Characteristics of cerebral ischemia in major rat stroke models of middle cerebral artery ligation through craniectomy

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Background **The refinement of experimental stroke models is important for further development of neuroprotective interventions.**

Aims and/or hypothesis **Our goal was to study the reproducibility of outcomes obtained in five rat models of middle cerebral artery (MCA) ligation in order to identify the optimal model for the preclinical studies.**

Methods **In Part 1 of the experiments, systolic blood flow velocity (sBFV) and cerebral area at risk (AR) were determined immediately after the onset of brain ischemia induced in different ways in Wistar rats. After that, another set of experiments was performed (Part 2 of the experiments), now aimed at the assessment of the delayed outcome of five different models of cerebral ischemia designated as Versions 1–5.**

The versions were: Version 1 – 40-minute left MCA (LMCA) occlusion with reperfusion; Version 2 – permanent LMCA ligation; Version 3 – permanent ligation of both LMCA and left common carotid artery (CCA); Version 4 – permanent LMCA and bilateral CCA (bCCA) ligation; Version 5 – permanent LMCA ligation and 40-minute bCCA occlusion. The infarct size (IS) was quantified using triphenyltetrazolium chloride staining. The severity of neurological deficit was assessed by the Garcia score. The extent of brain edema was determined by calculating the difference in volumes of affected and contralateral hemispheres.

Results **Within a relatively big AR, Versions 1 and 2 resulted in a small IS [0·2 (0·0; 0·4)% and 0·3 (0·0; 0·7)%, respectively, $P > 0·05$]. Unlike that and comparable with AR, Version 3 resulted in a greater, albeit more variable IS [5·9 (2·1; 8·3)%, $P < 0·0001$ vs. Version 2]. Also comparable with AR, Versions 4 and 5 produced greatest values of IS [14·5 (11·4; 17·9)% and 11·3 (10·1; 14·2)%, respectively]; this parameter was most reproducible in Version 5. A significant decrease in neurological deficit score was found in Versions 4 and 5. Again, the reproducibility of the data on neurological outcome was higher in Version 5 versus Version 4.**

Conclusions **Comparative analysis of several Versions of focal cerebral ischemia within a single study might be helpful in better understanding of the mechanisms underlying the development and aftermath of stroke. Permanent LMCA ligation plus transient bilateral CCA occlusion produced most consistent results and might be recommended for preclinical studies.**

Keywords: animal models, area at risk, collateral blood supply, focal cerebral ischemia, infarct size, middle cerebral artery

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Conflicts of interest: None declared.

DOI: 10.1111/j.1747-4949.2012.00947.x

Introduction

Stroke is a major health problem in the industrialized countries. In the United States, over 795 000 people experience either new or recurrent stroke annually, which accounts for 16·7% of cardiovascular mortality (1). This statistics implies an urgent need in the development of the therapies superior to those available, for which animal experiments using different types of focal cerebral ischemia (FCI) would be of primary significance. Among different animal species, rats are most commonly used in preclinical neuroprotection studies (2). Two main FCI models are currently recognized, including intraluminal middle cerebral artery (MCA) occlusion via a filament implantation, and MCA ligation through craniectomy. The filament implantation model described initially by Koizumi *et al.* (3) and further improved by Longa *et al.* (4) has serious limitation, that is, the dependence of the MCA occlusion effectiveness on filament diameter and on the precision of its positioning, which is unattainable without monitoring of regional cerebral blood flow (5). Moreover, the model is not reliable when used for induction of ischemia without reperfusion and, in any setup, for the experiments on aged animals due to the age-related anatomical changes interfering with the filament passage. Contrary to that, the direct MCA occlusion model, either transient or permanent, while being more invasive, is free of these limitations and, when transient, may be used in the experiments simulating cerebral reperfusion via thrombolysis and/or ischemic conditioning. Currently, at least five modifications of MCA occlusion model are used in the experimental studies of stroke. They include reversible (6) and irreversible (7) isolated MCA occlusion, simultaneous ligation of MCA and ipsilateral common carotid artery (CCA) (8), and MCA ligation accompanied by either permanent (9) or transient (10) bilateral CCA (bCCA) occlusion.

Aims

It is known that one of the main problems of rodent models of FCI is significant variability of the results, which can be reduced by combining various outcome measures. The primary objective of the present work was to study the reproducibility of outcomes obtained in five rat models of MCA ligation as well as the relationship between the reduction of blood flow, perfusion deficit area, infarct volume, and neurological deficit. Such an analysis of data had never been applied to the studies of experimental stroke previously. The overall objective of the study was to streamline the methodology and to identify the optimal ischemia protocol for highest consistency in outcomes.

Methods

Animals

All experiments were performed on male Wistar rats weighting 250–300 g. The animals were fed regular chow, and water was available *ad libitum*. The procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee. The animals were anesthetized with chloral hydrate given intraperitoneally at a dose of 550 mg/kg. Core body temperature was maintained at 37·0 ± 0·5°C by a feedback-controlled heating pad (TCAT–2LV controller, PHYSITEMP Instruments Inc., Clifton, NJ).

Study design

In the present study, we first focused attention on the determination of systolic blood flow velocity (sBFV) and potential cerebral area at risk (AR) immediately after different procedures with the arteries involved in blood supply to the brain (Part 1 of the experimental series). After that, another set of experiments was performed, now aimed at the assessment of the delayed outcome of these manipulations (Part 2 of the experimental series). In these experiments, infarct size (IS), brain edema, and neurological deficits were determined in five versions of FCI 48 hours after the onset of ischemia.

