Is Shorter Transient Middle Cerebral Artery Occlusion (t-MCAO) Duration Better in Stroke Experiments on Diabetic Female Sprague Dawely rats?

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Is shorter transient middle cerebral artery occlusion (t-MCAO) duration better in stroke experiments on diabetic female Sprague Dawely rats?

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Abstract

Aim: To determine optimal duration of transient middle cerebral artery occlusion (t-MCAO) for a stroke model in female diabetic Sprague-Dawley (SD) rats.

Methods: Streptozotocin-induced type-1 diabetic SD female rats (n = 25, 12 weeks old, five groups; n = 5 per group) were subjected to different duration of t-MCAO (20, 30, 45, 60 and 90 minutes) followed by reperfusion. A control group of rats without diabetes (n = 5) was subjected to 30 minutes of t-MCAO followed by reperfusion. Twenty-four hours after reperfusion, infarct volumes were evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining.

Results: Intra-ischaemic reductions of regional cerebral blood flow (rCBF) were similar in all groups (68–75% of baseline values). Reperfusion was significantly impaired in the 90-minute ischaemia group (56–62% vs 80–125% in other groups). Twenty minutes of t-MCAO induced a small infarct (3 ± 5% of ischaemic hemisphere). Thirty minutes of ischaemia produced a significantly larger infarct (46 ± 6%). In the 45 and 60 minute groups, ischaemia infarct was 52 ± 5% and 59 ± 3% of the ischaemic hemisphere, respectively. Ischaemia of 90 minutes led to a massive stroke (89 ± 6% of ischaemic hemisphere encompassing the whole striatum (22 ± 3%) and almost the whole MCA irrigated cortex area (67 ± 6%). Thirty minutes of t-MCAO did not produce stroke in the control group.

Conclusion: The diabetic rat stroke model should be different from the non-diabetic, because female type-1 diabetic SD rats are highly sensitive to brain ischaemia and it is necessary to significantly shorten the duration of t-MCAO, optimally to 30 minutes.

Introduction

Approximately 25% of all stroke patients have diabetes mellitus and 40% have hyperglycaemia, which is associated with worse neurologic outcome as well as higher risk of recurrence of stroke [1,2]. Diabetic patients, compared to non-diabetics, are known to be more sensitive to cerebral ischaemia. Thus, the same duration of ischaemia results in more severe neurologic deficits and larger brain infarcts in diabetic patients. Female patients with diabetes mellitus have 4.8-fold higher risk for developing ischaemic stroke than the general population (compared to 3.7-fold for men) and more often suffer fatal strokes (standardized mortality ratios of 3.1 for males and 4.4 for females) [3–5]. The outcome is frequently lethal, regardless of any therapy undertaken, including recombinant tissue plasminogen activator (rtPA). Moreover, diabetes is in some cases, such as treatment of recurrent stroke with thrombolysis, one of exclusion criteria [6].

The most commonly used experimental model of stroke in rats is a model of middle cerebral artery occlusion (MCAO) by intra-luminal suture. There are variations of this model in terms of use of permanent or transitory MCA occlusion-induced ischaemia. In rats without diabetes, duration of ischaemia in these models varies according to experiment from 60–120 minutes [7]. The duration of occlusion varies in models from permanent MCAO [8–10] to transitory MCAO (t-MCAO) of 180 [11], 120 [12–16], 105 [17] or 60 minutes [18]. Taking into account the observed differences in clinical presentation of diabetic vs non-diabetic patients with stroke, there are few issues that variations in experimental approach to stroke study are brought to light. For example, in diabetic rat stroke models, the same duration of MCAO as in non-diabetic rat models is used. Additionally, other models, e.g. the thromboembolic model, shows inconsistencies in the effects of occlusion which may be contributed to the unpredictable postponed reperfusion and consequently impossibility to predict with certainty the time when reperfusion will appear [19,20]. Such a model cannot simulate ischaemia in diabetic patients under internal carotid artery endarterectomy with accurately known duration of ischaemia and its localization in brain. On the other hand, the MCAO model with intra-luminal insertion of monofilament with LDF (laser Doppler flowmetry) monitoring in given time and known place,
mimics it perfectly and its complications alike [21,22]. With usual duration of t-MCAO used in non-diabetic rats, with all efforts and after significant time and animals used, it failed to establish stroke model in female rats with diabetes. Analysis found that the main reason for this was massive stroke with malignant brain oedema, producing devastating neurological deficits (such as inability to move, eat and drink) that became worse over time, leading to unconsciousness and death of animals within the first 24 hours. Taken all together, the aim of this study was to develop the adequate diabetic female rat model, using transitory middle cerebral artery occlusion (t-MCAO) that would produce treatable stroke conditions in rats with diabetes. To establish a reproducible model resulting with medium sized stroke, this study had to significantly shorten the duration of t-MCAO. The present study suggests that 30-minute t-MCAO could be a more appropriate stroke model than the usual 60–120 minute t-MCAO models.

