

Research Paper

Hyperbaric oxygenation and 20-hydroxyeicosatetraenoic acid inhibition reduce stroke volume in female diabetic Sprague–Dawley rats

Mihael Mišir^{1,2}, Marija Renić³, Sanja Novak², Martina Mihalj², Anita Ćosić², Monika Vesel² and Ines Drenjančević²

¹Clinical Hospital Center Osijek, Neurology Clinic, Osijek, Croatia

²University Josip Juraj Strossmayer Osijek, Faculty of Medicine Osijek, Department of Physiology and Immunology, Laboratory for Circulatory Physiology, Osijek, Croatia

³Croatian Institute for Brain Research, School of Medicine University of Zagreb, Zagreb, Croatia

Edited by: Karin Przyklenk

New Findings

- **What is the central question of this study?**

Is there a beneficial effect and what are the mechanisms of acute and multiple hyperbaric oxygenation (HBO₂) exposures on the outcome of cerebral tissue injury induced by a transient middle cerebral artery occlusion model in diabetic female rats? Are 20-hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids involved?

- **What is the main finding and its importance?**

Equal reduction of cortical and total infarct size in rats treated with HBO₂ and HET0016 (20-hydroxyeicosatetraenoic acid production inhibitor) and significant mRNA upregulation of epoxyeicosatrienoic acid-producing enzymes (Cyp2J3 and Cyp2C11) in treated groups suggest that HBO₂ and HET0016 are highly effective stroke treatments and that cytochrome P450 metabolites are involved in this therapeutic effect.

We evaluated the effects of acute and repetitive hyperbaric oxygenation (HBO₂), 20-hydroxyeicosatetraenoic acid (20-HETE) inhibition by *N*-hydroxy-*N'*-(4-butyl-2methylphenyl)-formamidine (HET0016) and their combination on experimental stroke outcomes. Streptozotocin-induced type 1 diabetic Sprague–Dawley female rats ($n = 42$; $n = 7$ per group), were subjected to 30 min of transient middle cerebral artery occlusion (t-MCAO)–reperfusion and divided into the following groups: (1) control group, without treatment; and groups exposed to: (2) HBO₂; (3) multiple HBO₂ (HBO₂ immediately and second exposure 12 h after t-MCAO); (4) HET0016 pretreatment (1 mg kg⁻¹, 3 days before t-MCAO) combined with HBO₂ after t-MCAO; (5) HET0016 treatment (1 h before, during and for 6 h after t-MCAO); and (6) HET0016 treatment followed by HBO₂ after t-MCAO. Messenger RNA expression of CYP2J3, CYP2C11, CYP4A1, endothelial nitric oxide synthase and epoxide hydrolase 2 was determined by real-time qPCR. Cortical infarct size and total infarct size were equally and significantly reduced in HBO₂- and HET0016-treated rats. Combined treatment with HET0016 and HBO₂ provided no significant additive effect compared with HET0016 treatment only. Messenger RNA of *Cyp2J3* was significantly increased in all study groups, and mRNA of *Cyp2C11* was significantly increased in the multiple HBO₂ group and the HET0016 treatment followed by HBO₂ group, compared with the control group. Expression of endothelial nitric oxide synthase

was significantly increased after HBO₂ treatments, and expression of epoxide hydrolase 2 was increased in all groups compared with the control group. In diabetic female Sprague–Dawley rats, HBO₂ and HET0016 are highly effective stroke treatments, suggesting the involvement of cytochrome P450 metabolites and the NO pathway in this therapeutic effect.

(Received 10 April 2017; accepted after revision 29 August 2017; first published online 21 September 2017)

Corresponding author I. Drenjančević: University Josip Juraj Strossmayer Osijek, Faculty of Medicine, Cara Hadrijana 10e, HR-31000 Osijek, Croatia. Email: ines.drenjancevic@mefos.hr

Introduction

The only effective pharmacological therapy of stroke in humans is thrombolysis with recombinant tissue plasminogen activator, but diabetes is sometimes an exclusion criterion in recurrent stroke treatment. The time window for the therapy is narrow, and no other pharmacological agents have demonstrated efficacy in improving outcomes after ischaemic stroke (Unfirer *et al.* 2008, Institoris *et al.* 2012; Jauch *et al.* 2013; Kernan *et al.* 2014). Thus, searches for alternative approaches are welcome. Hyperbaric oxygenation (HBO₂; Xu *et al.* 2016) improves oxygen delivery and post-ischaemic metabolism, restores ion pump function and allows time for collateral circulation to develop (Singhal 2007). In normal tissue it causes vasoconstriction, but in ischaemic brain tissue it increases microvascular flow and improves oxygen dissolution and transport (Singhal *et al.* 2005). The time window for HBO₂ application may be up to 6 h (Badr *et al.* 2001; Lou *et al.* 2004), which is longer than the time window for thrombolytic therapy. Hyperbaric oxygenation increases oxygenation of the ischaemic penumbra by 20% and improves mitochondrial function (Sunami *et al.* 2000; Liu *et al.* 2006). It has an anti-inflammatory effect by reducing expression of cyclooxygenase-2 and reduces the number of intercellular adhesion molecules and therefore reduces adhesion and infiltration of leucocytes (Hjelde *et al.* 2002). However, American Heart Association/American Stroke Association guidelines do not recommend HBO₂ treatment for acute ischaemic stroke because of somewhat inconclusive data. Some data imply that the intervention may be harmful (Jauch *et al.* 2013), whereas others found no evidence that HBO₂ improves clinical outcomes for acute stroke. However, the main disadvantage of these trials used in meta-analysis was the delay from stroke onset to initiation of HBO₂ and the need for delivery of care in a specialized chamber (Bennett *et al.* 2005).

20-Hydroxyecosatetreanoic acid (20-HETE), a cytochrome P450 (CYP) enzyme metabolite of arachidonic acid and a very potent vasoconstrictor produced in cerebral arterioles and brain tissue (Gebremedhin *et al.* 2016), is a well-described mediator of neural tissue damage in stroke (Dunn *et al.* 2008). The

inhibitors of 20-HETE, such as *N*-hydroxy-*N'*-(4-butyl-2methylphenyl)-formamidine (HET0016), reduce stroke volume and improve neurological outcome after stroke in animal models (Renic *et al.* 2009; Crago *et al.* 2011). Other metabolites of arachidonic acid, such as epoxyecosatrienoic acids (EETs), have the potential to alleviate the impairment of tissue perfusion and detrimental outcome of stroke (Kibel *et al.* 2015; Zuloaga *et al.* 2015).