Part 1 of the experimental series

To secure access to left MCA (LMCA), the inverse V-shaped scalp incision was done at the area between the left ear and the left eye, with the base of the skin flap located at the level of the left upper eyelid. After partial excision of the temporalis muscle, three burr holes of 1·5 mm in diameter were drilled through the squamosal bone at the upper caudal angle of the surgical field. Craniectomy between the holes was done, followed by incision of dura mater for visual inspection of LMCA. Under microscopic control, the distal LMCA was ligated with atraumatic prolene suture (Ethicon, 7–0, Johnson & Johnson, New Brunswick, NJ) above the rhinal fissure. Noteworthy, we preferred the LMCA ligation instead of cauterization since, according to our pilot experiments, the latter technique might inflict undesirable thermal damage.

CCAs were accessed through ventral midline cervical incision followed by blunt dissection of sternohyoid and sternomastoid muscles. Then, either left CCA (LCCA) or both arteries underwent occlusion.

A single measurement of sBFV was done in the LMCA immediately following the arterial occlusion or sham procedure in the following groups:

- Sham ($n = 13$) – LMCA was surgically exposed without ligation
- LMCAO ($n = 17$) – isolated LMCA occlusion
- LCCAO ($n = 9$) – LCCA occlusion
- bCCAO ($n = 17$) – bilateral CCA occlusion
- LMCAO + LCCAO ($n = 12$) – occlusion of LMCA plus LCCA
- LMCAO + bCCAO ($n = 15$) – occlusion of LMCA and both CCAs.

The AR was determined immediately after arterial ligation in the following groups of animals:

- LMCAO ($n = 6$) – isolated LMCA occlusion
- LMCAO + LCCAO ($n = 7$) – occlusion of both LMCA and LCCA
- LMCAO + bCCAO ($n = 7$) – occlusion of LMCA and both CCAs.

Determination of systolic blood flow velocity

Arterial blood flow was registered in the LMCA after craniectomy using the high–frequency ultrasound Doppler flowmetry (Minimax, Ltd., St. Petersburg, Russian Federation). sBFV was measured using 20 MHz probe positioned onto the wall of the artery and expressed in cm/s.

Determination of area at risk

In each case, the region of tissue with severe blood flow reduction due to arterial ligation(s) was referred to as the AR. In the present study, we used the technique of AR delineation with Evans blue, a method used previously for AR registration in the heart (11) and subsequently adopted for AR determination in the brain (12). Briefly, immediately after induction of cerebral ischemia 1 ml of 5% Evans blue was injected into the left ventricular cavity. Five seconds after that, the animals were euthanized, the brain was cut into five 3 mm-thick frontal slices, each photographed, digitized, and analyzed using Adobe Photoshop CS. The AR volumes were calculated by multiplication of Evans blue–negative area by slice thickness. The AR volume of the whole brain was calculated by summarizing the AR volumes of the slices to be finally expressed as a percentage of AR to the total brain volume.

Part 2 of the experimental series

The animals were randomly allocated to the following experimental groups (Fig. 1):

- Version 1 ($n = 6$) – LMCA occlusion for 40 minutes followed by reperfusion. For that, LMCA was cautiously pulled up with the thread for 40 minutes to abrogate blood flow followed by the release of the thread resulting in the microscopically controlled blood flow reinstitution
- Version 2 ($n = 6$) – permanent LMCA ligation without reperfusion
- Version 3 ($n = 13$) – permanent ligation of both LMCA and LCCA
- Version 4 ($n = 13$) – permanent LMCA and bCCA ligation
- Version 5 ($n = 8$) – permanent LMCA ligation and 40–minute bCCA occlusion followed by reperfusion. Temporal occlusion of CCAs was achieved by reversible microvascular clip fixation on the artery.

Sham-treated animals ($n = 5$) were subjected to similar surgical procedures with the exception of cerebral artery(ies) ligation. At the end of each procedure, the wounds were closed. Forty-eight hours after that, the neurological evaluation was performed and the animals were sacrificed for histochemical determination of IS and measurement of brain edema; the outcomes were determined by an investigator blinded to the group assignments.

Measurement of mean arterial pressure

The right femoral artery was cannulated for continuous monitoring of mean arterial pressure (MAP). MAP values were registered at baseline, prior to arterial occlusion(s), 20 and 40 minutes after LMCA ligation, and one hour after LMCA ligation in Versions