Materials and methods

Animals

Experiments were performed on 30 Sprague-Dawley (SD) female rats, 12 weeks old, weighing between 220–272 g, housed in the accredited animal care facility at the University of Osijek. Animals were kept in cages with a 12-hour light/dark cycle and were allowed free access to food and water throughout the study. All experimental procedures were approved by the Ethical committee of the Faculty of Medicine, University of Osijek.

Induction of diabetes

Type-1 diabetes mellitus in rats was induced by a single intraperitoneal injection of streptozotocin (STZ; 60 mg kg⁻¹, Sigma-Aldrich, St. Louis, MO). STZ was freshly prepared in 0.09 M citrate saline buffer and administered to rats at 6 weeks of age [23,24]. Fasting blood glucose levels in rats were repeatedly measured using a glucometer (Accu-Check Active, Roche, Germany).

Study design

Diabetic rats that had fasting glucose levels above 17 mmol L⁻¹ were randomly assigned to five groups with different t-MCAO duration (20, 30, 45, 60 or 90 minutes) and non-diabetic rats were subjected to 30-minute t-MCAO (n = 5 animals per treatment group).

Exclusion criteria were: death of the animal during the surgical procedure or within 24 hours after reperfusion, excessive bleeding during operating procedures, reduction of rCBF during ischaemia less than 65% of baseline values, absence of reperfusion after removal of suture monitored using LDF, presence of intracerebral and/or subarachnoid haemorrhage at post-mortem examination of brains. Animals excluded from the study were replaced. Quantitative analysis of rCBF and infarct size was done by a researcher who was blinded to the experimental protocol.

Regional cerebral blood flow (rCBF) monitoring by laser Doppler flowmetry (LDF)

By medial approach the periost of parietal bones was exposed, hemostasis achieved and left parietal bone was thinned using a dental drill until translucent leaving the dura intact, 2 mm posterior and 6 mm lateral to the bregma. A laser Doppler flow probe was placed in a probe holder and fixed above the dura on the skull with glue and dental cement to continuously monitor cerebral blood flow in the cerebral cortex (LDF, Moor Instruments Ltd, model MBF3D, Devon, UK) during experiment.

Transient middle cerebral artery occlusion (t-MCAO)