Although both HBO₂ and HET0016 were shown to be effective in treatment of rats with stroke, we did not find *in vivo* experiments on diabetic rats with stroke and experiments using their combinations.

The aim of the present study was to evaluate the effects and underlying mechanisms of acute and multiple HBO₂ exposures, as well as the potential effects of simultaneous or standalone 20-HETE inhibition and the role of EETs in a transient middle cerebral artery occlusion (t-MCAO) model in long-term diabetic female rats (Mišir *et al.* 2016).

Methods

Ethical approval

All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609). They were approved by the local ethical committee (Faculty of Medicine, University of Osijek) and the competent authority, the Ministry of Agriculture, Croatia (approval 525-06-1-0255/12-2). All procedures conducted in the present study were in accordance to the recommendations and requirements listed by Grundy (2015).

Animals

Experiments were performed on 42 healthy Sprague–Dawley female diabetic rats, 12 weeks old, weighting between 180 and 230 g, housed in an accredited animal care facility at the University of Osijek. Animals were housed in standard plastic cages in a temperature- and humidity-controlled environment, with a 12 h–12 h light–dark cycle, and were allowed access to food and water *ad libitum*.

Induction of diabetes

Type 1 diabetes mellitus was induced in the rats by a single i.p. injection of streptozotocin at 6 weeks of age (60 mg kg^{-1} ; Sigma-Aldrich, St Louis, MO, USA). Streptozotocin was freshly dissolved in 0.09 M sodium citrate buffer and induced by a single intraperitoneal injection to the rats (Akbarzadeh *et al.* 2007; Srinivasan & Ramarao, 2007). The fasting blood glucose concentrations were repeatedly measured using a glucometer (Accu-Chek Active; Roche, Mannheim, Germany).

Study design

Diabetic rats that had fasting blood glucose concentrations $>17 \text{ mmol l}^{-1}$ (Lan *et al.* 2008) were exposed to t-MCAO and reperfusion injury and randomly assigned to the following groups ($n = 7$ animals per treatment group): (1) control group (without pharmacological or HBO₂ treatment after t-MCAO and reperfusion); and experimental groups exposed to: (2) HBO₂ (HBO₂ immediately after t-MCAO); (3) multiple HBO₂ (first HBO₂ immediately, and second 12 h after t-MCAO); (4) HET0016 pretreatment + HBO₂ (single injection of HET0016 i.p. daily, 1 mg kg day^{-1} , for 3 days before t-MCAO, combined with HBO₂ after t-MCAO); (5) HET0016 treatment (injections of HET0016 i.p., 1 mg kg h^{-1} , every 1 h, starting from 1 h before t-MCAO, during and for 6 h after t-MCAO); and (6) HET0016 treatment + HBO₂ [HET0016 treatment as described in group 5; injections of HET0016 i.p., 1 mg kg h^{-1} , every 1 h, starting from 1 h before t-MCAO, during and for 6 h after t-MCAO, combined with HBO₂ after t-MCAO].

Exclusion criteria were: death of the animal during the surgical procedure or within 24 h after reperfusion; excessive bleeding during operative procedures; reduction of regional cerebral blood flow (rCBF) during ischaemia by $<55\%$ of baseline values; absence of reperfusion after removal of suture monitored using laser Doppler flowmetry (LDF); and presence of intracerebral and/or subarachnoid haemorrhage at post-mortem examination of brains. Overall, one animal from group 2 was excluded because of reduction of rCBF by $<55\%$ and one from group 6 because of excessive bleeding. Animals excluded from the study were replaced.

Anaesthesia

Rats were first premedicated with atropine (Atropini sulfas; Belupo, Koprivnica, Croatia), 0.1 mg kg^{-1} , i.p. (Zhang & Liu, 2004). Anaesthesia was induced by i.p. administration of a combination of midazolam (Dormicum; Roche Pharma AG, Grenzach-Wyhel, Germany), 0.5 mg kg^{-1} , and ketamine (Ketanest; PfizerPharma GmbH, Berlin, Germany), 75 mg kg^{-1} .

Regional cerebral blood flow monitoring by LDF

Under general anaesthesia, the periosteum of the parietal bones was exposed by a medial approach and haemostasis was achieved. Using a dental drill with a 1 mm tip, the left parietal bone was thinned until translucent, 2 mm posterior and 6 mm lateral to bregma. An LDF probe was placed in a probe holder and fixed with glue and dental cement above the thinned bone window and intact dura on the skull to monitor rCBF in the cerebral cortex continuously (LDF, model MBF3D; Moor Instruments Ltd, Axminster, UK) during the experiment.

Transient middle cerebral artery occlusion

Transient focal cerebral ischaemia in the region of the left middle cerebral artery (MCA) was induced as described by Koizumi *et al.* (1986) and modified by Longa *et al.* (1989). The body temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$. A 4–0 nylon monofilament (coated at the 0.25–0.3 mm diameter tip with silicone using silicone Xantopren and activator Elastomer; Heraeus Kulzer, Hanau, Germany) was introduced into the left internal carotid artery (ICA) and gently advanced to the origin of the MCA until rCBF decreased abruptly by $>55\%$ (Zuloaga *et al.* 2015). After 30 min, the filament was withdrawn to allow reperfusion. The rCBF was continuously monitored to confirm adequate occlusion of the MCA and reperfusion. After 20 min of reperfusion, the incision on the neck was sutured. The LDF probe was removed, and the animal was placed into the heated cage and monitored until completely recovered from anaesthesia. Only animals with a reduction of rCBF during t-MCAO by $>55\%$ and good reperfusion were analysed.

Hyperbaric oxygenation

After surgery, animals were put into a hermetically closed hyperbaric chamber (Đuro Đaković Holding d.d., Slavonski Brod, Croatia). Throughout 15 min, 100% oxygen pressure was increased to 2 ATA (2 atmospheres, 2 bars), and animals were treated for 2 h. Decompression was also done over 15 min (Kibel *et al.* 2015). The second HBO₂ in group (iii) was performed according to the same protocol, 12 h later (Zhang, 2007).

