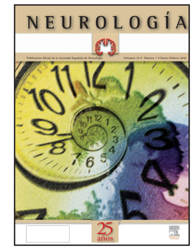
Neurología. 2011;**26(2)**:105-110

NEUROLOGÍA

ELSEVIER DOYMA

www.elsevier.es/neurologia



ORIGINAL ARTICLE

Recommendations guide for experimental animal models in stroke research

L. García-Bonilla, [a] **A. Rosell,** [a] **G. Torregrosa,** [b] **J.B. Salom,** [b] **E. Alborch,** [b] **M. Gutiérrez,** [c] **E. Díez-Tejedor,** [c] **R. Martínez-Murillo,** [d] **J. Agulla,** [e] **P. Ramos-Cabrer,** [e] **J. Castillo,** [e] **T. Gasull,** [f] **J. Montaner,** [a,*]

[a] *Laboratorio de Investigación Neurovascular, Institut de Recerca Vall d'Hebron, Hospital Universitario Vall d'Hebron, Universidad Autónoma de Barcelona, Barcelona, Spain*
[b] *Unidad de Circulación Cerebral Experimental, Centro de Investigación, Hospital Univesitario La Fe, Valencia, Spain*
[c] *Laboratorio de Investigación Cerebrovascular, Hospital Universitario de la Paz, Universidad Autónoma de Madrid, Madrid, Spain*
[d] *Departamento de Neurobiología Molecular, Celular y del Desarrollo, Instituto Cajal, CSIC, Madrid, Spain*
[e] *Laboratorio de Investigación en Neurociencias Clínicas, Hospital Clínico Universitario de Santiago, Santiago de Compostela, Spain*
[f] *Laboratorio de Investigación Básica en Neurociencia, Fundació Institut d'Investigació en Ciències de Salut Germans Trias i Pujol, Badalona, Spain*

Received on 11th January 2010; accepted on 19th March 2010.

KEYWORDS
Experimental design;
Translational research;
Cerebral ischaemia;
Animal models

Abstract
Introduction: The progress of effective therapies for stroke has become a challenging task for both researchers and clinicians. Some pitfalls in clinical trials might have their origins in the pre-clinical experimental ischaemic models for the evaluation of potential neuro-protective agents.
Methods: We aim to standardise the methods for the development of stroke animal models throughout Spain, to produce document with appropriate recommendations and best practice in order to improve experimental methods in the field of stroke research.
Results: Members of several experienced stroke research groups prepared a guide with recommendations in the application of focal cerebral ischaemic models. The main features of this guide are based on the selection of the most appropriate animal model, taking in account the objective of the study, the species, strain, age, sex of animals, as well as risk factors. The experimental design must include a sham control group and the sample size calculation. Animal randomisation and blind analysis, masked assessment of outcomes, monitoring of body temperature and cerebral blood flow, and the reporting of

*Corresponding author.
E-mail: 31862jmv@comb.cat (J. Montaner).

reasons for excluding animals from the study, as well as the mortality rate, are other main points to fulfil in the application of stroke models.
Conclusions: Standardised methods are essential to increase the success of the pre-clinical findings in the stroke neuroprotection field to be able to translate to the clinical practice.

PALABRAS CLAVE
Diseño experimental;
Investigación traslacional;
Isquemia cerebral;
Modelos animales

Guía de recomendaciones en la aplicación de modelos animales para el estudio del ictus