Transient focal brain ischaemia was induced in the region of left MCA using a silicon-coated monofilament, as previously described by Koizumi et al. [25] and subsequently modified by Zea Longa et al. [26]. Anaesthesia was induced with intraperitoneal (i.p.) administration of combination of midazolam (Dormicum, Roche Pharma AG, Grenzach-Wyhlen, Germany) 0.5 mg kg⁻¹ and ketamine (Ketanest, PfizerPharma GmbH, Berlin, Germany) 75 mg kg⁻¹. Atropine (Atropini sulfas, Belupo d.d. Koprivnica, Croatia) 0.1 mg kg⁻¹ was also administered i.p. to reduce possible salivation as a side-effect of anaesthesia [27]. Animals were shaved, disinfected and fixed on the operating table and body temperature was continuously monitored with a rectal probe and maintained at 37 ± 0.5°C with a thermostatically regulated heating pad. After a midline incision of the neck was made, muscles were dislocated and common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were carefully isolated from surrounding structures (nervus vagus and sympathetic plexus) under the operating microscope. CCA and ECA were ligated using 5-0 silk suture. A 4-0 nylon monofilament (coated at the tip with silicone 0.25–0.3 mm in diameter using silicone Xantopren and activator Elastomer, Heraeus Kulzer, Hanau, Germany) was introduced in the left ICA and gently advanced to the left MCA using a silicon-coated monofilament, as previously described by Zea Longa et al. [26]. Anaesthesia was induced with intraperitoneal (i.p.) administration of combination of midazolam (Dormicum, Roche Pharma AG, Grenzach-Wyhlen, Germany) 0.5 mg kg⁻¹ and ketamine (Ketanest, PfizerPharma GmbH, Berlin, Germany) 75 mg kg⁻¹. Atropine (Atropini sulfas, Belupo d.d. Koprivnica, Croatia) 0.1 mg kg⁻¹ was also administered i.p. to reduce possible salivation as a side-effect of anaesthesia [27]. Animals were shaved, disinfected and fixed on the operating table and body temperature was continuously monitored with a rectal probe and maintained at 37 ± 0.5°C with a thermostatically regulated heating pad. After a midline incision of the neck was made, muscles were dislocated and common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were carefully isolated from surrounding structures (nervus vagus and sympathetic plexus) under the operating microscope. CCA and ECA were ligated using 5-0 silk suture. A 4-0 nylon monofilament (coated at the tip with silicone 0.25–0.3 mm in diameter using silicone Xantopren and activator Elastomer, Heraeus Kulzer, Hanau, Germany) was introduced in the left ICA and gently advanced to the origin of the MCA until a slight resistance was felt and rCBF fell by ~ 70% of baseline value [28]. After a specified time of ischaemia (20–90 minutes) the filament was gently withdrawn to allow reperfusion. rCBF was continuously monitored to confirm adequate occlusion of the MCA and reperfusion after removal of the filament. After 20 minutes of reperfusion the incision on the neck was sutured, LDF probe removed and the animal was placed into the heated cage and monitored until recovered from anaesthesia. Animals had access to food and drink ad libitum.

Measurement of cerebral infarct volume

Twenty-four hours after reperfusion, the rats were deeply anaesthetized with ketamine and midazolam and killed by decapitation. The brains were quickly removed from the skull and placed into a steel brain matrix and were cut into 2 mm-thick coronal sections. The sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemie, Munich, Germany) at 37°C for 30 minutes in the dark. Viable brain tissue was stained dark brick red due to
reduction of TTC to formazan by mitochondrial enzymes, whereas necrotic brain areas were unstained and appeared white. The sections were fixed in 10% buffered formalin solution for 24 hours and anterior and posterior sides of each stained brain section were scanned. Infarct volumes were measured using Image J imaging software (v1.37, National Institute of Health, Bethesda, MD, USA) and expressed as a percentage of ischaemic hemisphere [29].

Statistical analysis

Data analysis was performed using SigmaPlot software (version 11.0, Systat Software Inc., San Jose, CA) and the graphs were created using GraphPad Prism (v5, GraphPad Software, Inc., La Jolla, CA). Shapiro-Wilks test was performed to assess normality of data distribution. Between-group analysis for normally distributed variables was tested using parametric one-way ANOVA and post-hoc Tukey test, whereas in the case of variables that violated assumption of normality differences were tested with non-parametric Kruskal–Wallis test followed by pairwise multiple comparison procedures (Dunn’s and Student-Newman-Keuls method). Cerebral blood flow data were compared by two-way ANOVA and Bonferroni multiple comparison. Data are presented as mean and standard error of the mean (mean ± SEM). Accepted statistical significance was for \( p < 0.05 \).

Results

Induction of diabetes

Induction of chronic high hyperglycaemia after injection of STZ (60 mg kg\(^{-1}\), i.p.) was confirmed in rats by repeatedly measuring of fasting blood glucose levels above 17 mmol L\(^{-1}\). During the t-MCAO procedure, fasting glucose levels in rats were measured before, immediately and 24 hours after t-MCAO. Fasting glucose levels in diabetic rats measured before and immediately after t-MCAO ranged from 22.6–31.2 mmol L\(^{-1}\), whereas fasting blood glucose levels measured 24 hours after t-MCAO ranged from 17–33 mmol L\(^{-1}\). In control non-diabetic animals fasting blood glucose levels measured at indicated time points were 5–9 mmol L\(^{-1}\). Rat body weight was similar in all experimental groups (220–270 g).