HET0016 [(*N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)-formamidine; Cayman Chemical Company, Ann Arbor, MI, USA] solution was prepared in vehicle (11% sulfobutylether-7- β -cyclodextrin in an isotonic mannitol solution) and kept protected from light in a refrigerator. HET0016 was administered as a pretreatment (preconditioning) in the HET0016 pretreatment + HBO₂ group (i.p., in a single injection, $1 \text{ mg kg}^{-1} \text{ day}^{-1}$, for 3 days consecutively before the experiment), or as a treatment after t-MCAO in groups that were exposed to

HET0016 treatment and HET0016 treatment + HBO₂ (I.P. injections, at a dose of 1 mg kg⁻¹ h⁻¹, administered every 1 h, starting from 1 h before t-MCAO and during the next 6 h; Renic *et al.* 2009).

Measurement of cerebral infarct volume

Twenty-four hours after reperfusion, the rats were anaesthetized with ketamine and midazolam and killed by decapitation. The brains were cut into 2-mm-thick coronal sections, stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich Chemie, Munich, Germany), fixed in 10% buffered formalin solution for 24 h and scanned. Infarct volumes were measured using ImageJ imaging software (v1.37; National Institutes of Health, Bethesda, MD, USA) and expressed as a percentage of the ischaemic hemisphere (Bederson *et al.* 1986). Quantitative analysis of rCBF and infarct size, statistical analysis, surgical procedures and treatment procedures were all done by different researchers blinded to the other procedures.

Analysis of mRNA expression

Brain samples of the ischaemic part of the hemisphere ipsilateral to t-MCAO were collected 24 h after t-MCAO, with rapid freezing in liquid nitrogen, and stored at –80°C until further processing. Total RNA was isolated using One Step RNA Reagent (Bio Basics Canada Inc., Markham, Ontario, Canada). The RNA concentration and purity were assessed using a P330 NanoPhotometer (Implen, Munich, Germany). Samples were treated with DNase I (Sigma) in order to remove any eventual gDNA, then 1 µg of RNA was transcribed to cDNA using PrimeScriptTM RT Reagent Kit (Takara, Shiga, Japan). Quantitative PCR was performed using Absolute qPCR SYBR Low ROX Mix (Thermo Scientific, Wilmington, DE, USA) on CFX96TM Real-Time System (Bio-Rad, Singapore). Relative mRNA expression of *Cyp2J3*, *Cyp2C11*, *Cyp4A1*, *Cyp4A3*, *eNOS* and *EPHX2* genes was calculated relative to a standard curve, where hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) was used as the reference gene. Primers were as follows: *Cyp2J3* (forward 5'-CCTTCTGTTCTGGCTGATTT-3', reverse 5'-AGGCCCTGGC GGGTAGT-3'), *Cyp2C11* (forward 5'-CAATCCGCAGTCTGAGTT-3', reverse 5'-TGCTGAGAATGGCATAAA-3'), *Cyp4A1* (forward 5'-GTTCTACCTGCAAAGGCAA TGG-3', reverse 5'-TGCCCAAAGAACCAGTGGAA-3'), *Cyp4A3* (forward 5'-TCTCAGGGAGCAAACACGA-3', reverse 5'-CAACAGGAGCAAACCATAACCA-3'), *HPRT1* (forward 5'-GAAAGAACGTCTTGATTGTTGAAGATAT-3', reverse 5'-GAGAGGTCCTTTTCACCAGCAA-3'), *eNOS* (forward 5'-CGAACAGCAGGAGCTAGAGG-3', reverse 5'-GAGGTGGATCTCTCCTGGGT-3') and *EPHX2* (forward 5'-TGGCTGAGGCTGAACTGG-3',

reverse 5'-GTGTCCAGTGACCACAGT-3'). Primers for *Cyp4a3* also recognize *Cyp4A2* owing to the high compatibility of these genes.

Statistical analysis

The reduction of brain infarct volume was calculated by dividing the brain infarct volume of the respective animal by the average brain infarct volume of rats from the t-MCAO control group and deducting this number from 1.0. Data are presented as mean values ± SD. The rCBF was expressed as a percentage of the baseline value measured immediately before t-MCAO. The distribution of data was tested by the Shapiro–Wilk test. The significance of differences in mean values between the groups was assessed using one-way ANOVA followed by the Holm–Sidak *post hoc* test. Cerebral blood flow at different time points was compared by two-way repeated-measures ANOVA followed by the Bonferroni *post hoc* test. Differences between two groups were compared by Student's unpaired *t* test. In the event of an abnormal data distribution, non-parametric tests were used. All calculations were performed with SigmaPlot v.11 (Systat Software Inc., San Jose, CA, USA) and GraphPad Prism v.5 software (GraphPad Software Inc., La Jolla, CA, USA). For mRNA, statistical analysis was done using GraphPad Prism v.5. The Kolmogorov–Smirnov test was used to determine the distribution of variables. Differences between groups were calculated using the Mann–Whitney non-parametric test. Values of *P* < 0.05 were treated as statistically significant.

Results

Induction of diabetes and fasting blood glucose concentrations

Before and after t-MCAO, fasting blood glucose concentrations ranged from 21.9 to 30.4 mmol l⁻¹, whereas 24 h after t-MCAO fasting blood glucose concentrations ranged from 24 to 32.1 mmol l⁻¹, and values were similar among groups. Experimental treatments did not significantly alter fasting blood glucose values (Table 1).

Regional cerebral blood flow during t-MCAO and reperfusion

Induction of t-MCAO. As shown in Fig. 1A, there was no statistically significant difference in the reduction of rCBF among the groups, suggesting that t-MCAO was equally successful in all study groups (rCBF fell by 59.6% to 72.2%, with no differences between groups at any time points). The rCBF during first 20 min of reperfusion did not differ significantly between control groups and groups treated only with HBO₂, ranging from 87 to 113% of baseline (Fig. 1A and B). Reperfusion was

