* 1997; Payne *et al* 2005), desflurane (Erdem *et al* 2005; Tsai *et al* 2004), halothane (Haelewyn *et al* 2003), xenon (David *et al* 2008), nitrous oxide (Abraini *et al* 2004; Haelewyn *et al* 2008), barbiturates (Warner *et al* 1996), propofol (Bayona *et al* 2004), ketamine (Proescholdt *et al* 2001), and the local anesthetic lidocaine (Siniscalchi *et al* 1998; Weber and Taylor 1994)..

Most anesthetics have been found to potentiate the inhibitory activity of the g-aminobutyric-acid (GABA)-A receptor. Volatile anesthetics are also antagonists of N-methyl-D-aspartate (NMDA) and amino-3-hydroxy-5-m ethyl-4-isoxazol-propionic acid (AMPA) receptors, and openers of K+ channels (Grasshoff *et al* 2005). Therefore, the working mechanism of anesthetics needs to be considered during the experimental design of neuroprotection studies (Maggi and Meli 1986; Sceniak and Maciver 2006). When an expected neuroprotection is likely via the opening of K+ channels, volatile anesthetics may have compounding effect and should be avoid when possible.

Hyperglycemic effects only occur in fed animals, and thus can be eliminated by fasting animals 18-24h before experimentation. Xylazine is an α2-adrenergic agonist, which decreases plasma insulin level and induces hyperglacemia (Greene *et al* 1987; Thurmon *et al* 1984). Hence, xylazine should be avoided when blood glucose level becomes a concern in experimental design. In addition to xylazine's hyperglycemic effects, some commonly used volatile anesthetics, such as isoflurane and halothane, also may cause a rapid increase in blood glucose levels (up to 230 mg/dl or 12.6 mmol/L) within 20min of induction. Ketamine/xylazine can result in hyperglycemia reaching 290 mg/dl (15.9 mmol/L) (Saha *et al* 2005)..

10. TEMPERATURE

A. THE NECESSITY OF MONITORING TEMPERATURE

GUIDANCE—Controlling animal body temperature in a normal range is necessary for eliminating the protective effect of hypothermia and potential harmful effect of hyperthermia (Zaremba 2004).

SUPPORTING DISCUSSION—Brain temperature during hypoxia affects brain metabolism significantly (Winn *et al* 1981). Hypothermia reduces (Florian *et al* 2008; Miyazawa *et al* 2003; Ohta *et al* 2007) and hyperthermia exacerbates (Kim *et al* 1996; Noor

et al 2003; Noor *et al* 2005) ischemic brain injury, hence, fluctuation in animal body temperature will increase the variability of stroke outcome. Ischemia itself also affects post-ischemic temperature regulation, which in turn influences the extent and severity of brain injury and functional deficits (Colbourne *et al* 2000)..

B. MONITORING BRAIN/CORE TEMPERATURE

GUIDANCE—Various methods may be used for monitoring body temperature in stroke animal models. The simplest way of monitoring temperature is by placing a temperature probe in the rectum of the anesthetized animal. Monitoring brain or pericranial temperature may be performed with caution in some experiments when a difference between brain and rectal temperatures is predicted. Temperature monitoring should commence before inducing anesthesia and there is also a need to monitor temperature after surgery.

SUPPORTING DISCUSSION—Monitoring rectal temperature assumes that there are no, or at least there are predictable, differences between brain and rectal temperatures. However, brain temperature may actually be considerably different from rectal temperature during the ischemic period (DeBow and Colbourne 2003; Marion 2004; McIlvoy 2004; Nussmeier 2005). Pericranial temperature can be monitored by placing a subcutaneous needle thermistor adjacent to the skull in the temporal muscle, (Xiong *et al* 2003; Yonekura *et al* 2004) although it should be noted that this measurement is very sensitive to small changes in needle position. Alternatively, for experiments studying the effects of temperature changes during cerebral ischemia, a thermistor can be inserted directly into the brain (DeBow and Colbourne 2003; Menzel *et al* 1998). However, the latter method involves a relatively complex and invasive surgery to insert the brain probe, and runs the risk of causing brain injury and infection.