Induction of t-MCAO

Successful t-MCAO was performed on 30 rats of a total of 36 rats used in this study. The remaining six animals were excluded from this study according to the exclusion criteria (see Methods section). Two diabetic rats in the 90-minute ischaemia group died within 24 hours after t-MCAO due to development of massive stroke. Furthermore, two animals in the 60-minute t-MCAO group were excluded from the study, one animal died of a large stroke and the other one was excluded due to subarachnoid haemorrhage, as confirmed at post-mortem examination of the brain. Two rats were excluded due to failure to reduce intra-ischaemic rCBF higher than 65% of baseline values, one diabetic animal in the 45-minute t-MCAO group and the other one in the control non-diabetic group.

Using LDF, rCBF was continuously monitored to confirm adequate reduction of intra-ischaemic rCBF (above 65% of baseline value) and reperfusion after removal of the occluding filament. Average intra-ischaemic reduction of rCBF was similar in all experimental groups, diabetic and non-diabetic, and ranged from 68–75% of baseline values (Figure 1(a)). rCBF monitored during the first 20-minutes of reperfusion did not significantly differ among the 30-minute t-MCAO control non-diabetic and 20, 30, 45 and 60-minute t-MCAO diabetic groups, ranging from 80–125% of baseline values (Figure 1(b)). Reperfusion was significantly impaired in all diabetic animals in the 90-minute t-MCAO group compared to all other groups and rCBF during reperfusion ranged from 56–62% of baseline values (Figure 1(b)).

Brain infarct volume in various durations of t-MCAO protocols

The volume of infarct was quantitatively measured by TTC staining 24 hours after t-MCAO. In the control group of rats without diabetes, 30-minutes of t-MCAO did not induce infarct of brain tissue on TTC staining, indicating that an ischaemia duration of 30-minutes is not sufficient to produce stroke in healthy animals (Table I, Figures 2 and 3).

In rats with diabetes, there was a significant increase in brain infarct volume expressed as a percentage of ischaemic
hemispheres, among the experimental groups with longer duration of ischaemia (Table I, Figures 2 and 3).

Twenty minutes of t-MCAO in diabetic rats resulted in a significantly smaller infarct volume compared to all other durations of t-MCAO (Figures 2(a–c) and 3), producing only 3 ± 5% of an ischaemic hemisphere. The affected area was mostly striatal and only a small part was cortical infarct (2 ± 3% vs 1.5 ± 2% of an ischaemic hemisphere, respectively; Table I, Figures 2 and 3). Thirty minutes of t-MCAO produced a significantly larger infarct that extended over 46 ± 7% of the ischaemic hemisphere, encompassing 12 ± 2% of striatum and 34 ± 7% of cortical area, which represents approximately half of the whole striatal area and half of the ipsilateral MCA irrigated cortex area (Figures 2(a–c) and 3, Table I).

Increasing ischaemia to 45 and 60 minutes resulted in spreading of infarct to almost the whole striatum that covers 22% of the hemisphere (17 ± 2% in the 45-minute t-MCAO and 19 ± 3% in the 60-minute t-MCAO groups) and a large portion of the cortex (35 ± 10% in the 45-minute t-MCAO and 40 ± 5% in the 60-minute t-MCAO group; cortical area irrigated by MCA is ~ 66% of the hemisphere). Total infarct size was 52 ± 5% in the 45-minute t-MCAO and 59 ± 3% of the ischaemic hemisphere in the 60-minute t-MCAO (Table I, Figures 2(a–c) and 3).

The longest ischaemia duration of 90-minutes in diabetic rats led to massive hemispheric stroke (89 ± 6% of hemisphere; the whole striatum (22 ± 3%) and the whole MCA territory (67 ± 6%)) (Table I, Figures 2(a–c) and 3).

