

Global brain ischemia in Mongolian gerbils: Assessing the level of anastomosis in the cerebral circle of Willis

Nelvys Subirós Martínez^{1**}, Jorge Martín Machado¹, Héctor Pérez Saad², Rosa M. Coro Antich³, Jorge A. Berlanga Acosta¹, Sandra Rodríguez Salgueiro⁴, Gerardo García Illera¹, José Suárez Alba¹, and Diana García del Barco Herrera^{1#}

¹Department of Pharmaceutics, Center for Genetic Engineering and Biotechnology, La Habana, Cuba, *Email: nelvys.subiros@cigb.edu.cu; ²Department of Experimental Neurology, Institute of Neurology and Neurosurgery, La Habana, Cuba; ³National Center for Medical Information (INFOMED), La Habana, Cuba; ⁴Electron Microscopy Laboratory, National Center for Scientific Research (CNIC), La Habana, Cuba

Both Authors contributed equally to this work

The Mongolian gerbil has been widely used as a global brain ischemia model because of its incomplete cerebral circle of Willis. However, the inter-individual anatomic variability of this vascular structure interferes with the reliability of the model. The aim of this work was to introduce modifications to the protocol of global brain ischemia experiments in Mongolian gerbils in an attempt to increase the reliability and usefulness of this model. Our study focused on the assessment of the level of anastomosis of the cerebral circle of Willis in order to evaluate its contribution to clinicopathological outcomes in this model. Sham-operated, Ischemic, and Ischemic + Hypothermia animals were subjected to a 15-minute occlusion of the common carotid arteries. Transcardiac perfusion with bromophenol blue / gelatin solution was performed 72 hours after ischemia. Brains were processed for anatomopathological analysis. Tissue damage was observed in the hippocampus, caudate-putamen nucleus, neocortex, and thalamic nuclei of animals from the Ischemic group. The circles of Willis of the Sham-operated animals showed bilateral (38%), unilateral (48%) or no posterior communicating arteries (14%). A negative correlation between infarct volume and the level of anastomosis was revealed for the Ischemic, but not for the Ischemic + Hypothermia group. Additionally, Analysis of covariance (ANCOVA) was performed to assess the contribution of the level of anastomosis to the clinicopathological outcomes. It was confirmed that the infarct volume decreased in the Ischemic + Hypothermia group when compared to the Ischemic group. Since the level of anastomosis cannot be predicted, this variable should necessarily be considered when analyzing the results of global brain ischemia in Mongolian gerbils.

Key words: global brain ischemia, cerebral circle of Willis, Mongolian gerbil, animal model

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*) has been widely used as a model of global brain ischemia for both the elucidation of the underlying mechanisms of the disease and the preclinical evaluation of neuroprotective agents (Kirino 1982, Traystman 2003). Levine and Sohn were the first to describe that most Mongolian gerbils lack the posterior communicating arteries (PComA) which connect the vertebrobasilar

and the carotid arterial systems (Levine and Sohn 1969). The vascular structure resulting from these anastomoses is known as the cerebral circle of Willis, and is located at the base of the brain (Kenney 1998). Thus, a bilateral carotid occlusion is expected to result in global brain ischemia. These ideas have prevailed as a dogma and the Mongolian gerbil transcended due to its incomplete cerebral circle of Willis.

The model of brain ischemia in gerbils is commonly based on a 5-minute bilateral carotid occlusion, which restricts histopathological changes to the CA1 region of the dorsal hippocampus (Kirino 1982). However, the major problem with this model is its lack of reproducibility, probably owed to the

Correspondence should be addressed to N. Subirós Martínez
Email: nelvys.subiros@cigb.edu.cu

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anatomical variability of the cerebral circle of Willis found in different populations of Mongolian gerbils (Laidley et al. 2005, Seal et al. 2006). That might be the main difficulty in obtaining reproducible bilateral hippocampal damage, especially if short-time carotid occlusion is used (Laidley et al. 2005). Thus, it might be reasonably assumed that increasing occlusion time and monitoring the level of anastomosis in the cerebral circle of Willis would result in a more robust and reliable model of global brain ischemia. The relative simplicity of the procedure deserves this recommendation to be observed.