Resumen
Introducción: La búsqueda de una terapia neuroprotectora efectiva para el ictus sigue siendo un reto para investigadores y clínicos. Una de las causas principales por la que a nivel clínico han fracasado terapias eficaces en ensayos experimentales reside probablemente en el desarrollo y modo de evaluación en estudios preclínicos de los agentes neuroprotectores en los modelos animales de isquemia cerebral.
Métodos: Para unificar la metodología en la aplicación de los modelos experimentales a nivel nacional y mejorar la investigación en este campo, se ha elaborado un documento entre varios grupos españoles expertos en investigación neurovascular que constituye una guía de recomendaciones para el uso de los mismos.
Resultados: Sus aspectos fundamentales se basan en la selección del modelo más adecuado en función del objetivo del estudio, teniendo en cuenta el tipo de especie y la cepa animal, la edad, el sexo y los factores de riesgo. La realización del diseño experimental incluye un grupo *sham* control y el cálculo previo del tamaño muestral. Otros aspectos muy importantes a seguir son la aleatorización en la asignación de los animales en cada grupo, el análisis ciego de los parámetros estudiados, el registro de la temperatura y flujo sanguíneo cerebral, así como la notificación y causas de animales excluidos en el estudio y la tasa de mortalidad.
Conclusiones: Es esencial adquirir compromisos metodológicos para la optimización del empleo de los modelos animales de isquemia cerebral que incremente el rendimiento de hallazgos positivos en la fase preclínica y puedan trasladarse a la práctica clínica.

Introduction

As strokes are one of the most common causes of permanent disability and the first cause of death among Spanish women,[1] the search for effective therapies to treat ictus currently constitutes an enormous challenge for both basic and clinical researchers. Despite the evaluation of over 50 neuroprotective agents in Phase III clinical trials that have revealed therapeutic effects in cerebral ischaemia models with rats, mice or gerbils, none has been shown to have conclusive effects on humans.[2,3] Currently the only approved therapy is treatment with tissue plasminogen activator in the hyperacute phase of the ischaemic ictus.[4,5] However, despite their great efficacy, the fact that it must be administered within a narrow therapeutic window of no more than 4 and a half hours from the start of symptoms and the risk of an increase in cerebral bleeding[6] have given rise to fewer than 5% of stroke patients benefiting from thrombolytic therapy.[7] All of this means that it is fundamental to identify new neuroprotective or neuroreparative agents that may be useful for treating ictus and to understand why we have witnessed so many therapeutic failures when promising drugs are transferred from the laboratory to clinical practice.

Some of the reasons the neuroprotective drugs have not been shown to be effective in clinical trials are probably due to a failure in the development and evaluation of the animal models for cerebral ischaemia. The critical factors to be optimized because they affect the success rate and the variability of the models include, among others, the selection of each experimental model depending on the purpose of the study (for a review of the types of experimental models, please refer to the bibliography[8]), the use of appropriate anaesthesia, the maintenance and monitoring of animals in a physiological setting and the observation and analysis of the results eliminating any kind of bias.[9-11]

The present paper reviews the most critical aspects for the development of animal experimentation models for the study of ictus and publishes the most important recommendations in their application.

Table 1 Recommendations of the cerebral focal ischaemia model to be used by study type

Model	Study	Recommendations for application	Recommendations for "non-use"
Intraluminal suture model; proximal occlusion of the MCA	Neuroprotection in acute phase (studies over 24-72 h)	*Transient*: use occlusion for 1 to 2 hours *Permanent:* it is advisable to perform an angiography using magnetic resonance to confirm the occlusion of the artery throughout the study period. It is advisable to have an extra group to confirm the protective action of the agent in the long term (23 weeks)	Do not use when using thrombolytic therapy with tPA
	Neurorepair (studies over several weeks)	Monitoring for at least 21 days	
Embolic Model	Application of thrombolytic drugs and co-treatments with these medicines	If the study also includes the assessment of the incidence of haemorrhagic transformation, it is advisable to use hypertensive rats	
Endothelin model	Animal simulation of a lacunar infarction		Do not use any light source other than cold light to induce thrombosis
MCA distal occlusion model	Studies of neurorepair, neuroplasticity, angiogenesis, etc. requiring medium- or long-term monitoring		If mice are used, do not apply unilateral or bilateral occlusion of the carotid arteries. Do not use if the oedema is the target for the study, as craniotomy is necessary for application of this model

Purpose

The purpose of the "Recommendations guide for experimental animal models in stroke research" is to unify the methodology used in the development of experimental models, clarify and establish the critical factors to optimize the results and improve research in the field of cerebral ischaemia nationwide. It sets out the common action lines of the members of the different Spanish animal experimentation laboratories that took part. The guide was produced following the meeting entitled *Lost in (stroke) translation. Barcelona Think Tank Project (July 17th-18th, 2009)* with the support of the RENEVAS network and constitutes a document for the future consensus on actions and recommendations based on the aspects dealt with at the meeting and the discussions of the committee members. The following section describes the main points it contains.