**Discussion**

The most important findings in the present study are: (a) diabetic female SD rats are highly sensitive to ischaemia in a t-MCAO stroke model; (b) 60 and 90-minute t-MCAO currently used in non-diabetic female rats seems to be too long for diabetic ones, producing massive strokes in rats with diabetes; (c) lesser reperfusion was found in longer ischaemia in the 90-minute group (which possibly could be explained as a result of already irreversible brain infarct with brain vascular derangement). This brain vascular sign could be a marker of point of no return in stroke treatment; (d) 30-minute t-MCAO seems to be an adequate duration of occlusion for the diabetic rat female stroke model, consistently producing medium sized stroke which affects 30–50% of ischaemic
Diabetic rats, t-MCAO duration

Non-diabetic rats

Diabetic rats, t-MCAO duration

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Figure 3. Brain infarct in the control group and after various duration of transitory middle cerebral artery occlusion in experimental groups. Typical brain infarct in control group and after 20, 30, 45, 60 and 90 minutes of transitory middle cerebral artery occlusion in experimental groups (t-MCAO, n = 5/group) in diabetic (DM) rats and 30 minutes t-MCAO in healthy control (non-DM) rats (t-MCAO, n = 5/group). While 30 minutes t-MCAO in healthy controls did not produce cerebral infarction and 20 minutes of t-MCAO was not successful in inducing brain infarction or resulted in infarction of minimal size; 30, 45 and 60 minutes of t-MCAO resulted in significantly larger infarction in DM rats.

hemisphere; (e) 20-minute t-MCAO in rats with diabetes produced small striatal strokes of inconsistent size; and (f) it is questionable to compare results of occlusion for rats with and without diabetes, even if the duration of t-MCAO is equal.

Sprague-Dawley rat strain (SD) is the most widely used strain in rat MCAO experiments (41% of researchers have done experiments on this strain, vs 34% on Wistar, 10% on SHR, 2% on Long-Evans and 2% other strains [30]). SD rats have lower complication rates including subarachnoid haemorrhage (SAH) compared to Fisher and Wistar rats, due to anatomical course of ICA in petrous segment [18]. Previous experiments suggested that oestrogen relatively protected female rats from diabetes-exacerbated stroke damage [18]. However, present experiments showed that the diabetic female rats had extremely high susceptibility to brain infarction. When establishing a model for stroke in diabetic rats based on a published model in rats without diabetes, at first it was found that the usual duration of ischaemia from 60–120 minutes has produced large strokes, with massive oedema and devastating neurological deficits, such as inability to feed, immobility and alteration of consciousness. These strokes were very often lethal in the first 24 hours after the experiment, mostly due to massive oedema and a rise in intracranial pressure. Similarly, patients with the most severe strokes of the whole MCA territory and high NIHSS (National Institute of Health Stroke Score) are poor candidates for treatment with thrombolysis and mostly die due to brain oedema and complications of dysphagia and immobility, but also have higher risk of secondary haemorrhage. Having this in mind, in the present experiments, duration of MCA occlusion was shortened variably in the groups to determine MCAO duration that will result in moderate stroke, already described in non-diabetic patients/rats, i.e. 30 and 50% of volume of hemisphere [31–34]. In the present experiments longer ischaemia inevitably produced a larger brain infarct (Figures 2(c) and 3, Table I) and usual duration of ischaemia in rats without diabetes (in earlier studies mostly 90 minutes, but also 60–120 minutes) was too long for diabetic ones [7]. Ischaemia of 60 and 90 minutes produced massive infarct with lesser probability for survival as well as any kind of treatment.

Diabetes worsens stroke outcome; diabetic patients have 2–4-times higher risk for stroke compared to non-diabetic patients [13]. Possible underlying causes are chronic hyperglycaemia, which leads to free oxygen radicals and cytokines production and increases ischaemic brain cells pre-disposition to apoptosis [35]. In addition, the intimal artery thickening and arteriolar occlusion occurs in diabetes, contributing by impaired vascular function to inadequate tissue perfusion [12]. In the past research, animal models of longer ischaemia and longer time before treatments of stroke starts were accompanied with negative or poor results in outcome of treatments [31]. For example, rtPA treatment after 2 hours of t-MCAO showed no effect on brain infarct size and neurological deficit, but had negative effects on the risk of intracerebral haemorrhage, blood–brain barrier breakdown and production of inflammatory mediators [10]. Longer ischaemia inevitably worsens brain infarct and augments inflammatory response that is related to vascular disease and diabetes [36]. Higher inflammatory response leads to brain oedema and consequently to lower brain perfusion and incurable damage [37]. Taking into account all these facts, a diabetic rat model of stroke should be modified to suit the purpose, i.e. ischaemia duration needs to be shortened to produce a medium-sized brain infarct that would mimic treatable human stroke. Otherwise, the false conclusion can be drawn from massive strokes due to an inadequate experimental design—that no therapy is useful in stroke treatment in diabetics. In clinical practice, the majority of strokes are effectively treated in a short period after they occur and, in fact, they are of medium and smaller size. On the other side, a longer duration of ischaemia and, consequently, larger strokes are mostly resistant to treatment and detrimental to patients.
In the present study, a 20-minute ischaemia duration did not produce infarction in two out of five rats with diabetes, while in the remaining animals the infarct was rather small (3 ± 5% of ischaemic hemisphere) and located almost only in the striatum (Table I, Figures 2(a) and (b) and 3).