The aim of this work was to modify the model of global brain ischemia in gerbils in order to obtain more reliable results. Specifically, the occlusion time was increased to 15 minutes, and the contribution of the level of anastomosis in the cerebral circle of Willis to the clinicopathological outcomes was considered for the interpretation of the results. A positive control

group subjected to hypothermia was included, since this procedure is well recognized as a neuroprotective strategy.

METHODS

Procedures involving animals and their care were conducted in conformity with the guidelines established by the Program for the Use and Management of Laboratory Animals for Experimental Purposes in the Center for Genetic Engineering and Biotechnology (CIGB, La Habana, Cuba). Experiments were approved by the Animal Use and Care Committee of the CIGB (La Habana, Cuba).

Adult male Mongolian gerbils (25 to 30-week-old *M. unguiculatus*, 73 ± 5 g body weight) were obtained from the National Center for Laboratory Animal Breeding (CENPALAB, Cuba). Animals were individually housed and maintained under a light-dark cycle of 12 hours, with free access to

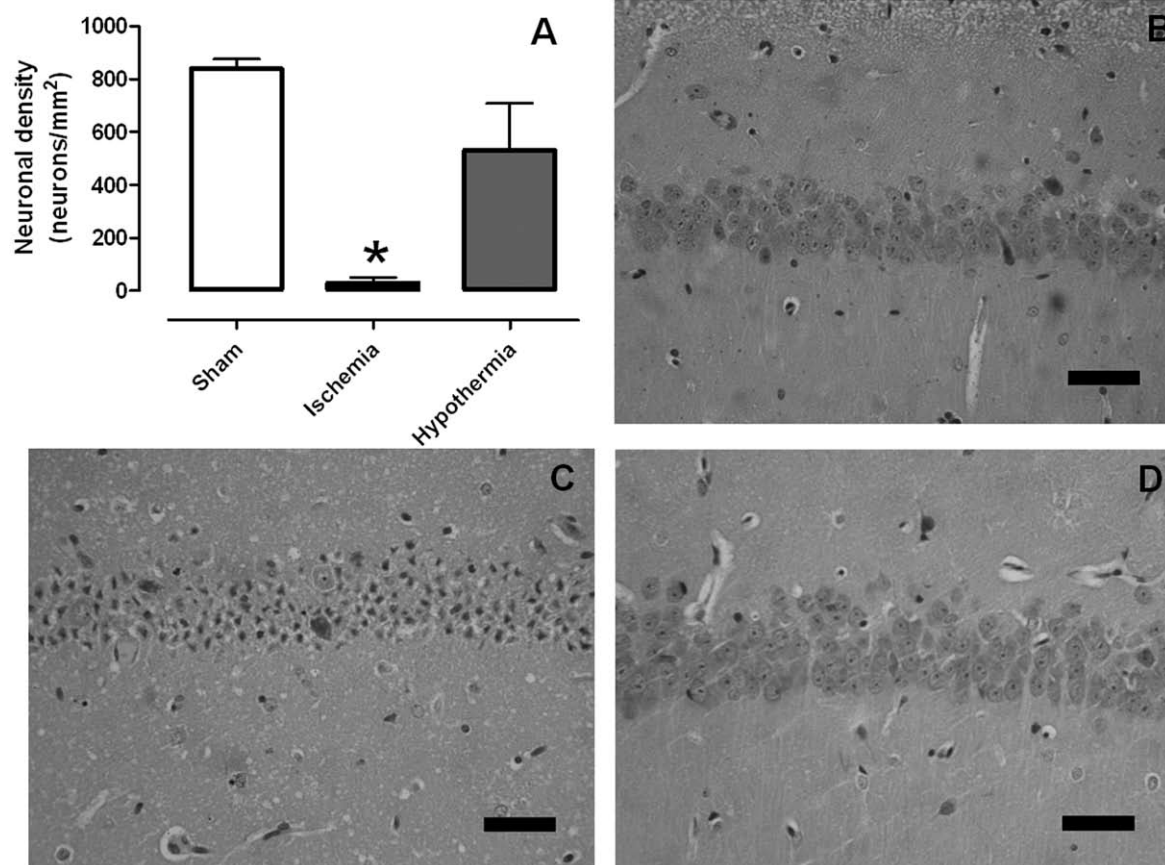


Fig. 1. (A) Neuronal density in the hippocampal CA1 region. (B) CA1 region of a Sham-operated animal. (C) CA1 region of an Ischemic animal. (D) CA1 region of an Ischemic + Hypothermia animal, showing a normal morphology. Scale bars are 50 μ m.

food and water. Gerbils were allowed to acclimate to laboratory conditions for 1 week prior to surgery.

Ischemia/Sham insult

Animals were randomly distributed in three experimental groups: (1) Sham-operated ($n=29$), (2) Ischemic ($n=53$), and 3) Ischemic + Hypothermia, ($n=11$). Gerbils

were anesthetized with chloral hydrate (400 $\mu\text{g/kg}$) and a ventral midline neck incision was performed in order to expose the common carotid arteries. Care was taken to avoid damaging the vagus nerve (Colbourne et al. 1998). The ischemic groups were subjected to 15 minutes of bilateral carotid artery occlusion by using micro-aneurysm clips. The Sham-operated animals were subjected to the same surgical procedure, except for the carotid artery occlusion. During surgery and