Table 1. Fasting blood glucose concentrations

Time point	Fasting blood glucose (mmol l ⁻¹)					
	Control group	HBO ₂	Multiple HBO ₂	HET0016 pretreatment + HBO ₂	HET0016 treatment	HET0016 treatment + HBO ₂
12 weeks, before t-MCAO	25	28.8	22.7	22.8	24.4	30.4
	30.2	23.5	27.6	27.1	28.7	26
	22.2	22.9	24.6	23.5	21.9	26.5
	23.3	30.4	28.9	28.5	27.3	22.3
	29	28.4	30.1	27.3	30.1	27.9
	29.8	29.9	29.3	30.3	26.1	28.3
	24.6	24.9	24.1	29.7	22.3	29.4
	Average	26.30	26.97	26.76	27.03	25.83
After t-MCAO	25.7	26.4	23.1	23.6	25.6	29.1
	28.8	23.9	29.6	26.1	30.1	26.8
	24.5	23.1	26.3	22.5	22.3	27.7
	23.7	28.1	28.1	25.1	25.9	28.8
	28.7	27.3	28.8	26.4	28.8	24.2
	29.5	29.5	29.6	27.3	26.7	30.3
	30.1	27	22.7	29.2	23	28.6
	Average	27.29	26.47	26.89	25.74	26.06
24 h after t-mCAO	27.2	24.8	25.4	30.2	25.9	25.2
	26.1	26.8	28.6	24	32.1	31.5
	28.2	29.8	28.1	30.7	31.9	28.3
	24	30.6	29	27.2	24.8	32.1
	29.2	28	31.8	26.8	30.3	27.9
	31.9	27.6	30.8	28.4	28.3	28.7
	31.7	31.6	27.9	30.4	27.5	26.2
	Average	28.33	28.46	28.80	28.24	28.69

Abbreviations: HBO₂, hyperbaric oxygenation; HET0016, *N*-hydroxy-*N'*-(4-butyl-2methylphenyl)-formamidine; and t-MCAO, middle cerebral artery occlusion.

significantly impaired in all groups treated with HET0016 at each measured time point compared with the control t-MCAO group, ranging from 67 to 83% of baseline values ($P < 0.0001$; Fig. 1B). Similar differences were found when HET0016-treated groups were compared with HBO₂-treated groups ($P < 0.05$; Fig. 1B).

Hyperbaric O₂ treatment alone reduces brain infarct volume after t-MCAO. Cortical infarct size and the total infarct size were significantly reduced in rats treated with HBO₂ compared with the control group (Fig. 2B and C); however, the subcortical infarct size was not reduced by HBO₂ (single HBO₂ $P = 0.966$, multiple HBO₂ $P = 0.078$; Fig. 2A). There was no significant difference in infarct volumes between the groups treated with different HBO₂ protocols (Fig. 2). Hyperbaric oxygenation reduced the total infarct volume by $44.9 \pm 7.41\%$ in the HBO₂ group and by $47.6 \pm 12.07\%$ in the multiple HBO₂ group, compared with the control group.

Treatment with HET0016 alone reduces brain infarct volume after t-MCAO. Treatment with HET0016 significantly reduced total, subcortical and cortical brain

infarct volume (total infarct volume was reduced by $63.1 \pm 3.34\%$, cortical infarct volume by $71.6 \pm 4.74\%$ and subcortical infarct volume by $44.1 \pm 5.45\%$, compared with the control group; Fig. 2). In addition, HET0016 treatment was more efficient in reducing subcortical and total infarct volume than single HBO₂ treatment (Fig. 2B and D).

Treatment or pretreatment with HET0016 combined with HBO₂ provides no significant additive effect in reducing brain infarct size compared with exclusive HET0016 treatment. Hyperbaric oxygenation in addition to pretreatment (HET0016 pretreatment + HBO₂ group) or treatment (acute perioperative administration) with HET0016 (HET0016 treatment + HBO₂) significantly reduced total, subcortical and cortical brain infarct size compared with the control group, but showed no superiority to HET0016 treatment alone (Fig. 2).

Although there was no difference in cortical infarct volumes among any of the treated groups, all three groups treated with HET0016 had significantly greater subcortical and total infarct size reduction compared with single HBO₂ treatment (Fig. 2). Multiple HBO₂ treatments were

equally effective in reducing infarct size as HET0016 treatment (Fig. 2).

Messenger RNA expression of CYP450 enzymes

Although we performed the *Cyp4A3* mRNA expression analysis, results are not displayed because the expression was extremely low (highest value was 2.1×10^{-4}). *Cyp4a1*, although present in the tissue, did not show significant differences among groups (Fig. 3C). In contrast, mRNA of *Cyp2J3* was significantly increased in all groups compared with the control group and in HBO₂ and multiple HBO₂ groups compared with the HET0016 pretreatment + HBO₂ group (Fig. 3B). *Cyp2C11* mRNA was significantly increased in multiple HBO₂ and HET0016 pretreatment + HBO₂ groups compared with the control group (Fig. 3A). The relative expression of *eNOS* was significantly increased in the HBO₂ group compared with all other groups, and in the HET0016 treatment + HBO₂ group compared with the control, HET0016 pretreatment + HBO₂ and HET0016 treatment groups, and in the multiple HBO₂ group compared with the control group (Fig. 4B). Expression of *EPHX2* was

significantly increased in all groups compared with the control group, and in HET0016 pretreatment + HBO₂ group compared with the HET0016 treatment + HBO₂ group (Fig. 4A).

Discussion

The most important findings of the present study are as follows: (i) if administered shortly after the stroke, HBO₂ (used as a single exposure or multiple exposures to HBO₂) is a highly effective treatment of stroke in diabetic female rats, even in the presence of long-term untreated diabetes; (ii) treatment with HET0016 alone is equally effective; (iii) HET0016 (treatment or pretreatment) combined with HBO₂ provides no significant additive effect in reducing brain infarct volume compared with exclusive HET0016 treatment; and (iv) there is significantly increased expression of EET-forming enzymes in the brain tissue in groups after HBO₂ or HET0016 treatment, suggesting that EETs contributed to the reduction of brain infarct volume.