Development

Selection of the cerebral ischaemia animal model most suitable for each study

The correct cerebral ischaemia animal model to be used will depend on the purpose of the study. The choice of model will mainly be based on the ischaemic physiopathology process the study is focusing on and will take into account the reproducibility of the variables to be studied for each model. The selection of the model will depend, among other factors, on the permanent or transient mode of ischaemia applied, depending on whether or not there is an interest in the pathophysiology of the of ischaemic reperfusion; on the intensity of the cerebral ischaemia and the involvement of the brain areas desired for the study; on the duration of the experimental study (acute or chronic study) and on application in drug administration. Table 1 describes the most important recommendations on the type of model to be used depending on the purpose of the study.

Selection of species/strain, age, sex and risk factors

To select the best model, it will be essential to consider the type of animal to be used, with the following factors having great relevance: i) species, ii) strain, iii) sex, iv) age and v) risk factors. The main characteristic to bear in mind is that the neurovascular anatomy differs greatly from one strain to another. The result in terms of the size of the ischaemic lesion will vary greatly depending on the existence of a larger or smaller number of collateral ramifications irrigating the territory of the MCA. The rodent species used by our groups are rats and mice. The most appropriate rat strains for these models are the Wistar strain, although the use of rats from the Wistar Kyoto sub-strain is not advised as they develop very small infarction sizes, and the Sprague Dawley (SD) strain. Problems have been detected in the SDOFA substrain with regard to the variability between batches affecting the size of the infarction.

The importance of the use of mice is determined by the use of genetically modified animals. The specific use of a particular strain is not recommended, although it is important to remember that the volume of cortical infarction may vary enormously between strains. For example, the infarction in FVB mice may be up to three times smaller than in BALB/C mice.

With respect to sex, the recommendation is to replicate the study in a particular group using the other sex and to evaluate at least the main *end-point* of the study. This recommendation is aimed basically at works designed to establish the therapeutic effect of an agent.

On the other hand, our groups have used young adult animals (250-350 g in rats; 812 weeks in mice, 2530 g) for the study of cerebral ischaemia. For the study of ictus risk factors streptococci was applied in the case of diabetes, while for hypertension spontaneously hypertensive rats (SHR) should be used or the "2K1C" model should be contemplated for the induction of hypertension.[12]

Realization of the experimental design

The preparation of the experimental design prior to the execution of the study is essential for the correct development of the models. The points described below must be considered in the experimental design:

Experimental study groups

The realization of a sham control group will be included in the experiment. In addition, the use of old controls for the comparison of results should be avoided and, where appropriate, a control group of animals should be used to confirm that the study end-points are reproducible between the old animals and the new ones operated on.

Sample size

It is recommended to calculate the sample size for use in each study (using a 1 β power of 0.8 and a level of significance α of 0.05). Once the exclusion criteria have been applied, there must be at least n = 10 valid animals in each experimental group.

Randomization

The allocation of each animal to an experimental group will always be done on the basis of a random choice, and the use of computer software is recommended.

Blinding of the allocation during the experimental process

It is recommended that the researcher responsible for inducing the ischaemia/reperfusion and for taking decisions on the end-point criteria should be unaware of the experimental group the animal belongs to.

Blinding of treatment

It is advisable for the administration of treatment by a researcher to be done blind. In order to achieve this goal, the use of treatment encoding strategies is proposed.