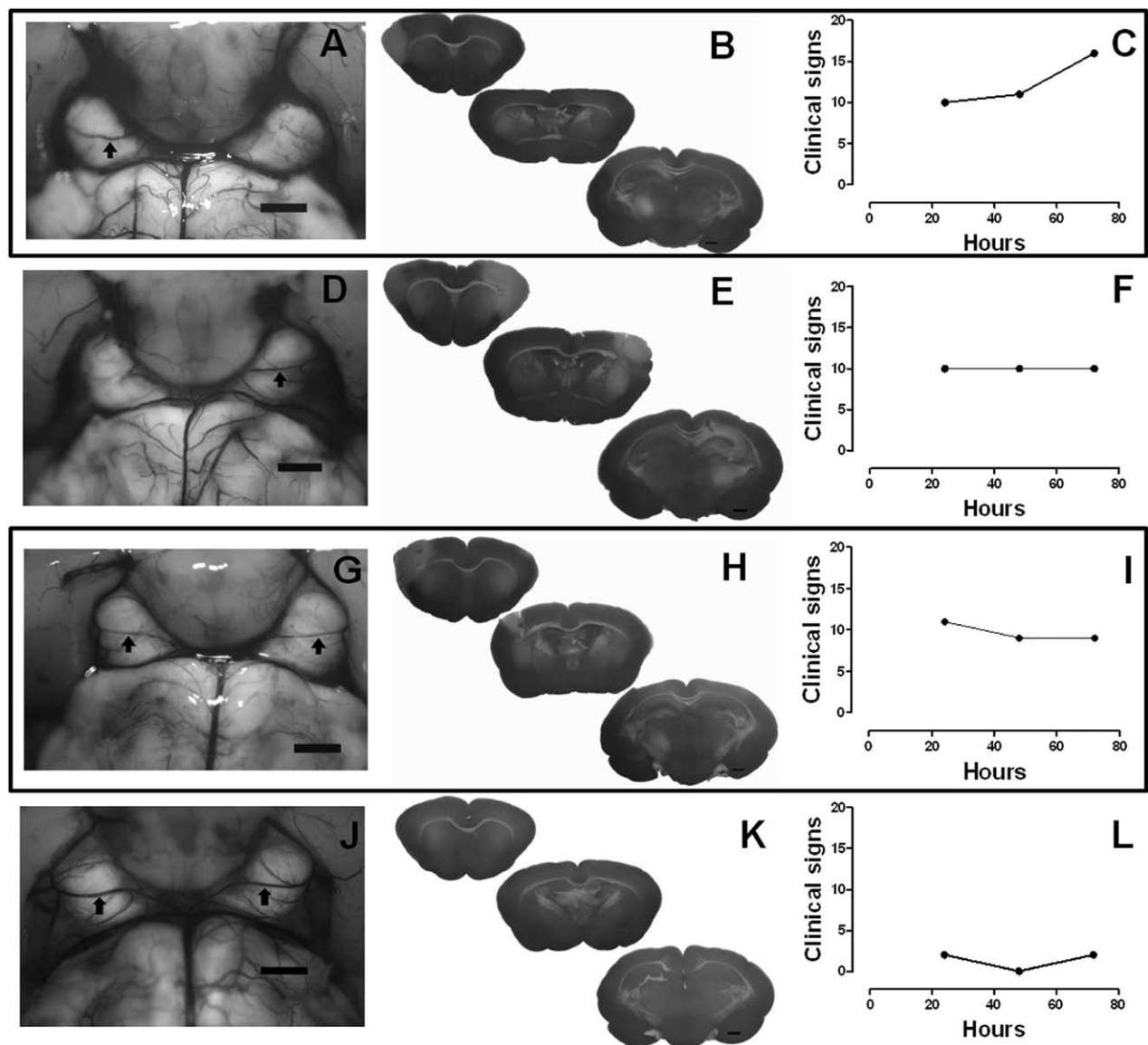


Fig. 2. Influence of the characteristics of the cerebral circle of Willis on infarct volume and clinical signs in four representative Ischemic animals. Each row corresponds to one animal. A, D, G, J: Posterior cerebral vasculature. B, E, H, K: TTC-stained serial slices. C, F, I, L: Clinical evolution of infarct signs. In the Ischemic group, all animals lacking both posterior communicating arteries (PComAs) died before 72 hours. Unilateral infarcts were found in the hemispheres with PComA deficiency (rows 1 and 2). In the cases of bilateral communication, the PComAs diameter influenced both the extent of injury and the clinical score (rows 3 and 4). The arrows indicate PComAs connecting both arterial systems. Scale bars are 1 mm.

Table I

Distribution of posterior communicating arteries (PComAs)			
Experimental groups	NoPComAs	Unilateral PComA	Bilateral PComAs
Sham	14%	48%	38%
Ischemic	0%	56%	44%
Ischemic + Hypothermia	9%	64%	27%

Percentages are referred to the total number of animals of each group

for the whole anesthetic time (2 hours), groups 1 and 2 were maintained at $37 \pm 0.3^\circ\text{C}$, by means of a heating blanket. Rectal temperature was systematically monitored using a digital thermometer (Digi-sense, Cole-Parmer, Barnant Company, USA). Animals from group 3 were operated without any warming procedure, and body temperature was maintained at $32 \pm 0.8^\circ\text{C}$ during the whole anesthetic time. Room temperature was $26 \pm 1^\circ\text{C}$.

Neurological tests

To reveal clinical signs of infarction, animals were examined at 24, 48 and 72 hours after reperfusion, according to the criteria proposed by Lawner and coauthors (1979), with some modifications. The score assigned to each animal was called neurological grade and included the evaluation of palpebral ptosis, grip strength, flexor reflex, body posture, gait pattern (including walking speed and circling), exploring activity and handling-associated seizures. The normal condition was assigned a score of 0. When the neurological grade involved continuous seizures or extreme prostration the animals were euthanized in order to avoid unnecessary suffering.