Only 30-minutes of ischaemia in rats with diabetes consistently produced medium sized infarcts that include ~ 50% of the cortex and ~ 50% of striatum irrigated by MCA (Figures 2(a) and (b) and 3, Table I). That corresponds well with large artery atherosclerotic stroke [38]. At the same time, 30 minutes of occlusion was not devastating or lethal for rats, making it an optimal duration of ischaemia in a stroke model for future research. Brain infarcts of the same size were used successfully in rats without diabetes [8,31].

Longer ischaemia durations of 45 and 60 minutes in rats with diabetes produced larger stroke in the MCA territory, encompassing almost the whole striatum and a large portion of the cortex (Table I, Figures 2(a) and (b) and 3). Ischaemia of 90 minutes led to massive hemispheric stroke (Table I and Figures 2(b) and 3). Such large strokes would be in clinical practice almost always incompatible with survival due to development of brain oedema, raised intracranial pressure (ICP) with secondary low arterial perfusion and subsequent spreading of infarction, brain herniation, etc. This type of stroke occurs after sudden and permanent MCA occlusion, with no reperfusion and without preformed arterial collaterals. One significant finding of this study was also lesser reperfusion in longer ischaemia. All animals in the 90-minute group had significantly lower reperfusion (56–62% of baseline compared to 80–125% of baseline in other groups, Figure 1(b)) and brain vascular derangement in which normal post-ischaemic reperfusion as a salvation mechanism does not appear. It is speculated that this brain vascular sign is important and could be a marker of point of no return in stroke treatment.

If compared to humans, strokes classified of small size have a range between 4.5–14% of the ipsilateral hemisphere [39,40]. Infarct is considered malignant when it covers more than 50% of the cerebral hemisphere, but some studies report that malignant infarction occurs when the stroke size is greater than 39% of the hemisphere [41,42].

For every experimental model that could represent human conditions, it is important to simulate average situation. The model has to simulate most common types of stroke in humans which are treatable strokes—those of medium and smaller size. Based on the results of the present study, the best model for mimicking human condition of stroke would be 30-minute occlusion that led to infarct of approximately half of the striatum and half of the cortex irrigated by MCA. All other longer models produced total or almost total striatum devastation and the cortex was almost totally destroyed in the 90-minute model, which is incurable in human medicine and there is no purpose to further explore them.

Conclusion
The present study tends to impact the experimental design of stroke model in basic research and consequently influence clinical research in the field of cerebrovascular disorders. It is proposed that duration of ischaemia in a female rat model has to be adjusted depending on which type of stroke one wants to produce. Not all strokes are treatable the same way and not all of them have the same prognosis; smaller and medium size strokes have the best chances for improvement by therapy. Almost any treatment of massive stroke or treatments started after certain time points after stroke tend to be ineffective. The present study, although restricted to female SD rats, aims to alter the current point of view on duration of t-MCAO used in the experiments. The main application of the results of the present study are in finding the optimal duration of MCA occlusion in rats with diabetes in stroke model that could be effectively treated. In addition, by establishing the optimal duration of occlusion, results of different studies would be comparable and testing of new therapeutic options in rat stroke experiments would be easier and more effective, finally leading to better understanding of mechanisms of prevention of neurological deficits. In the present study, the 30-minute t-MCAO model in female rats with diabetes seems to produce a stroke of medium size, most likely the best for the experimental and clinical research on new treatment options.

Declaration of interest
The authors report no conflicts of interest. Support came from a grant by the Ministry of Science, Education and Sport of Republic of Croatia #219-2160133-2034 (principal investigator: Ines Drenjančević) and by EU FP7 grant GlowBrain (REGPOT-2012-CT2012-316120).

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