Hyperthermia or hypothermia can occur because of the surgery, anesthetic, ischemia, and/or unexpected infection. The use of telemetric temperature probes for monitoring post-ischemic body temperature has a significant advantage in its capacity to measure temperature continuously in conscious animals. In addition, telemetry can be used with an automated feedback system for temperature control. Disadvantages of this method are the high cost of telemetric systems and the need for additional surgery to implant probes.

C. MAINTAINING BRAIN/CORE TEMPERATURE

GUIDANCE—Maintaining core temperature within an appropriate range during ischemia can be achieved by using a water heating pad, electric heating blanket, heat lamp and/or heating fan. PID temperature controller equipped heating devices provide fast response and precise temperature control. Electric blankets are not recommended if a telemetric system is being used as they may interfere with the probe signal. Maintaining body temperature after surgery is necessary. This may be done by placing animals in a humidified warm chamber for a few hours.

SUPPORTING DISCUSSION—Since the thermal conduction of a water heating pad may not be rapid, an overhead incandescent lamp or heating fan may serve as a complementary heating source to further control temperature. It must be taken into consideration that the overhead heating source may interfere with the operation by heating the surgical tools and the operator's hands. Temperature control when using electric heating pads and blankets can be improved by using a proportional integral derivative (PID) temperature controller to allow *ex vivo* or *in vivo* feedback from the sensor that monitors heating pad or animal body temperature. The electric current to the heating pad can be either direct current (DC) or alternating current (AC), depending on the experimental design and budget. DC powered heating devices have less electric noise and are well suited for electrophysiological studies.

AC powered heating devices may be used in most preclinical stroke trials that do not need electrophysiological monitoring.

Maintaining body temperature after surgery is just as important as during the ischemia period because the full recovery of animal body temperature regulation needs time (Chang *et al* 2008; Jia *et al* 2006). The most popular method of maintaining body temperature is by using a warm chamber that keeps the environmental temperature at 28-32°C. The considerable advantage of the thermo-controlled temperature regulation system with an in vivo feedback system, as described by Colbourne et al (Colbourne *et al* 2000), is that the investigator can regulate the temperature of conscious animals with precision during the postischemia period. Precise temperature regulation can be achieved with the automated telemetry system, which uses fine water mist with overhead fans for cooling and infrared lamps for heating.

11. MECHANICAL VENTILATION AND BLOOD GAS/GLUCOSE MONITORING

A. PROPER USE OF MECHANICAL VENTILATION

GUIDANCE—The importance of using mechanical ventilation should be determined by the anticipated impact of the surgical/anaestheic procedure on respiratory function. The potential confounding effects from respiratory functional deficits can be minimized by the use of mechanical ventilation. Unnecessary use of mechanical ventilation should be avoided when a particular MCAO model is not likely to cause respiratory problems.

Ventilation may be needed when the operation lasts long (>1 hour) and when the ischemia affects brain stem function. A mixture of 30%:70% (O_2:N_2 or N_2O) may be used for preclinical stroke trials combined with individualised adjustment of ventilator parameters. The concentration of inspired oxygen and ventilator parameters (tidal volume, airway pressure, respiratory rate, inspiratory/expiratory duration) can be roughly determined by a pilot experiment with periodic measurements of arterial blood gases. The respiratory rate and stroke volume can be set differently in accordance with the different “dead space” of each ventilator and anesthetic circuit.

SUPPORTING DISCUSSION—Hypoxia is injurious to the CNS, especially the adult brain. Normobaric hyperoxia (Singhal *et al* 2002) and hyperbaric oxygen treatment (Iwatsuki *et al* 1994; Takahashi *et al* 1992; Wallsh *et al* 1986) have been demonstrated to be neuroprotective during ischemia and reperfusion, but also can have deleterious effects on the normal and injured central nervous system (CNS) (Bostek 1989; Bulte *et al* 2007; Diringer 2008). Similarly, as carbon dioxide is a potent cerebral vasodilator and causes increased cerebral blood flow (CBF) (Kontos *et al* 1977a; Kontos *et al* 1977b; Rusyniak *et al* 2003), hypercarbia may have a protective effect during ischemia and reperfusion (Vornov *et al* 1996). On the other hand, extracellular acidosis caused by hypercapnia may inhibit neuronal functions (Velisek 1998) and cause adenosine triphosphate (ATP) depletion (Yamamoto *et al* 1997). In addition to the above, intubation and mechanical ventilation are commonly used to improve control of blood oxygen and carbon dioxide levels. However, the intubation procedure itself and control of the mechanical ventilation process are technically demanding and may cause tissue damage even in experienced hands. The use of mechanical ventilation will mostly depend on the nature of the experiments. If the experiment is not likely to cause respiratory failure, intubation and mechanical ventilation may not be necessary.