Vasculature and injury assessment

At day 3, animals were deeply anesthetized with diethyl ether and trans-cardially perfused with 7 ml of phosphate-buffered saline (PBS) followed by 0.5 ml of blue bromophenol / 7% gelatin solution in order to dye the cerebral arteries (Wang et al. 2002, Seal et al. 2006). The brains were quickly removed, cooled in PBS at $0-4^\circ\text{C}$ and immediately sectioned into 1-mm coronal serial slices. The most caudal cut was made at -1.8 mm

from Bregma. Isolation and slicing of the brain were completed within 10 min after perfusion. These serial slices were immediately incubated in complete darkness at 37°C for 20 minutes in a flat-bottomed covered dish with 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) solution. After staining, the slices were washed with PBS (three washes, 1 min each) and fixed in 4% formaldehyde at room temperature for histological evaluations.

The stained slices and the cerebral circle of Willis (studied in the caudal region, from -1.8 mm to -6 mm from Bregma) were photographed under a stereomicroscope (Zeiss, Stemi 2000, Germany). The PComAs, if any, were visually assessed and photographed to confirm the anastomosis between carotid and verte-brobasilar arterial systems. Afterwards, all brain regions were processed for histological analysis.

Image analysis and histological evaluation

The diameter of communicating arteries was measured by means of the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Multiple measurements of artery diameters were made and the smallest artery diameter was recorded for analysis.

The infarct area was also measured using the ImageJ software. Data were collected specifically for the following areas: caudate-putamen nucleus, cerebral cortex, hippocampus and thalamic nuclei. Finally, total infarct volume was calculated by multiplying the infarct area by the slice thickness. After TTC staining, all the slides were immersed in paraffin, cut and stained with hematoxylin-eosin for histological examination.

Neuronal density was determined in hippocampal CA1 sections. A regular field of CA1 hippocampus

from both hemispheres was digitalized (40× magnification). The total area of each image was 118 604 μm^2 . Viability was assessed according to the following criteria: sharply delineated nucleus with ellipsoid or round shape; clearly distinguishable nucleolus located centrally within the nucleus; nucleus slightly darker than surrounding neuropil; neuronal cytoplasm clearly demarcated from surrounding neuropil and less than one third of the neuron surrounded by confluent vacuolization (Stummer et al. 1994).

Statistical analysis

The softwares GraphPad Prism (Graphpad Software Inc., San Diego, CA, USA) and Statistica 8.0 (StatSoft, 2007) were used. Survival data were analyzed using the log-rank test. For each group, the relationship

between the level of anastomosis and infarct volume or neurological grade was studied by calculating the Spearman's rank correlation coefficient. Differences in infarct volume among groups were assessed using analysis of covariance (ANCOVA); the level of anastomosis was used as a covariate. All tests were performed at a significance level of 5% ($\alpha=0.05$).

RESULTS

Clinical and histopathological results

Three days after the induction of global brain ischemia with a 15-minute carotid occlusion, there was a 42% mortality rate in the Ischemic group, contrasting with no deaths in the Ischemic + Hypothermia group. Most of the deaths occurred within the first 24 hours