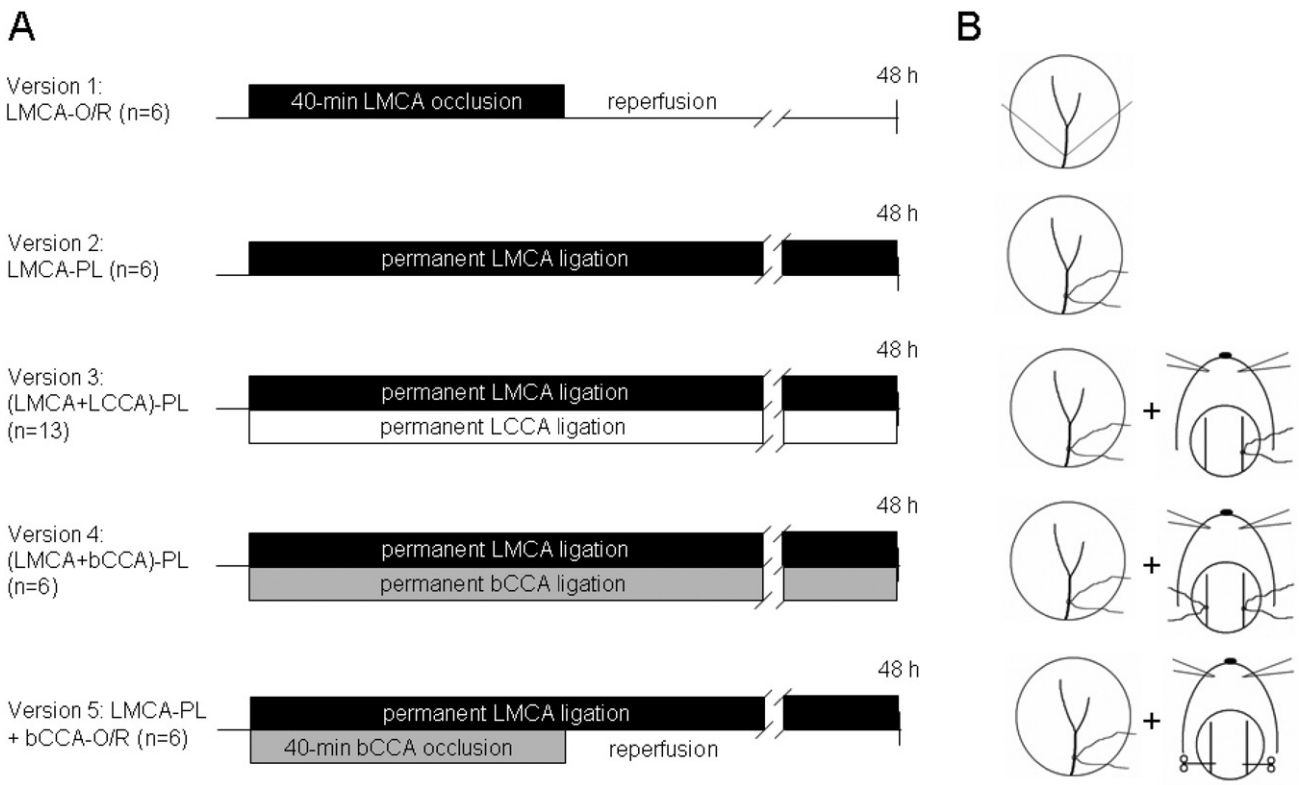

Fig. 1 Schematic and visual presentation of the experimental protocol. (a) Schematic presentation. The animals were allocated to five groups (Versions), each subjected to distinct ways of focal cerebral ischemia. Briefly, Version 1 (LMCA–O/R, $n = 6$): 40 minutes of LMCA occlusion followed by reperfusion; Version 2 (LMCA–PL, $n = 6$): permanent ligation of LMCA; Version 3 ((LMCA + LCCA)–PL, $n = 13$): permanent ligation of both LMCA and LCCA; Version 4 ((LMCA + bCCA)–PL, $n = 13$): simultaneous permanent ligation of LMCA and both CCAs; Version 5 (LMCA–PL + bCCA–O/R, $n = 8$): permanent ligation of LMCA plus 40-minute bilateral occlusion of CCAs. (b) Visual presentation. For detail, see Materials and Methods.

2–5. In Version 1, MAP values were registered in the same time points with the last measurement done 20 minutes after LMCA reperfusion.

Neurological tests

Examination of neurological status was performed according to Garcia *et al.* (13), with six tests applied and the degree of injury scored from 18 (no deficit at all) to 3 (the most severe deficit).

Determination of infarct size

IS was determined after incubation of the brain slices in 0·1% 2·3.5–triphenyltetrazolium chloride (TTC, MP Biomed., St. Louis, MO) at 37°C (pH 7·4) for 15 minutes followed by taking photographs, digitizing, and calculation of infarct volume as discussed earlier (for detail, see section '*Determination of area at risk*'). After correction for edema, IS was expressed as a percentage of infarct volume to that of the total brain.

Evaluation of brain edema

The extent of brain edema was calculated using the formula (14): [volume of ischemic hemisphere–volume of contralateral hemisphere]/volume of contralateral hemisphere × 100.

Data analysis

Statistical analysis was performed with use of SPSS 12·0 software package. The Kruskal–Wallis test was used to determine differences in outcomes, followed by pairwise inter-group comparisons performed using non–parametric Mann–Whitney *U*-test. p values $\leq 0{\cdot}05$ were considered significant. All data in the text were expressed as 'median (25 quartile; 75 quartile)'. Neurological scores were presented as box plots with median and quartiles as well as whiskers to express minimum and maximum values.

Results

Mortality

At the end of the study, results could be obtained from 138 of 149 rats (92·6%). The mortality rates, therefore, were 0/6, 0/6, 3/13, 6/13, and 2/8 in Versions 1–5, respectively.

Part 1 of the experimental series

Systolic blood flow velocity

As presented in Fig. 2, sBFV in sham-operated animals differed significantly between the individual experiments and averaged 42·0 (31·0; 57·1) cm/s. Also, LMCA ligation resulted in significant reduction of blood flow [10·2 (7·1; 13·2) cm/s, $P < 0{\cdot}0001$ vs. baseline]. Unlike the LMCA, the ligation of LCCA caused only an insignificant decrease in sBFV [31·4 (18·1; 41·0) cm/s, $P = 0{\cdot}071$ vs. baseline]. bCCA ligation resulted in a dramatic decrease in sBFV [9·0 (6·3; 12·0) cm/s, $P < 0{\cdot}0001$ vs. baseline, Fig. 2a]. In the group with ligation of LMCA plus LCCA the reduction in sBFV was higher, that is, 6·1 (3·0; 9·0) cm/s ($P < 0{\cdot}05$ vs. bCCA ligation). The lowest sBFV in the LMCA was observed when LMCA ligation