Since respiratory function might be suppressed by anesthesia, endotracheal intubation and mechanical ventilation may be necessary in order to maintain blood gases within the normal

range. This is especially relevant during long operations (>1 hour) and when the ischemia affects brain stem function. The components and concentrations of the inspired gas are essential for maintaining arterial blood gases within a normal range when the airway is secured. Oxygen and nitrous oxide are traditionally used in a mixture of 30%:70% (O_2:N_2O) in rodent stroke models. Because nitrous oxide has been shown to be neuroprotective in ischemia-induced brain injury (Abraini *et al* 2004; Haelewyn *et al* 2008), it may be preferable to use nitrogen mixed with oxygen. Since hyperoxia has been shown to have a neuroprotective effect in brain ischemia (Liu ne*et al* 2006; Singhal *et al* 2005; Singhal 2007), oxygen concentration and pressure in the inspired air should be controlled at a stable level so as to avoid hypoxia and hyperoxia. Therefore, a mixture of 30%:70% (O_2:N_2) may be used for preclinical stroke trials combined with individual adjustment of ventilator parameters. Note, however, that substituting N_2 for N_2O may slow induction and recovery times, and will generally require that the concentration of volatile anesthetics be adjusted upwards.

Several studies (Bottiger *et al* 1999; Olsson *et al* 2003; Yang *et al* 1997; Yonekura *et al* 2004) have shown that with an inspired gas mixture of 30% O_2 and 70% N_2O, the pre-ischemia levels of partial oxygen pressure (PaO_2), partial carbon dioxide pressure ($PaCO_2$), and pH varied from 93.4 ± 21.1 to 208 ± 45 mmHg, from 28.1 ± 4.6 to 40 ± 5 mmHg, and from 7.12 ± 0.04 to 7.39 ± 0.08, respectively. One likely reason for these large variations may be that different tidal volume and respiratory rates were used throughout these studies. For example, the respiratory rate and the stroke volume were set at 120 breaths /min and 0.25 ml in the studies of Olsson et al (Olsson *et al* 2003) whilst they were set as 130 breaths /min 0.7 ml in those by Sheng et al (Sheng *et al* 1999). In addition, the normal resting tidal volume of animals ordinarily will increase, and the respiratory rate will decrease, in proportion to increases in body weight.

B. MONITORING GLUCOSE

GUIDANCE—It is necessary to monitor blood glucose levels before, during and after ischemia, especially in models causing severe brain damage, or in certain newly acquired genetically modified strains. A glucose meter may be preferable to the integrated glucose measurement function of a standard blood gas analyzer. During the post-surgery stage, hypoglycemia can be prevented by proper care. An animal's appetite, food consumption, and body weight should be monitored, and supplemental administration of glucose by gavage or intraperitoneal injection may be needed. Hyperglycemia can be prevented by fasting the animal overnight before surgery. Any observed hyperglycemia is usually not treated, but it may be used as a criterion for subgrouping or excluding animals in data analyses.

SUPPORTING DISCUSSION—Since hyperglycemia can cause exacerbation of ischemic damage, glucose should be routinely measured during experimental stroke (Lovblad *et al* 2003; Parsons *et al* 2002). Many commonly used volatile anesthetics such as isoflurane and halothane cause a rapid increase in blood glucose (Saha *et al* 2005). Some transgenic animals (Rajkumar *et al* 1995; Rajkumar *et al* 1996) might have congenital diabetes or have a tendency to suffer hyperglycemia after an ischemic insult. In contrast, the loss of appetite or inability to access food may also cause hypoglycemia in animals and may potentially affect survival rates and outcomes, especially in small rodents (mice and gerbils).