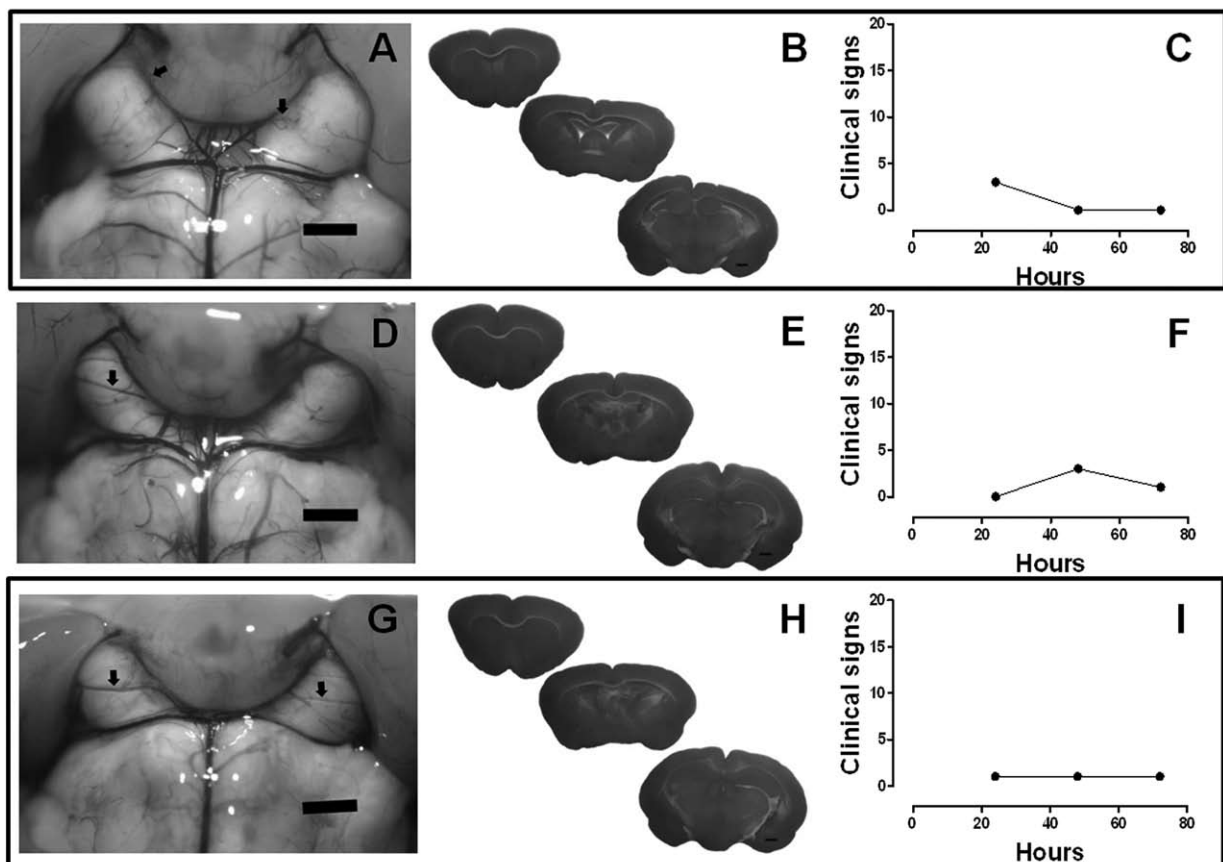


Fig. 3. Posterior cerebral vasculature (A, D, G), infarct lesions (B, E, H) and clinical evolution of infarct signs (C, F, I) of three representative Ischemic +Hypothermia animals. Each row corresponds to one animal. The hypothermic treated animal represented in the first row had no anastomosis between the arterial systems; the arrows indicate vessels that do not reach the carotid system. Second and third rows correspond to hypothermic animals with unilateral or bilateral anastomosis, with no infarct lesions and low clinical scores. The arrows indicate posterior communicating arteries connecting both arterial systems. Scale bars are 1 mm.

Table II

Clinical and morphological data of experimental groups			
Experimental groups	Mortality (%)	Neurological grade (Mean \pm SD)	Infarct volume (mm ³) (Mean \pm SD)
Ischemic	42	8.1 \pm 4.7	15.8 \pm 14.3
Ischemic + hypothermia	0*	1.2 \pm 1.3*	0.007 \pm 0.02*

of ischemia, mainly as a consequence of generalized tonic-clonic seizures. This mortality rate is similar to those of previous reports (Stummer et al. 1994).

As for neurological symptoms, some animals from the Ischemic group were hyperexcitable on handling during the first 24 hours post-reperfusion. At 72 hours post-reperfusion the major neurological signs were related to gait abnormalities (circling, slow movements), decreased grip strength and body weight reduction. None of these signs were observed in the Ischemic + Hypothermia or the Sham-operated animals.