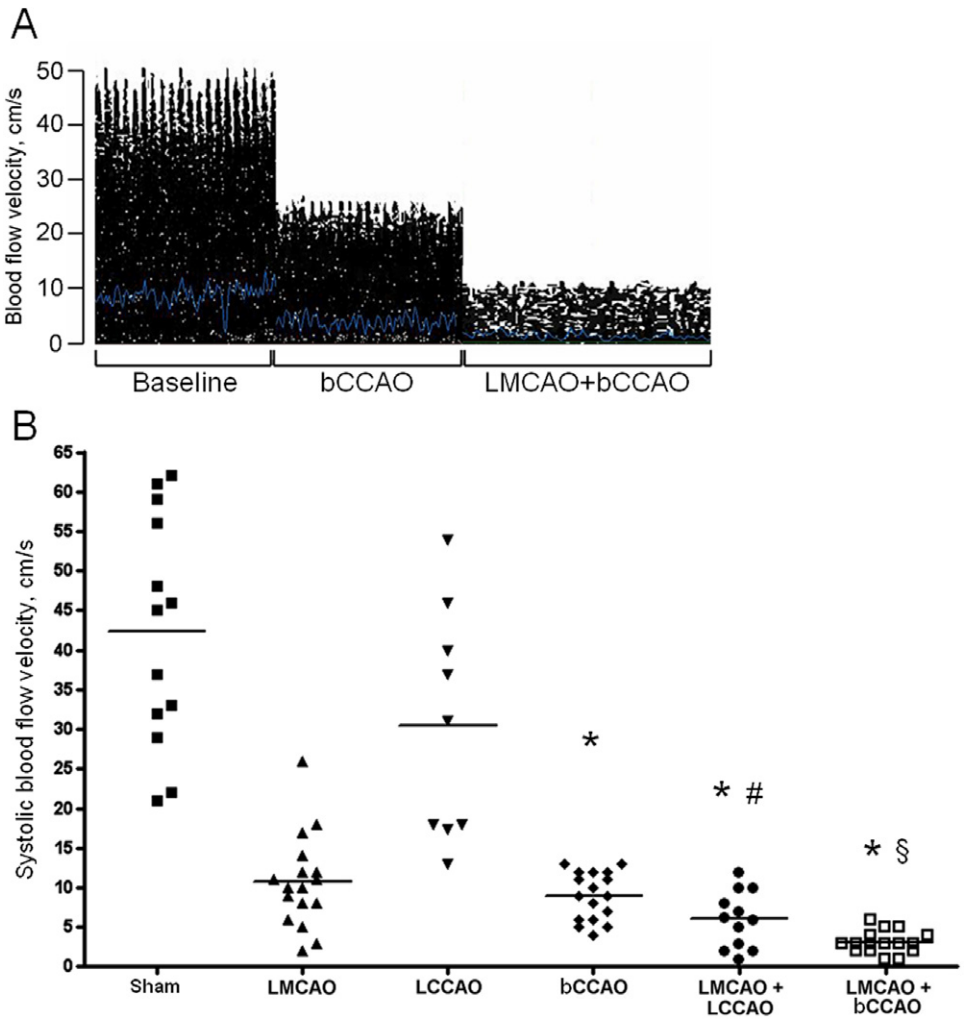

Fig. 2 The systolic blood flow velocity in the LMCA measured in the Part 1 of experimental series. (a) A representative recording of systolic blood flow velocity in the LMCA registered at the sequential stages of the procedure in the LMCAO + bCCAO group, that is, at baseline, after bilateral CCA occlusion, and after LMCA plus bilateral CCA occlusion. (b) Systolic blood flow velocity in the LMCA immediately after arterial occlusion. Occlusion of either LMCA, or bilateral CCA occlusion, or occlusion of both LMCA and LCCA, or occlusion of LMCA and bilateral CCA occlusion, all invariably resulted in significant reduction of systolic blood flow velocity in comparison with sham-operated rats while the unilateral CCA occlusion did not. The data are expressed as dot plots with median values. LMCAO – left middle cerebral artery occlusion; LCCAO – left common carotid artery occlusion; bCCAO – bilateral common carotid artery occlusion. * – $P < 0{\cdot}01$ versus baseline value; # – $P < 0{\cdot}05$ versus bCCAO; § – $P < 0{\cdot}05$ versus LMCAO + LCCAO.

was accompanied by bCCA occlusion [3·0 (2·1; 4·0) cm/s, $P < 0{\cdot}05$ vs. ligation of LMCA and LCCA].

Area at risk

As to the AR (Fig. 3), the ligation of LMCA alone resulted in its value of 4·7 (3·8; 6·0)%. The ligation of LMCA and LCCA resulted in a somewhat greater AR [7·6 (4·8; 11·4)%], although the difference between this and the previous group was not significant ($P = 0{\cdot}1$). LMCA and bCCA ligation was accompanied with a significantly greater AR [15·6 (12·8; 17·0)%, $P < 0{\cdot}01$ vs. both isolated LMCA ligation and LMCA plus LCCA ligation].

Part 2 of the experimental series

Mean arterial pressure

Both preischemic and postischemic values of MAP within or between groups were not different (Table 1).

Neurological status

The data on neurological deficit after induction of ischemia are presented in Fig. 4. In sham-operated animals, neurological deficit score averaged 17·1 (16·3; 18·0). In Versions 1–3, the score was not different from that in sham-treated animals [17·2 (16·1; 18·3), 17·1 (16·4; 17·9), 16·0 (15·7; 17·5), respectively, $P = 0{\cdot}94$, 0·95, 0·41 vs. shams, respectively]. The greatest deficit was observed after permanent ligation of LMCA plus both CCAs (Version 4) [11·4 (8·5; 13·6), $P = 0{\cdot}002$ vs. shams]. The same was true for Version 5 (permanent LMCA ligation plus reversible bCCA occlusion) – [14·2 (12·5; 14·4), $P = 0{\cdot}139$ vs. Version 4], although the reproducibility was higher in the last of the versions.