For blood glucose assay, a glucose meter may give more precise readings than the integrated glucose measurement function incorporated into a blood gas analyser. In addition, a glucose meter uses much less blood than a blood gas analyser and is usually quicker.

C. BLOOD SAMPLING FOR BLOOD GAS ANALYSES

GUIDANCE—Blood sampling is necessary for periodic measurement of arterial blood gas and frequency of measurement should be selected with reference to animal size. Although pulse oximetry for measuring oxygen saturation has been widely used in clinics, its value in middle cerebral artery occlusion (MCAO) models is not clear. It may be considered as an alternative option when blood sampling from mice/gerbils is not possible.

SUPPORTING DISCUSSION—In larger animals, it is feasible to adjust the stroke volume and the respiratory rate of the ventilator based on the periodic measurement of arterial blood gas. However, frequent blood sampling is not possible in small animals like gerbils and mice, due to their limited blood volume. In our experience, two blood samples (0.08ml per time) can be taken in mice using a capillary tube without affecting the survival rate and ischemic outcome. The first sample could be taken 10 minutes after ventilation and before ischemia; the second sample can usually be taken right after ischemia has ended. The first sample is preferably used to determine whether the ventilator is set properly because physiological parameters may change significantly in the post ischemia period (Bottiger *et al* 1999).

12. BLOOD PRESSURE

GUIDANCE

Monitoring blood pressure during experiments is needed because blood pressure fluctuation affects stroke outcomes. Blood pressure can be monitored by non-invasive and invasive methods. Use non-invasive methods for experiments that cause minimal blood pressure fluctuation and require a neurological evaluation. Use invasive methods for experiments that require constant blood pressure monitoring. Blood pressure fluctuation due to cerebral ischemia is usually not corrected during the experiment, although it can be used as a guide to anesthetic depth and the concentration of inspired anesthetic gas can be adjusted if appropriate.

SUPPORTING DISCUSSION

In stroke models blood pressure can fluctuate due to anesthetic depth and changes in animal body temperature. In addition, during the ischemic period, blood pressure may be elevated due to the systemic response in attempting to maintain a normal brain perfusion pressure. The change of blood pressure affects regional cerebral blood flow and hence stroke outcome (Drummond *et al* 2000; Kawaguchi *et al* 2004) and clinical trials (Cole *et al* 1990; Rordorf *et al* 1997; Wise 1970; Zhu and Auer 1995). Therefore, blood pressure should be monitored during experiments, especially when a significant fluctuation of blood pressure is expected.

Non-invasive blood pressure monitors are equipped with tail-cuff devices for artery occlusion and oscillometric pulse detectors for readings. Because of the limited accuracy and the relatively long interval (in minutes) between two sequential measurements, this method is not suitable for experiments that need constant blood pressure monitoring.

Invasive blood pressure monitoring provides constant readings throughout the experiment. A disadvantage of invasive blood pressure monitoring, however, is the need to establish an arterial line to connect to a pressure transducer. Additionally, when cannulation of the femoral artery is utilized, the cannulating process and the wound associated with the arterial line may interfere with subsequent neurological function evaluation because of pain, impaired blood flow and potential nerve injury in the affected limb (Zlotnik *et al* 2008). However, if the arterial line used for blood sampling is also used for blood pressure monitoring the problems are minimized.

Blood pressure is a sensitive indicator for assessing anesthetic depth, and is also an indicator for ventilation efficiency. Anesthetic dose and ventilation can be modified accordingly before the systemic blood gas changes occur. With the exception of experiments specifically designed for studying the effects of blood pressure on brain injury, blood pressure manipulation is usually not suggested when the blood pressure fluctuation is a result of an ischemic insult. In addition, the blood pressure information obtained during experiments may serve as evidence for exclusion or inclusion of animals for the final analyses.

13. THE IMPORTANCE OF A PILOT STUDY

GUIDANCE

A pilot study should be performed before the implementation of a preclinical stroke trial. Stroke model success rate, mortality rate, outcome variation, and sample size should be determined through the pilot study.