The histopathological analysis of the Sham-operated animals showed that all cerebral regions maintained their normal morphology; even the CA1 pyramidal neurons of the hippocampus were fully preserved (Fig. 1A, B). In all Ischemic gerbils, areas of tissue damage were observed in the caudate-putamen nucleus and in the hippocampus (Fig. 1A, C and Fig. 2). Fifty-two percent of the animals showed well-defined areas of necrosis in at least one of the following regions: neo-

cortex, caudate-putamen nucleus, thalamic nuclei or hippocampus. The rest of the Ischemic gerbils showed selective neuronal death in the hippocampus and caudate-putamen nucleus, whereas glial and endothelial cells were partially preserved. The Ischemic + Hypothermia group did not exhibit any necrotic area (Fig. 1A, D and Fig. 3).

Evaluation of the anastomosis in the cerebral circle of Willis

The posterior communicating arteries had a mean diameter of $36.4 \pm 12.4 \mu\text{m}$. The distribution of the animals with unilateral, bilateral or no PComA in the cerebral circle of Willis is shown in Table I. It is noteworthy that all the survivors of the Ischemic group had at least one PComA, i.e. there were no animals in this group lacking both PComA at the time of the vasculature assessment.

A negative correlation between the total infarct volume and the diameters of the PComAs was revealed for

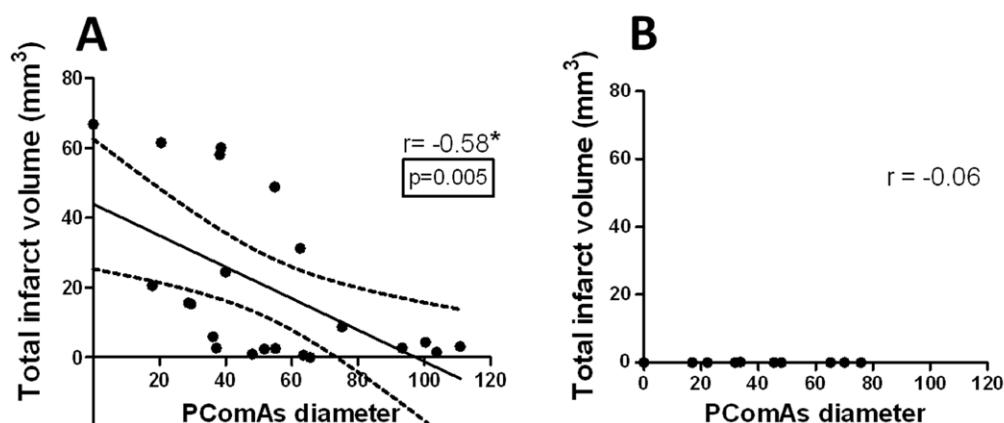


Fig. 4. Spearman's rank correlation between total infarct volume and diameter of the posterior communicating arteries in ISCH (A) and ISCH+Hypothermia (B) animals. (r) Spearman r .

the Ischemic group (Fig. 4A). On the contrary, no correlation was found in the Ischemic + Hypothermia group (Fig. 4B). When comparing the infarct volume in the Ischemic and the Ischemic + Hypothermia groups with ANCOVA (to consider the level of anastomosis as a covariate), a smaller injured area was demonstrated for the latter group ($F_1=33.2$; $P=0.000003$). (Table II).

DISCUSSION

The cerebral circle of Willis is a major source of collateral blood flow supply in the human brain, and developmental morphologic variants are very frequent in this vascular structure, particularly those regarding the anastomoses in the posterior circulation (Cucchiara and Detre 2008, Manninen et al. 2009). Krabbe-Hartkamp and coauthors (1998) found an incomplete posterior circle in 72 of 150 (48%) healthy volunteers using magnetic resonance imaging. This anatomic variability is also evident in rodents (Laidley et al. 2005, Seal et al. 2006).