To our knowledge, these are the first experiments using HBO₂ and HET0016 in *in vivo* experiments on diabetic

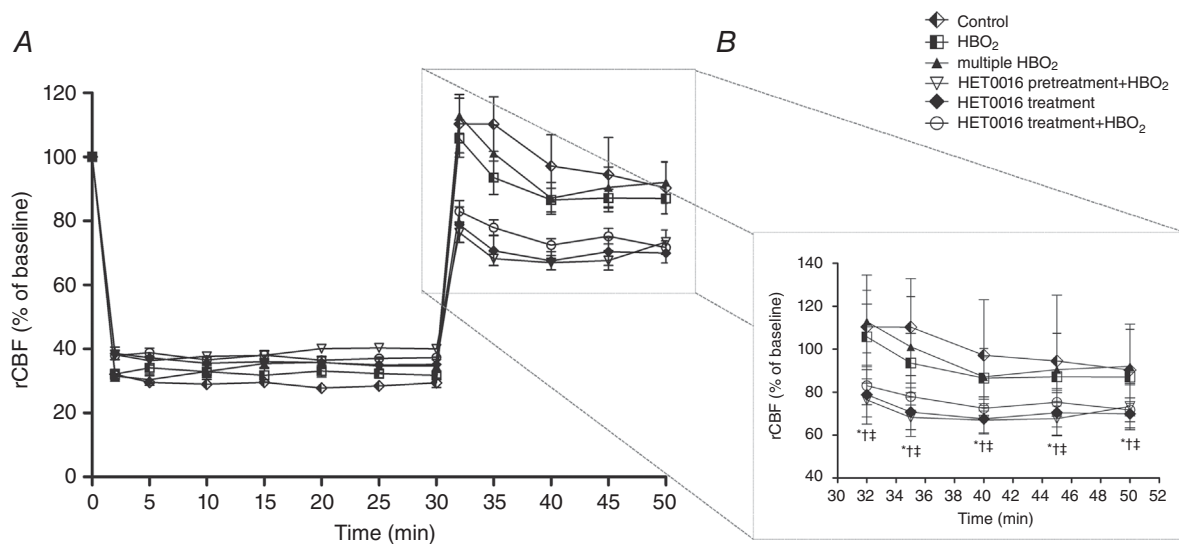


Figure 1. Changes in regional cerebral blood flow (rCBF) before, during and after transitory middle cerebral artery occlusion (t-MCAO) in all study groups

Groups are as follows: (1) control, t-MCAO for 30 min; (2) HBO₂, t-MCAO for 30 min followed by one hyperbaric oxygen treatment (HBO₂); (3) multiple HBO₂, t-MCAO for 30 min followed by two HBO₂ (immediately after t-MCAO and after 12 h); (4) HET0016 pretreatment + HBO₂, t-MCAO for 30 min in rats treated with HET0016 (*N*-hydroxy-*N'*-(4-butyl-2methylphenyl)-formamidine, 3 days before the experiment) followed by one HBO₂; (5) HET0016 treatment, t-MCAO for 30 min in rats treated with HET0016 (administered every 60 min, starting from 1 h before experiment, up to 6 h after experiment); and (6) HET0016 treatment + HBO₂, t-MCAO for 30 min in rats treated with HET0016 (administered in the same manner as in the previous group) followed by one HBO₂. The rCBF values at different time points are expressed as a percentage of the corresponding baseline value measured at time 0 (100%). The rCBF of all groups was compared with the control group. **P* < 0.05 for HET0016 pretreatment + HBO₂ group. †*P* < 0.05 for HET0016 treatment. ‡*P* < 0.05 for HET0016 treatment + HBO₂ group.

female rats, and the first experiments to explore their combined administration in treatment of stroke in the available literature. An additional value of the present study is that it was intentionally performed on the more difficult and rarely used female long-term diabetic rat model (Alkayed *et al.* 2000; Toung *et al.* 2000). Female patients with diabetes mellitus have 4.8-fold higher risk for

developing ischaemic stroke than the general population (compared with 3.7-fold for men) and more often suffer fatal strokes (standardized mortality ratios of 3.1 for males and 4.4 for females; Kernan *et al.* 2014).

Published experiments in non-diabetic rats have demonstrated that HBO₂ reduces stroke volume after t-MCAO. In the t-MCAO model, ischaemia produces