SUPPORTING DISCUSSION

Although information regarding the suture size, coating length, insertion length, and related surgical procedures is available in the literature, these parameters may not be optimal for your own experiment. In addition, the intraluminal stroke model demands delicate surgical skill; inexperienced surgeons need a period of time to command the necessary skills for producing acceptably consistent results, which is referred to as the “surgeon's learning curve” (Renzulli and Laffer 2005). For these modeling reasons, a pilot study is needed to find out the optimal parameters for the MCAO suture and the lab settings for any new study. The pilot study will also be helpful for study design because it may provide the closest information for the expected infarct variation, success rate, and mortality rate.

14. IMPLEMENTATION OF THE PRECLINICAL STROKE TRIAL

A. ADHERE TO “GOOD LABORATORY PRACTICE”

The implementation of a preclinical stroke trial should be conducted with high standards so that experimental bias can be minimized. Some journals have set “Good Laboratory Practice” standards for publishing preclinical trials, and only studies fulfilling these standards will be accepted for publication in these journals. As stated in “Good Laboratory Practice” (Macleod *et al* 2009a; Macleod *et al* 2009b; Macleod *et al* 2009c), a preclinical stroke trial should be conducted with a clear methodology which includes at least the following items,:

- Detailed information on animals used;
- Sample Size Calculation;
- Inclusion and Exclusion Criteria;
- Randomization;
- Allocation Concealment;
- Reporting of Animals Excluded From Analysis;
- Blinded Assessment of Outcome;
- Reporting Potential Conflicts of Interest and Study Funding.

B. USE APPROPRIATE STATISTICAL METHODS FOR DATA ANALYSES

It is very important to use the correct statistical method for data analyses. Scaled data (such as neurological evaluation and semi-quantitative data) and categorical data (such as

mortality rates) should be treated with caution because incorrect statistical methods may lead to invalid conclusions. As discussed in the *Functional Evaluation* section, scaled neurological scores may not always conform to a normal distribution and non-parametric statistical analyses should be used if the data can't pass a normality test. For example, one should use a *Mann-WhitneyU*-test (Estevez and Phillis 1997) for two group comparison and a *Kruskal-Wallis* analysis of ranks (Meden *et al* 2002; Onal *et al* 1997) for multiple group comparison. The mortality rate is a type of categorical data; therefore, its analysis should use the *Chi-Square* test (Lu *et al* 2009), not *Student'st*-test (Tang *et al* 2005).

15. FACTORS BEYOND SCIENCE

A. THE NEED FOR SOPS FOR STROKE MODELS

Stroke model procedures, especially those steps that influence model quality, have not yet been standardized. Investigators in each laboratory implement the stroke model with their own lab-settings. In addition, many models have been conducted using suboptimal procedures. This situation makes the results less comparable between laboratories. Standard operational procedures (SOP) for stroke models, if available, may help to improve inter-lab comparability and reduce outcome variations. Relevant organizations, such as the Society for Experimental Stroke (SFES), National Institutes of Health (NIH), National Stroke Association, American Stroke Association, and International Society for Cerebral Blood Flow and Metabolism (ISCBFM) may take a leadership role in promoting development of an SOP for stroke models.

B. TECHNICAL CHALLENGES IN STROKE MODELS

Some variations in stroke model outcomes are due to technical difficulties in stroke model procedures. The following technical challenges comprise a wishlist for the improvement of stroke model quality:

- Non-invasive blood pressure monitor with constant reading.
- Minimally invasive remote brain temperature monitoring.
- Warm chamber equipped with PID temperature controller and in vivo feedback.
- Remote/in vivo blood gas analyser, glucose monitor.
- Light weight microclips for MCAO.
- Micromanipulator for applying microclips, bipolar coagulator on MCA.
- Uniformly shaped and lysing-controllable emboli for embolic MCAO model.
- Optimized surgical tools for MCAO models.
- Method for in vivo detection of cerebral arterial structure variation.