Since 1969, Mongolian gerbils were considered the paradigm of an incomplete cerebral circle of Willis, given the absence of posterior communicating arteries in this vascular structure. This characteristic promoted the extensive use of this species as a model of global brain ischemia by considerably simplifying the surgical procedure. However, some studies published in the last decade have questioned the reproducibility of the model after the finding of a higher incidence of posterior communicating arteries in different populations of Mongolian gerbils. Laidley and coauthors (2005) have even suggested that the Mongolian gerbil should be disesteemed as an experimental animal model of brain ischemia. Furthermore, Wang and colleagues (2002) established a sieve method in which gerbils with medium to large diameters of the PComAs were excluded from the analysis. However, we consider that this procedure may not be suitable for gerbil populations with a high number of animals with PComAs. Here we have demonstrated that even gerbils with a complete cerebral circle of Willis exhibited tissue injury after 15 minutes of carotid occlusion.

In an attempt to increase the reliability and the usefulness of this model, we implemented two main modifications: firstly, the occlusion time of the carotid arteries was increased from 5 to 15 minutes, since the duration of the ischemic insult is strongly associated with the severity of the ischemic cerebrovascular dis-

ease (Castillo et al. 1999, Shuaib and Hussain 2008); and secondly, the monitoring of the level of anastomosis in the cerebral circle of Willis was incorporated to the experimental routine and this information was considered for the interpretation of the results. The outcome of this approach was a more reproducible and severe model of brain ischemia that might mimic a clinical situation.

Contribution of the anastomosis in the cerebral circle of Willis to the clinicopathological outcomes in acute global brain ischemia in gerbils

When considering the presence of posterior communicating arteries in the gerbil population used in this study, it is striking that Ischemic animals lacking anastomosis did not survive 72 hours post-ischemia (Table I). The degree of blood flow reduction is one of the variables that determine the extent of damage after cerebral ischemia (Lipton 1999). In ischemic gerbils lacking PComAs the decrease of blood flow is more remarkable than in the rest of the animals, thus greater tissue damage is expected.

A low level of anastomosis in the cerebral circle of Willis has been associated to high hippocampal neuronal damage after 5 minutes of carotid occlusion (Laidley et al. 2005, Seal et al. 2006). We too found a negative correlation between the anastomosis in the cerebral circle of Willis and the total infarct volume in the Ischemic group. In addition, we demonstrated that also with this longer occlusion time, the level of anastomosis in the cerebral circle of Willis determines the severity of damage in brain regions other than hippocampus (Fig. 4A).

The assessment of the contribution of the level of anastomosis in the cerebral circle of Willis to the clinicopathological outcomes could allow more realistic analyses when studying neuroprotective approaches. To support this idea, we evaluated the effects of hypothermia in the 15-minute model of global brain ischemia, as this strategy elicits strong endogenous mechanisms of neuroprotection (Linares and Mayer 2009, Macleod et al. 2010). In the Ischemic + hypothermia group, no correlation was found between the level of anastomosis and the neurological grade or the infarct volume.

Regarding the use of the ANCOVA, this procedure has the advantage of considering the effect of variables

other than the treatments on the clinical and pathological outcomes and it is a very useful tool in the evaluation of neuroprotective strategies in this model of global brain ischemia.

The modifications introduced to the global brain ischemia in Mongolian gerbils increased the severity of the model and allowed us to upgrade the interpretation of the results. Neuroprotection in this context would represent an additional proof of principle in support of any other potential therapeutic strategy.

CONCLUSION

In our opinion, the global brain ischemia model in Mongolian gerbils should continue to be used, considering the simplicity of its surgical procedure. However, in order to override the possible inconveniences associated with this model it is essential to assess the level of anastomosis in the cerebral circle of Willis of each animal and include this information in the analysis of the results.

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