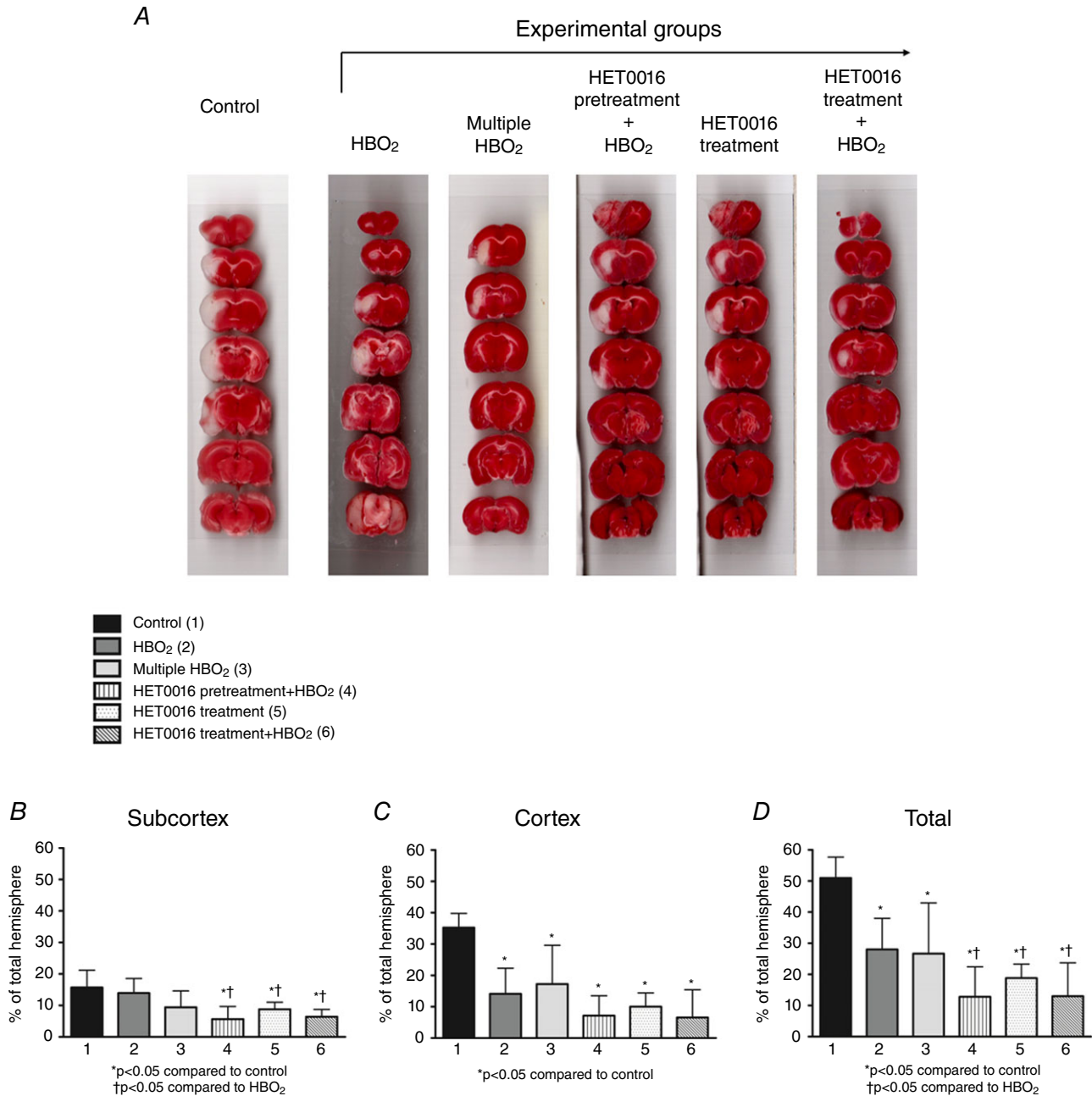


Figure 2. Representative brain slices (A) and infarct sizes of different experimental groups [subcortical (B), cortical (C) and the total brain infarct volume (D)] 24 h after transitory middle cerebral artery occlusion (t-MCAO) in all study groups
Groups are the same as in Fig. 1. The infarct size is expressed as a percentage of the total hemisphere size (volume). *P < 0.05 compared with control group. †P < 0.05 compared with HBO₂ group.

infarction initially in the area of the striatum (because this is an area with almost no collateral flow; Garcia *et al.* 1995) and later in the dorsolateral cortex above it. Striatal infarcts are therefore mostly resistant to any therapy. In

our recent experiments, we established a 30 min t-MCAO model in female diabetic Sprague–Dawley rats with the intention of producing on average, moderate-sized stroke (with stroke volume affecting between 30 and 50% of

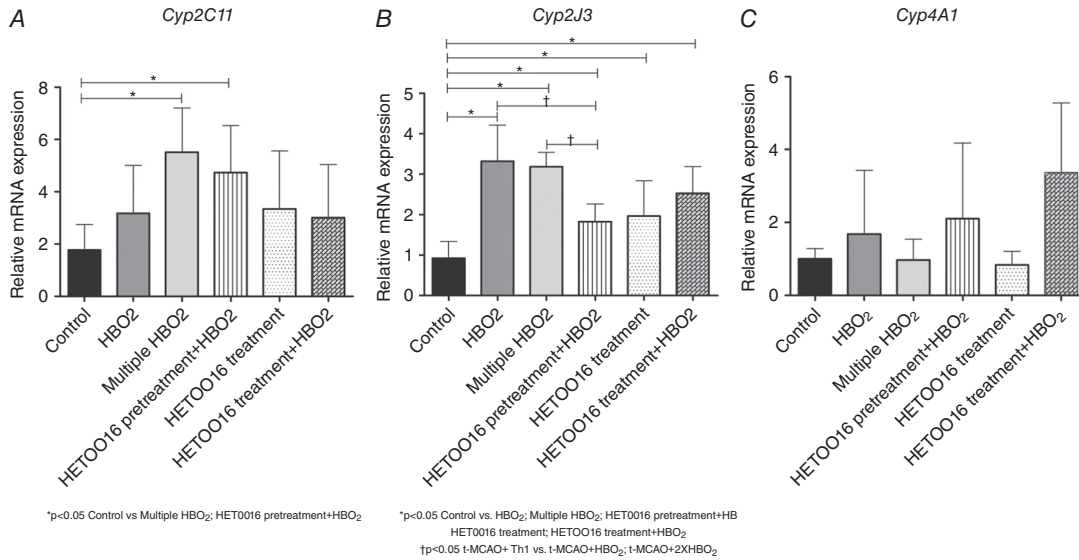


Figure 3. Relative mRNA expression levels of *Cyp2C11* (A), *Cyp2J3* (B) and *Cyp4A1* (C) genes in brain tissue
 Groups are the same as in Fig. 1. Data are presented as means + SD, and the level of significance was determined at $P < 0.05$ ($n = 5$).

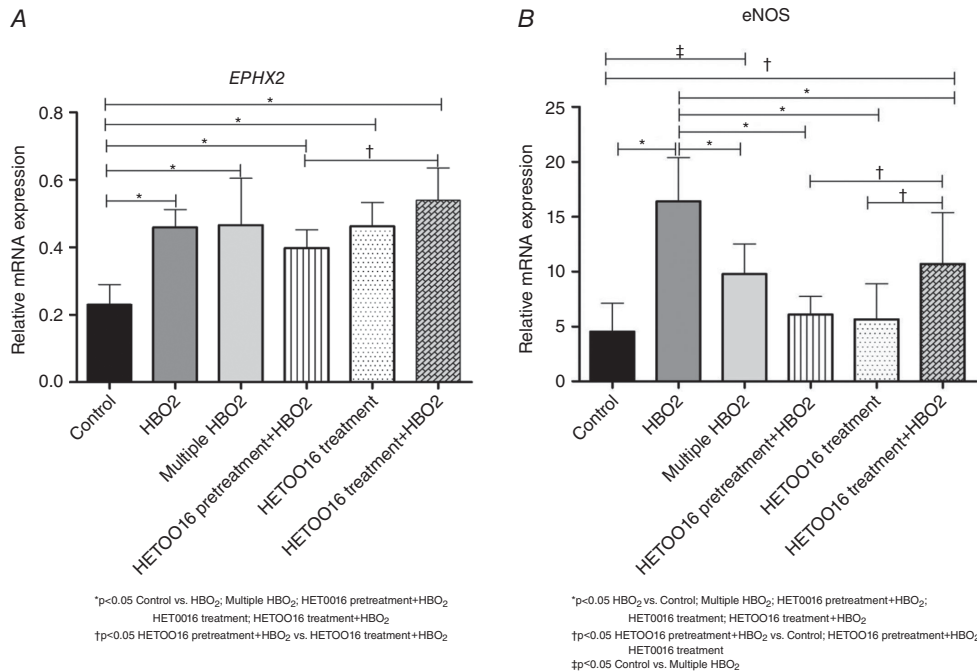


Figure 4. Relative mRNA expression levels of *EPHX2* (A) and *eNOS* (B) genes in brain tissue
 Groups are the same as in Fig. 1. Data are presented as means + SD, and the level of significance was determined at $P < 0.05$ ($n = 5$).

the ischaemic hemisphere; Dunn *et al.* 2008), that would allow observation of treatment efficiency, and not massive stroke that would be lethal within the first 24 h owing to complications such as brain oedema or dysphagia.