Blind evaluation and analysis of the study variables

It has been established that, when assessing the various study parameters (for instance, infarction volume, neurological deficit, …), the researcher in charge of conducting this assessment should not know the treatment group the study subject belongs to.

Inclusion and exclusion criteria

It is of significant importance to define these prior to starting the study. It is established that each group will take this decision with regard to the definition of these criteria (for example, the exclusion of animals that fail to develop a minimum size of ischaemic lesion or a minimum score in the neurological deficit analysis).

Registration of variables in the preparation of the models

The measurement of cerebral perfusion in the region irrigated by the MCA using Doppler laser flow metering must unavoidably be performed when using cerebral ischaemia models to ensure the occlusion and reperfusion of the artery and to corroborate the success of the operation. In addition, its use is recommended in permanent models to confirm homogeneous occlusion. The cerebral flow thresholds established for considering occlusion and reperfusion of the MCA to be acceptable are indicated in table 2, and these should be treated as inclusion/exclusion criteria.

Body temperature (use of a rectal probe) will be recorded at all times and must remain at 37 ± 0.5°C during the surgical procedure. The use of a cranial probe in the temporal muscle is advisable. As for gasometry, the measurement of arterial pressure and heart rate, these will be recorded whenever intubation of the animal is required, during anaesthesia or ischaemia. It is not strictly necessary if the surgery is not so aggressive (for example, without intubation).

Anaesthesia and analgesia

It is advised to replace the use of inhaled anaesthesia using isoflurane by sevoflurane. For analgesic treatment, magnesium metamizole is recommended, and notice must always be given of which drug has been used.

Stabulation conditions

Twelve-hour light and dark cycles must be used and the animals must be kept in a controlled environment with optimal temperature (22°C) and humidity (50%). The network has experience with the differences that dietary composition may entail for some experiments, so it is recommended to feed animals with a known diet and to ensure water is freely available. Suspending food the day prior to surgery is something most of our groups do not do. The possibility of determining glycaemia prior to performing the surgical procedure should also be taken into account.

Notification

All the aspects contemplated in the preceding paragraphs will be notified in the publications arising out of a study. In addition, notice must be strictly provided for the mortality rate in the study together with the number of animals excluded and why.

Table 2 Cerebral blood-flow thresholds established as inclusion/exclusion criteria in the occlusion and reperfusion of the MCA*

Doppler laser model	Reduction in flow during occlusion	Increase in flow during reperfusion
Perimed	65%of baseline	80%of baseline
Moor-Lab	75%of baseline	80%of baseline

*Thresholds established in rats.

Evaluation of the study parameters, expression of the outcomes and statistical applications: infarction size and neurological assessment

To avoid variability, it is desirable for the various analyses and procedures for the assessment of these parameters to be conducted by the same person insofar as this is possible. The calculation of the infarction volume must also be made taking into account the correction for cerebral oedema and it is recommended to calculate and express the result in terms of cortical infarction and striate or sub-cortical infarction separately, as well as the total volume infarcted.

The authors feel that there are currently no adequate neurological tests. Of those available and depending on the study phase, it is recommended to use the Bederson test[13] or tests based on the Bederson test (for example, a 09 scale[14]) for studies of neuroprotection in the acute phase and to assess the possibility of performing functional tests in the short term. For chronic phase studies (neurorepair studies), it is recommended try out neurological scales (for example, the Bederson test) and to perform functional tests; the *Corner test* and *Sticky Label*[15] tests are advised. The functional tests must be evaluated at the same time of day for all animals in the study.

With respect to the expression of the data obtained, the recommendation is that, in the case of those with a normal distribution, they should be reflected as mean ± standard deviation or by providing the 95% confidence interval; for non-normal datasets, it is advisable to sue the median and the interquartile range. In either case, a check must be made that the distribution is normal before using parametric statistical tests. On the other hand, it is recommended that the graphic representation of the infarction volume should also be given in a bar chart, using representation by points, so as to be able to observe the individual values of each animal.