Infarct size

No infarction was detected in sham-operated rats. All of the versions were aimed at and resulted in the development of neocortical infarction (Fig. 5). A 40-minute LMCA occlusion followed

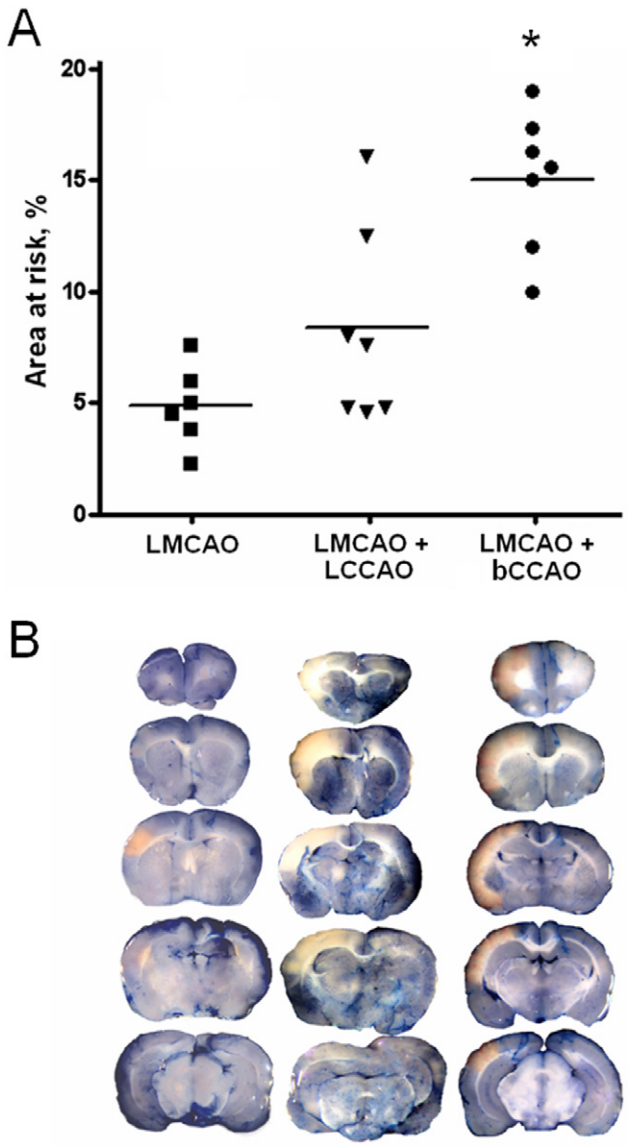

Fig. 3 Quantitative and visual presentation of area at risk size defined immediately after arterial occlusion as a percentage of Evans blue-negative cerebral tissue. (a) Quantitative presentation. Area at risk is significantly greater after occlusion of LMCA plus bilateral CCA occlusion than after either isolated LMCA occlusion or occlusion of LMCA plus LCCA. The data are expressed as dot plots with median values. * – $P < 0{\cdot}01$ versus LMCA occlusion and LMCA plus LCCA occlusion. (b) Evans blue-stained brain slices selected from representative animals of each group.

by reperfusion (Version 1) caused a relatively small IS [0·2 (0·0; 0·4)%]. Permanent LMCA ligation (Version 2) was not different significantly from Version 1 by both IS and its variability [0·3 (0·0; 0·7)%, $P = 0{\cdot}93$ vs. Version 1]. Simultaneous permanent ligation of LMCA and LCCA (Version 3) resulted in significantly greater IS, that is, 5·9 (2·1; 8·3)%, $P < 0{\cdot}0001$ vs. Versions 1 and 2. The greatest IS was observed after permanent ligation of LMCA plus both CCAs in Version 4 [14·6 (11·4; 17·9)%, $P = 0{\cdot}019$ vs. Version 3]. Unlike Versions 1–3, a significant variability in IS was observed in Version 4. In Version 5 (permanent ligation of LCMA along with transient bCCA occlusion), although both CCAs were occluded for 40 minutes only, the IS was essentially the same as in Version 4 [11·3 (10·1; 14·2)%, $P = 0{\cdot}2$ vs. Version 4]. Notably, in Version 5, IS was less variable in comparison with Versions 3 and 4.

Brain edema

There was no cerebral edema in sham-operated rats (data not shown), Versions 1 and 2 (Fig. 6). Permanent occlusion of both LMCA and LCCA (Version 3) resulted in an appreciable edema [8·2 (6·0; 12·1), $P = 0{\cdot}003$ vs. this parameter in Version 2], whereas permanent ligation of LMCA plus both CCAs (Version 4) as well as permanent ligation of LMCA along with 40-minute bilateral occlusion of CCAs (Version 5) both resulted in edema roughly equal to that in Version 3 [12·0 (4·2; 16·0) and 8·6 (3·9; 14·9), respectively; $P = 0{\cdot}017$ and 0·014 vs. Version 2, respectively].

Discussion

Five generally accepted modifications of rat stroke model with transcranial MCA ligation were described previously (6–10). In the present work, all five models (Versions 1–5) were utilized within a single study to ensure a more precise comparative evaluation of distinct patterns of individual models of brain infarction.

Systolic blood flow velocity

This parameter varied significantly depending on the procedure applied based on the results of high-frequency Doppler ultrasound flowmetry. Thus, unilateral CCA ligation did not cause significant reduction in sBFV consistent with the data of Herz *et al.* (15), likely due to the contribution of blood flow through the collateral vessels at the base of the brain. Notably, the inhomogeneity in the sBFV (Fig. 2b) might be explained by natural variability in the number and/or caliber of collateral vessels that supply the affected area. Unlike that, a significant and more homogenous sBFV reduction after bCCA ligation could be explained by a cessation of the collateral blood supply from the circle of Willis, which might have been compensated by the unaltered blood supply to the affected area from vertebral circulation through the system of leptomeningeal anastomoses. Furthermore, although either LMCA ligation alone or together with uni- or bilateral CCA ligation both resulted in a dramatic reduction of sBFV, the variability of the effects was different due to the variability in the collateral blood supply discussed above (Fig. 2b), along with the anatomical characteristics of collaterals, sBFV value after arterial occlusion might be influenced by the perfusion pressure. Since MAP was not measured in the Part 1 of the experiments, we can not rule out the possibility that the observed variability in sBVF and AR size might be at least partially explained by the differences in blood pressure.