In the present study, in long-term diabetic female rats, HBO₂ was not efficient in reducing the subcortical infarct volume. In other words, although the duration of ischaemia was shortened significantly, the ischaemic core went over the point of no return. In contrast, the infarcted volume of the cortex and, consequently, total brain infarct was reduced significantly (Fig. 2). Although the effects of repeated HBO₂ treatments on brain infarct volume were studied in different models of stroke (Wang *et al.* 2010; Yin & Zhang 2005), there is a paucity of data in studies on diabetic rats with stroke. The present study suggests that the strongest effect of HBO₂ is accomplished by its first use (as soon as possible after stroke), and repeated applications are not superior to single application (Fig. 2). Earlier experiments that did not show effectiveness of HBO₂ were unsuccessful because of the lack of recognition of the vulnerability of neurons, prolonged ischaemia and the use of HBO₂ too late after stroke onset (Xu *et al.* 2016).

In earlier experiments, inhibitors of 20-HETE (TS-011 or HET0016) were used as a treatment or pretreatment and reduced the stroke volume in non-diabetic rats (with 70% reduction of cortical stroke volume and 55% reduction of total infarct volume, owing to the lesser effect on subcortical area; Renic *et al.* 2009). So far, we have found no such experiments on diabetic rats. Our results in diabetic female rats suggest that treatment with HET0016 alone significantly reduced stroke volume (Fig. 2). Rats pretreated or treated with HET0016 had a similar reduction of rCBF during ischaemia, but significantly lower rCBF values during reperfusion, compared with the control group (Fig. 1). It has yet to be determined whether this is a consequence of smaller stroke, an effect of 20-HETE on the vasculature, or some other protective mechanism.

In the present study, we tested also the impact of combined administration of HET0016 and HBO₂, but also differences between combinations of HBO₂ with HET0016 pretreatment and HBO₂ with HET0016 treatment during t-MCAO. The 20-HETE could increase oxidative stress in cells through activation of reduced NADP oxidase phosphorylation, uncoupling of nitric oxide synthase, and through a NADP oxidase-independent pathway. Given that intermittent HBO₂ reduces oxidative stress (Rossignol *et al.* 2007), one may speculate that HBO₂ could interact with HET0016 on that matter. Contrary to our expectations, such combinations provided no significant additive effect in reducing brain infarct size compared with exclusive HET0016 treatment (Fig. 2). The results show equal potency of HBO₂ and 20-HETE inhibitors in reducing infarct size.

Increased levels of eNOS have been observed in response to rhGLP-1 and granulocyte-colony stimulating factor (Zhao *et al.* 2015; Liew *et al.* 2015, respectively), in the model of ischaemia–reperfusion injury in rats with diabetes mellitus type 2, suggesting an involvement of the NO pathway in the neuroprotective effects of these treatments. In agreement with these studies, the results of the present study demonstrated increased eNOS mRNA expression in all treated groups (Fig. 4B).

The contribution of CYP metabolites to the autoregulation of cerebral blood flow still needs to be defined more accurately, yet it is already clear that they could play an important role as new targets for drug development for managing brain damage that occurs with cerebral ischaemia and stroke (Imig *et al.* 2011). *Cyp4A3* mRNA expression was extremely low in the present study, which is in accord with very low or undetectable levels of *Cyp4a2* and *Cyp4a3* mRNA in brain tissue of Wistar rats reported previously (Strömstedt *et al.* 1994; Kawasaki *et al.* 2012). Surprisingly, the *EPHX2* gene expression was also significantly increased in all treated groups, compared with the control t-MCAO-untreated group, and significantly higher in the HET0016 treatment + HBO₂ group compared with the HET0016 pretreatment + HBO₂ group (Fig. 4A). However, the therapeutic effects of treatments on infarct volume were similar among groups, and expression of *Cyp2J3* and *Cyp2C11* in the present study very much confirms previous reports and the observations that EETs have an important neuroprotective role (Imig *et al.* 2011; Fig. 3). Considering enzyme expression analysis and the effects of HET0016, it is feasible to speculate that increasing the level of EETs and decreasing the level of 20-HETE pharmacologically, supplemented with HBO₂, could be the future of stroke treatment.

Conclusion

The present study indicates that in female Sprague-Dawley diabetic rats, HBO₂ is a highly effective treatment for stroke even in the presence of long-term untreated diabetes, when performed early after the stroke. Inhibition of 20-HETE production, alone or in combination with HBO₂, was equally effective, but contrary to our expectations, did not show superiority to single and multiple HBO₂ treatments. The results suggest that both inhibition of 20-HETE and HBO₂ treatment are promising, equally effective, new therapeutic options in stroke complicated with diabetes treatment.

References

- Akbarzadeh A, Norouzzian D, Mehrabi MR, Jamshidi Sh, Farhangi A, Allah Verdi A, Mofidian SMA & Lame Rad B (2007). Induction of diabetes by streptozotocin in rats. *Indian J Clin Biochem* **22**, 60–64.