Conclusions

One of the most important causes of bias in clinical evidence on the efficiency of neuroprotective strategies in the experimental study of ictus lies in the design, methodology and notification of results when using animal models. In order to ensure translational success in the advance of stroke treatment, effectively reduce the number of animals used and avoid unnecessary financial expense, teamwork is required in the scientific community. It is essential to undertake methodological commitments for the optimization of the use of cerebral ischaemia animal models so as to increase the yield of positive findings in the field of science. Following the recommendations set out in this guide, such as the choice of a suitable model, monitoring temperatures, measuring cerebral blood flow using Doppler laser flow meters and the notification of the mortality rate when publishing the results, will improve the quality of the studies and generate extremely useful pre-clinical findings that can then be put into practice in the clinical setting.

Funding

RENEVAS (RD06/0026/0010) and Neurovascular Research Laboratory, "Institut de Recerca Vall d'Hebron", Barcelona.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to Dr. Anna Planas, Dr. Xiaoying Wang, Dr. Malcom McLeod, Dr. Dennis Vivien and all the members of the "Red de Investigación Cooperativa Neurovascular" (RENEVAS, Neurovascular Co-operative Research Network) who took part in the meeting on *Lost in (stroke) translation. Barcelona Think Tank Project (July 17th-18th, 2009)* for their interesting contributions.

References

1. Álvarez-Sabin J. Ictus isquémico. Madrid: Internacional de Ediciones y Publicaciones S.A; 1998.
2. Fisher M. El objetivo del tratamiento en la enfermedad cerebrovascular aguda y los enfoques terapéuticos neuroprotectores. Rev Neurol. 1999;29:536-44.
3. Cheng YD, Al-Khoury L, Zivin J.A. Neuroprotection for ischemic stroke: two decades of success and failure. NeuroRx. 2004;1:36-45.
4. Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med. 1995;333:1581–7.
5. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5hours after acute ischemic stroke. N Engl J Med. 2008;359:1317-29.
6. The NINDS t-PA Stroke Study Group. Intracerebral hemorrhage after intravenous t-PA therapy for ischemic stroke. Stroke. 1997;28:2109-18.

7. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al., American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2010 update: a report from the American Heart Association. Circulation. 2010;121:e46-e215.
8. Prieto-Arribas R, Moreno-Gutiérrez A, Simal-Hernández P, Pascual-Garvi JM, Matías-Guiu JM, Roda G, et al. Experimental models of cerebral ischemia. Rev Neurol. 2008;47:414-26.
9. Liu S, Zhen G, Meloni B, Campbell K, Winn R.H. Rodent Stroke Model Guidelines for Preclinical Stroke Trials (1st Edition). Exp Stroke Transl Med. 2009;2.
10. STAIR. Recommendations for standards regarding preclinical neuropro-tective and restorative drug development. Stroke. 1999;30:2752-8.
11. Macleod MR, Fisher M, O'Collins V, Sena ES, Dirnagl U, Bath PM, et al. Good laboratory practice: preventing introduction of bias at the bench. Stroke. 2009;40:e50-2.
12. Thuiston H, Bing RF, Swales J.D. Reversal of two-kidney, one clip renovascular hypertension in the rat. Hypertension. 1980;2:256-65.
13. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke. 1996;17:472-6.
14. Perez-Asensio FJ, Hurtado O, Burguete MC, Moro MA, Salom JB, Lizasoain I, et al. Inhibition of iNOS activity by 1400W decreases glutamate release and ameliorates stroke outcome after experimental ischemia. Neurobiol Dis. 2005;18:375-84.
15. Schallert T, Woodlee MT, Fleming S.M. Disentangling multiple types of recovery from brain injury. In: Krieglstein J, Klumpp S, editors. Pharmacology of cerebral ischemia. Stuttgart: Medpharm Scientific Publisher; 2002. p. 201-16.