Area at risk and infarct size

Two distinct approaches to registration of cerebral response to ischemia were used in the present study: immediate labeling for evaluation of AR size (Part 1) as related to the ultimate infarct-producing effect of different manipulations on the artery(ies) (Part 2; Versions 1–5). That the IS was significantly smaller than mean AR in the animals subjected to isolated LMCA ligation [0·3 (0·0; 0·7)% for Version 2 and 4·7 (3·8; 6·0)% for the results

Table 1 Pre- and postischemic values of mean arterial pressure (MAP, mm Hg) in different groups

	Sham	Version 1	Version 2	Version 3	Version 4	Version 5
Baseline	86 ± 9	87 ± 6	91 ± 5	89 ± 7	93 ± 5	85 ± 3
Before ischemia	88 ± 8	89 ± 7	92 ± 6	85 ± 4	95 ± 6	89 ± 5
After LMCA ligation:						
20 min	83 ± 6	85 ± 8	89 ± 7	81 ± 5	87 ± 3	88 ± 4
40 min	85 ± 8	86 ± 7	78 ± 9	76 ± 6	85 ± 5	84 ± 6
60 min	82 ± 10	77 ± 5	82 ± 6	79 ± 4	80 ± 7	78 ± 5

Data are mean ± standard error of mean. LMCA – left middle cerebral artery.

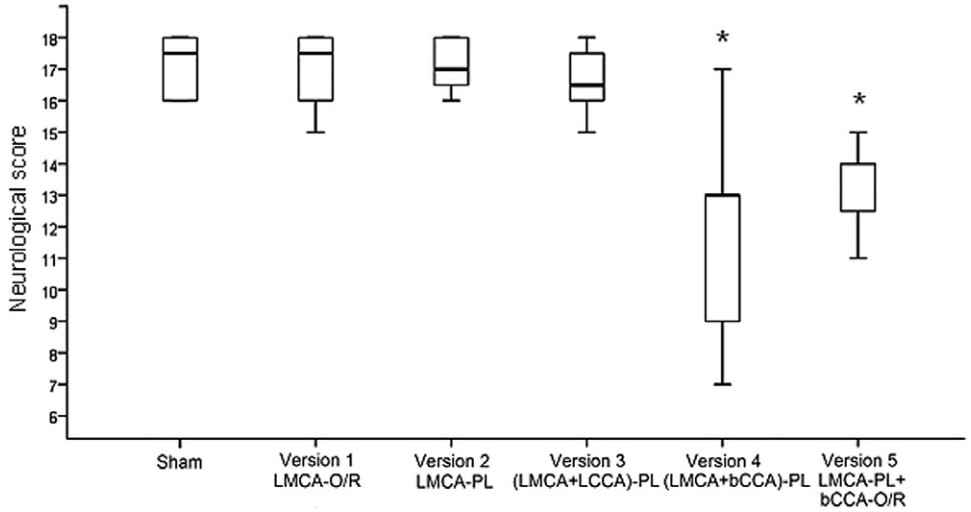

Fig. 4 Neurological deficit scores 48 hours after induction of focal cerebral ischemia. Permanent ligation of LMCA plus two CCAs (Version 4) and permanent ligation of LMCA with 40-minute bilateral CCA occlusion (Version 3) both resulted in a significantly greater deficit compared to that parameter in sham-operated rats. Data are presented as box plots with median and quartiles; whiskers denote minimal and maximal values. * – $P < 0{\cdot}01$ versus sham-treated animals.

obtained in Part 1, respectively], can be explained by the extensive collateralization of the LMCA territory leading to minimization of irreversible injury. In contrast, IS and AR size did not differ statistically either after both LMCA and LCCA occlusion [5·9 (2·1; 8·3)% for Version 3 and 7·6 (4·8; 11·4)% for the results obtained in Part 1, respectively, $P = 0{\cdot}165$] or in the animals with simultaneous occlusion of LMCA and both CCAs [14·5 (11·4; 17·9)% for Version 4 and 15·6 (12·8; 17·0)% for the results obtained in Part 1, respectively, $P = 0{\cdot}372$]. It appears, therefore, that the results obtained in the two latter groups of Part 1 favor the idea that the AR size remained practically unchanged during the 48-h observation period (Versions 3, 4) due to absence or insufficient collateralization. Supportive of this idea might be recent experimental and clinical data on the extent of cerebral tissue collateralization after arterial occlusion in stroke as the major predictor of final IS (16,17).

Reduction of cerebral IS is considered to be a 'gold standard' for the preclinical studies of neuroprotective interventions, and reproducibility of infarct parameters should be of crucial importance in the relevant experimental settings. Consistent with the histochemical findings obtained by the others, IS, along with other factors, is dependent on time interval after induction of ischemia (18). We chose 48 h as an optimal period for determination of infarct parameters. Concerning evaluation of our present results, the ligation of LMCA alone (Version 2) resulted in a relatively small infarction, which is in accord with the results of prototype trials using the same model (19,20). Moreover, in some cases the authors found no infarction at all, although in more technically refined experiments such cases were a rarity (20). A considerable inter-animal variability in IS was documented in the previously mentioned experiments as well as in Versions 3 (ligation of LMCA plus LCCA) and 4 (ligation of LMCA plus both CCAs) of our study. The major source of IS variability most likely would be a significant animal-to-animal difference in the development of vasculature and therefore collateral local blood supply of the affected brain area from vertebrobasilar system through leptomeningeal anastomoses. A robust support of this idea comes from our results obtained under the conditions that anatomically exclude the probability of collateralization: LMCA ligation accompanied by temporal (40 minute) bCCA occlusion makes the IS variability very low (Version 5). In line with our observations, the opening of leptomeningeal anastomoses was reported to begin not immediately but following 40 or more minutes after bCCA occlusion (21). The models of reversible FCI are also of interest because of their relevance to the routine clinical approach of cerebral reperfusion via thrombolysis. In the present study, two experimental protocols of reversible cerebral ischemia were used, that is, Versions 1 (40-minute LMCA occlusion) and 5 (perma-