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ABBREVIATION

AC	Alternating Current
ACA	Anterior Cerebral Artery
AComA	Anterior Communicating Arteries

AMPA	Amino-3-Hydroxy-5-M Ethyl-4-Isoxazol-Propionic Acid
ATP	Adenosine Triphosphate
BBB	Blood-Brain-Barrier
CBF	Cerebral Blood Flow
CCA	Common Carotid Artery
CNS	Central Nervous System
DC	Direct Current
dMCAO	Distal MCA Occlusion
ECA	External Carotid Artery
GLP Good Laboratory Practice GABA	γ-Aminobutyric-Acid
H&E	Hematoxylin And Eosin
ICA	Internal Carotid Artery
ISCBFM	International Society For Cerebral Blood Flow And Metabolism
LDF	Laser Doppler Flowmetry
MAP2	Microtubule-Associated Protein 2
MCA	Middle Cerebral Artery
MCAO	Middle Cerebral Artery Occlusion
NMDA	N-Methyl-D-Aspartate
$PaCO_2$	Partial arterial Carbon Dioxide Pressure
PaO_2	Partial arterial Oxygen Pressure
PComA	Posterior Communicating Arteries
PID	Proportional–Integral–Derivative
PLL	Poly-L-Lysine
pMCAO	Proximal MCA Occlusion
pSDM	Percentage Of Standard Deviation To Mean
PTA	Pterygopalatine Artery
rCBF	Regional Cerebral Blood Flow
SAH	Subarachnoid Hemorrhage
SD	Standard Deviation
SEM	Standard Error Of Mean
SFES	Society For Experimental Stroke
SMG Stroke Model Guidelines SOP	Standard Operational Procedures
STAIR	Stroke Therapy Academic Industry Roundtable
tCCAO	Temporary Common Carotid Artery Acclusion

TTC	2,3,5-Triphenyltetrazolium Chloride

DEFINITION

pSDM	percent of SD to mean

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TABLE 1

SUMMARY OF STROKE MODEL SELECTION

Stroke model	Preferred application	Reference
Photochemical model	Neuroprotection is associated with a particular brain cortex region. Free radicals and endothelial injury play major roles in lesion development.	(Chen et al. 2004; De Ryck et al. 1996; De Ryck et al. 1989; De Ryck et al. 2000; Eichenbaum et al. 2002; Futrell et al. 1988; Lozano et al. 2007; Ostrovskaya et al. 1999)
Autologous clot model	Clot-related protection mechanisms.	(Beech et al. 2001; Busch et al. 1997; Harada et al. 2005; Kano et al. 2003; Kano et al. 2005; Kilic et al. 1998; Krueger and Busch 2002; Orset et al. 2007; Romanos et al. 2007; Tejima et al. 2001; Toomey et al. 2002; Vosko et al. 2003)
Endothelin-1 model	Transient to semi-permanent focal cerebral ischemia that need outcome measurements of both histology and neurofunctions	(Biernaskie et al. 2001; Horie et al. 2008; Nikolova et al. 2009; Windle et al. 2006)
Tamura model	Permanent focal cerebral ischemia. Transient focal cerebral ischemia in which functional assessment is not needed.	(Bederson et al. 1986b; Buchhold et al. 2007; Chen et al. 1986; Roof et al. 2001)
Intraluminal model	Permanent and transient focal cerebral ischemia that need outcome measurements of both histology and neurofunctions	(Candelario-Jalil et al. 2008; Khan et al. 2006; Liu et al. 2006; Shimamura et al. 2006a; Shimamura et al. 2006b; Solaroglu et al. 2006; Tsubokawa et al. 2007; Tsubokawa et al. 2006a; Tsubokawa et al. 2006b)

TABLE 2

COMPARISON OF THE INTRALUMINAL MODEL AND THE TAMURA MODEL

Model	Intraluminal	Tamura
Need for craniotomy	No	Yes
Incur brain trauma	No	Yes
Occlusion methods	Monofilaments: heat/flame blunted PLL coated Silicone-rubber coated	Microclips Suture tying Suture/wire retraction Electrocoagulation
Success rate for transient MCAO	60-100%	40-75%
Infarct distribution	Basal ganglia and cortex	Depending on occlusion site. Only cortical infarct occurs if occlusion site is lateral to the olfactory tract
Reported best pSDM	10-20% in rats 5-10% in mice	6.6-16% in rats 10-13% in mice
Functional deficit	Severe and consistent	Mild and inconsistent, especially when the infarct is limited to the cortex