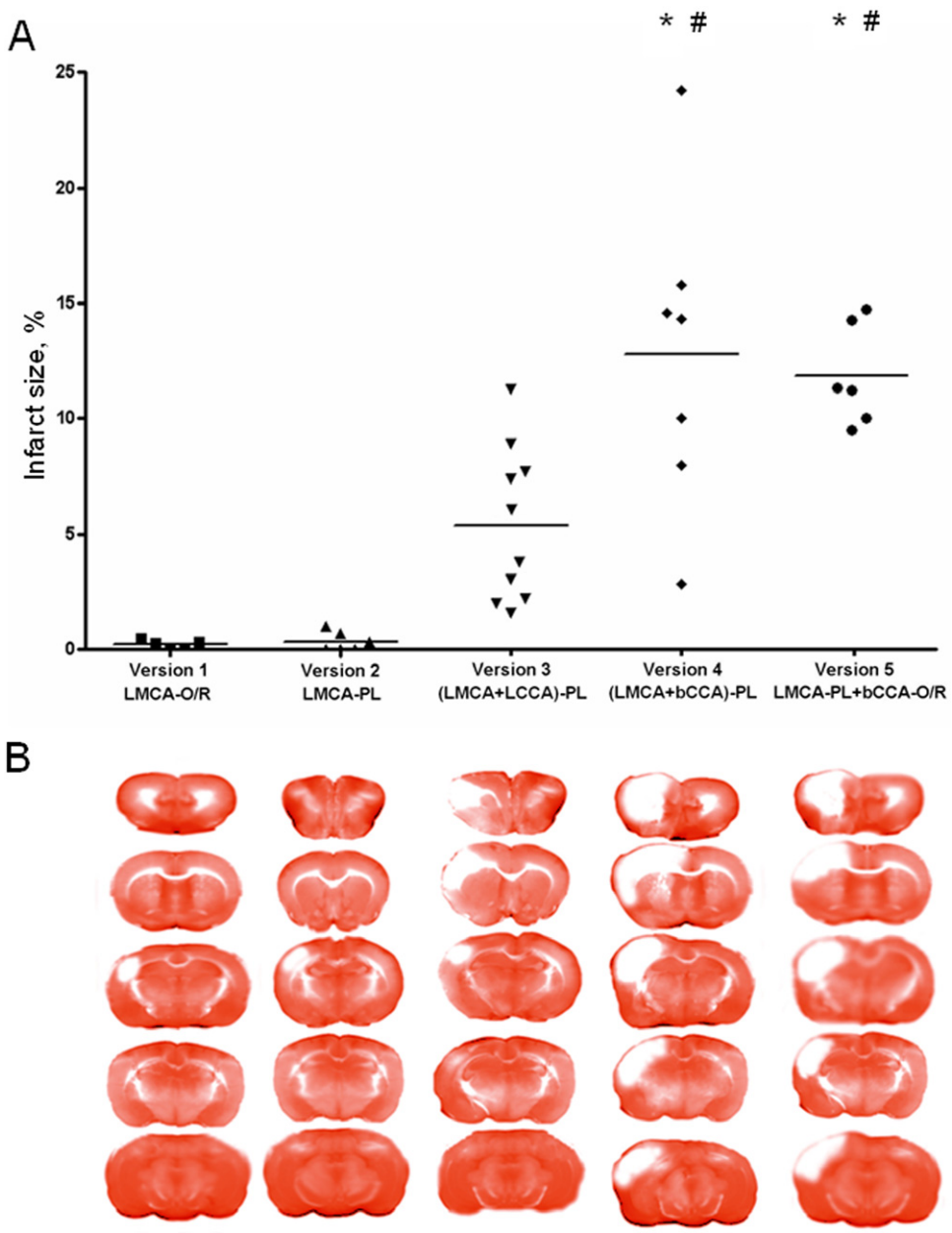

Fig. 5 Quantitative and visual presentation of cerebral infarct size 48 hours after induction of focal cerebral ischemia. (a) Quantitative presentation. Infarct size is significantly greater in Version 3 compared to Versions 1 and 2. Infarct size is greater in Versions 4 and 5 compared to Version 3. The data are presented as dot plots with median values. * – $P < 0{\cdot}01$ vs. Version 2; # – $P < 0{\cdot}05$ vs. Version 3. (b) 2,3,5–triphenyltetrazolium chloride-stained brain slices selected from representative animals of each group.

nent LMCA plus 40-minute bCCA occlusion) with complete and only partial reperfusion, respectively. Clearly, the former approach should rescue some brain tissue, although blood flow restoration could cause an additional injury (for review, see Reza Noorian *et al.* (22) and the references therein). The lack of neuroprotective effect of reperfusion in Version 1 might account for by prolonged ischemia and/or the progressive irreversible reperfusion injury overrunning the benefits of revascularization.

Based on our morphological data, it seems justified to determine first the initial AR (Part 1) followed by the sets of experiments designated as Part 2 (Versions 1–5). Furthermore, the permanent LMCA ligation with 40-minute bCCA occlusion (Version 5) offers the most reliable and reproducible method for producing neocortical infarction. Next, the permanent ligation of LMCA and both CCAs (Version 4), which also caused generally similar effects, resulted in a significantly greater variability in all the parameters studied, as well as in greater mortality. Furthermore, the results obtained after either permanent or temporal LMCA occlusion (Versions 1 and 2, respectively) are of special interest because both were characterized by relatively small infarction with significant variability in its size without substantial complications, such as edema and neurological deficit. That, at least in part, might be accounted for by the individual variability in the efficiency of the collateral blood flow. A considerable vari-

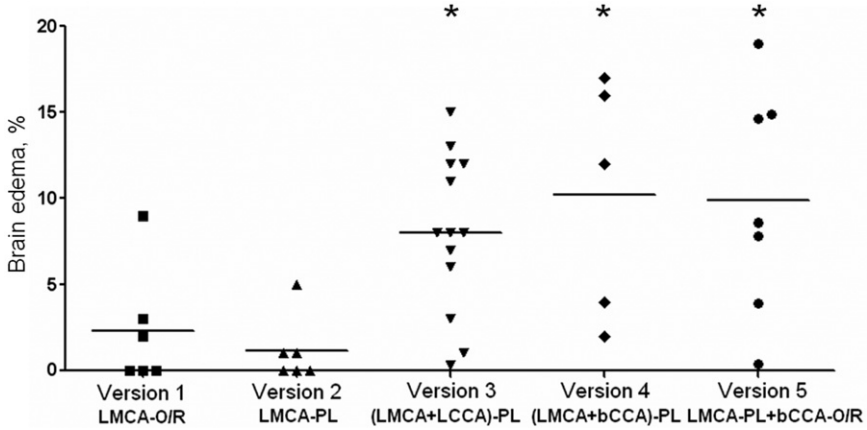

Fig. 6 Brain edema 48 hours after induction of focal cerebral ischemia. Edema is significantly greater in Versions 3–5 than in Versions 1 and 2. The data are presented as dot plots with median values. * – $P < 0{\cdot}01$ vs. Version 2.

ability of IS after a partial blockage of collateral blood supply (Version 3), despite a significantly greater brain injury, also lends support to this notion. Although MAP values were not statistically different within and between groups in Part 2 of the experiments, the baseline MAP values were slightly different among the Versions. These differences in MAP should be considered as one of the factors contributing to the observed variability in regional cerebral blood flow, AR, and IS.

Neurological outcome

In addition to cerebral IS, evaluation of neurological outcome is commonly considered to be a useful complementary end point of cerebral protection against ischemia. As in many similar studies (e.g., (23)), our experiments established a direct correlation between neurological deficits and infarct volume with a single exception. The exception was Version 3 (permanent ligation of both LMCA and LCCA) in which neurological deficit was not different from sham-operated animals despite significantly greater IS vs. Versions 1 and 2. In this particular case, the lack of the above-mentioned correlation might be explained by IS variability, which proved to be unpredictably higher compared to that of neurological deficit (Fig. 5a cf. Fig. 4). The lack of such a correlation was also noted in the experiments using certain neuroprotective agents (24,25), probably reflecting the selective beneficial effect of these particular drugs on functional rather than morphological manifestation of cerebral injury.

Utility of stroke models for the preclinical testing of neuroprotective agents

Rodent stroke models are increasingly used for testing of novel neuroprotective therapies. Although more then 1000 distinct therapies were shown to be neuroprotective in the preclinical studies, the results of clinical trials have been largely disappointing (26). In an attempt to facilitate translation of novel drug candidates to clinical settings, the Stroke Therapy Academic Industry Roundtable (STAIR) group elaborated (27) and recently updated (28) the criteria needed to be met in order to improve the quality of experimental studies on neuroprotection. Unfortunately, a systematic review by O'Collins *et al.* (29) showed in 2006 that only 5 out of 550 drugs with experimentally proven neuroprotective effect(s) fully met the STAIR criteria. It should be noted that some of STAIR criteria specify the protocol of drug administration and underline the importance of dose–response curves, while the others deal with the characteristics of experimental model and study end-points. It is suggested that the rat models of permanent MCA ligation through craniectomy could be useful tools in identifying the efficacy of novel neuroprotectants, at least for the screening stage (30). Randomization of the animals and blinded assessment of both histological and functional outcomes might further improve quality of preclinical neuroprotection studies. Better reproducibility of the outcomes, observed in the present study in Version 5 (permanent LMCA ligation with 40-minute bCCA occlusion), strongly contributes to a lower variance resulting in a lesser numbers of animals per group needed to obtain the statistically significant results.

Study limitations

The present study has several methodological limitations. First, both AR size and sBFV were determined at a single time point just after the induction of cerebral ischemia in the special series of acute experiments. It would be more informative to analyze the time course of changes in both cerebral blood flow and perfusion deficit area throughout the entire occlusion period, rather than looking at a cross-sectional picture of the parameters measured. Technically, this goal might be accomplished using neuroimaging modalities, such as diffusion/perfusion MRI scanning (31). Second, the neurological deficit scoring system applied is becoming increasingly criticized for its relative failure to test the disorders of rodent species-specific reflexes and, consequently, insufficient sensitivity in determining functional outcomes. Therefore, the development and validation of new species-specific neurological deficit scoring systems is warranted. Third, the extent of brain swelling was determined indirectly by calculating the difference in volumes of affected and contralateral hemispheres instead of using wet-dry cerebral weight measurements. However, the former approach might be more advantageous in the sense that it does not preclude IS determination, thereby avoiding the necessity of increasing the number of animals used. Fourth, blood gases and glucose levels were not routinely mea-

sured in all experiments but only in the pilot ones. These measurements showed that both blood gases and glycemia were maintained within normal range during the entire experiment. Lastly, functional and morphological outcomes of cerebral ischemia were determined only at 48 hours after reperfusion. According to STAIR recommendations, the outcomes should be also assessed at 2–3 weeks after the onset of ischemia. Future studies will address these important issues.

Conclusions

Investigation of several patterns of FCI within a single study would provide further insight in both technical details of the procedures applied and in the understanding the mechanisms underlying experimental stroke. The model of permanent LMCA ligation accompanied by reversible bCCA occlusion was shown to be optimal in terms of both lesion size and reproducibility of outcomes. The use of reliable and reproducible models of FCI could potentially contribute to better translation of novel neuroprotective strategies to the clinical arena.

Acknowledgements

The authors are grateful to Professor A. Nevorotin for helpful suggestions during preparation of the manuscript. This work was supported by the grant of the President of the Russian Federation for support of leading scientific groups 2359·2012·7.

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