- Alkayed NJ, Murphy SJ, Traystman RJ, Hurn PD & Miller VM (2000). Neuroprotective effects of female gonadal steroids in reproductively senescent female rats. *Stroke* **31**, 161–168.
- Badr AE, Yin W, Mychaskiw G & Zhang JH (2001). Dual effect of HBO on cerebral infarction in MCAO rats. *Am J Physiol Regul Integr Comp Physiol* **280**, R766–R770.
- Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL & Bartkowski HM. (1986). Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* **17**, 1304–1308.
- Bennett MH, Weibel S, Wasiak J, Schnabel A, French C & Kranke P (2005). Hyperbaric oxygen therapy for acute ischaemic stroke. *Cochrane Database Syst Rev* **3**, CD004954.
- Crago EA1, Thampatty BP, Sherwood PR, Kuo CW, Bender C, Balzer J, Horowitz M & Poloyac SM (2011). CSF 20-HETE is associated with delayed cerebral ischemia and poor outcomes after aneurysmal subarachnoid hemorrhage. *Stroke* **42**, 1872–1877.
- Dunn KM, Renic M, Flasch AK, Harder DR, Falck J & Roman RJ (2008). Elevated production of 20-HETE in the cerebral vasculature contributes to severity of ischemic stroke and oxidative stress in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* **295**, H2455–H2465.
- Garcia JH, Liu KF & Ho KL (1995). Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex. *Stroke* **26**, 636–642.
- Gebremedhin D, Zhang DX, Carver KA, Rau N, Rarick KR, Roman RJ & Harder DR (2016). Expression of CYP 4A ω -hydroxylase and formation of 20-hydroxyeicosatetraenoic acid (20-HETE) in cultured rat brain astrocytes. *Prostaglandins Other Lipid Mediat* **124**, 16–26.
- Grundy D (2015). Principles and standards for reporting animal experiments in *The Journal of Physiology and Experimental Physiology*. *Exp Physiol* **100**, 755–758.
- Hjelde A, Hjelstuen M, Haraldseth O, Martin D, Thom R & Brubakk O (2002). Hyperbaric oxygen and neutrophil accumulation/tissue damage during permanent focal cerebral ischaemia in rats. *Eur J Appl Physiol* **86**, 401–405.
- Imig JD, Simpkins AN, Renic M & Harder D (2011). Cytochrome P450 eicosanoids and cerebral vascular function. *Expert Rev Mol Med* **13**, e7.
- Instititoris A, Lenti L, Domoki F, Wappler E, Gáspár T, Katakam PV, Bari F & Busija DW (2012). Cerebral microcirculatory responses of insulin-resistant rats are preserved to physiological and pharmacological stimuli. *Microcirculation* **19**, 749–756.
- Jauch EC, Saver JL, Adams HP Jr, Bruno A, Connors JJ, Demaerschalk BM, Khatri P, McMullan PW Jr, Qureshi AI, Rosenfield K, Scott PA, Summers DR, Wang DZ, Wintermark M & Yonas H; American Heart Association Stroke Council; Council on Cardiovascular Nursing; Council on Peripheral Vascular Disease & Council on Clinical Cardiology (2013). Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* **44**, 870–947.
- Kawasaki T, Marumo T, Shirakami K, Mori T, Doi H, Suzuki M, Watanabe Y, Chaki S, Nakazato A, Ago Y, Hashimoto H, Matsuda T, Baba A & Onoe H (2012). Increase of 20-HETE synthase after brain ischemia in rats revealed by PET study with ^{11}C -labeled 20-HETE synthase-specific inhibitor. *J Cereb Blood Flow Metab* **32**, 1737–1746.
- Kernan WN, Ovbiagele B, Black HR, Bravata DM, Chimowitz MI, Ezekowitz MD, Fang MC, Fisher M, Furie KL, Heck DV, Johnston SC, Kasner SE, Kittner SJ, Mitchell PH, Rich MW, Richardson D, Schwamm LH & Wilson JA; American Heart Association Stroke Council, Council on Cardiovascular and Stroke Nursing, Council on Clinical Cardiology & Council on Peripheral Vascular Disease (2014). Guidelines for the prevention of stroke in patients with stroke and transient ischemic attack: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* **45**, 2160–2236.
- Kibel A, Novak S, Cosic A, Mihaljevic Z, Falck JR & Drenjanecvic I (2015). Hyperbaric oxygenation modulates vascular reactivity to angiotensin-(1-7) in diabetic rats: potential role of epoxyeicosatrienoic acids. *Diab Vasc Dis Res* **12**, 33–45.
- Koizumi J, Yoshida Y, Nakazawa T & Ooneda G (1986). Experimental studies of ischemic brain edema. I.: a new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J Stroke* **8**, 1–8.
- Lan X, Qu H, Yao W & Zhang C (2008). Granulocyte-colony stimulating factor inhibits neuronal apoptosis in rat model of diabetic cerebral ischemia. *Tohoku J Exp Med* **216**, 117–126.
- Liew HK, Kuo JS, Wang JY & Pang CY (2015). Granulocyte-colony stimulating factor increases cerebral blood flow via a NO surge mediated by Akt/eNOS pathway to reduce ischemic injury. *ScientificWorldJournal* **2015**, 657932.
- Liu S, Liu W, Ding W, Miyake M, Rosenberg GA & Liu KJ (2006). Electron paramagnetic resonance-guided normobaric hyperoxia treatment protects the brain by maintaining penumbral oxygenation in a rat model of transient focal cerebral ischemia. *J Cereb Blood Flow Metab* **26**, 1274–1284.
- Longa EZ, Weinstein PR, Carlson S & Cummins R (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* **20**, 84–91.
- Lou M, Eschenfelder CC, Herdegen T, Brecht S & Deuschl G (2004). Therapeutic window for use of hyperbaric oxygenation in focal transient ischemia in rats. *Stroke* **35**, 578–583.
- Mišir M, Renić M, Mihalj M, Novak S & Drenjančević I (2016). Is shorter transient middle cerebral artery occlusion (t-MCAO) duration better in stroke experiments on diabetic female Sprague Dawley rats? *Brain Injury* **30**, 1390–1396.
- Renic M, Klaus JA, Omura T, Kawashima N, Onishi M, Miyata N, Koehler RC, Harder DR & Roman RJ (2009). Effect of 20-HETE inhibition on infarct volume and cerebral blood flow after transient middle cerebral artery occlusion. *J Cereb Blood Flow Metab* **29**, 629–639.

- Rossignol DA, Rossignol LW, James SJ, Melnyk S & Mumper E (2007). The effects of hyperbaric oxygen therapy on oxidative stress, inflammation, and symptoms in children with autism: an open-label pilot study. *BMC Pediatrics* **7**, 36.
- Singhal AB (2007). A review of oxygen therapy in ischemic stroke. *Neurol Res* **29**, 173–183.
- Singhal AB, Lo EH, Dalkara T & Moskowitz MA (2005). Advances in stroke neuroprotection: hyperoxia and beyond. *Neuroimaging Clin N Am* **15**, 697–720.
- Srinivasan K & Ramarao P (2007). Animal models in type 2 diabetes research: an overview. *Indian J Med Res* **125**, 451–472.
- Strömstedt M, Warner M & Gustafsson JA (1994). Cytochrome P450s of the 4A subfamily in the brain. *J Neurochem* **63**, 671–676.
- Sunami K, Takeda Y, Hashimoto M & Hirakawa M (2000). Hyperbaric oxygen reduces infarct volume in rats by increasing oxygen supply to the ischemic periphery. *Crit Care Med* **28**, 2831–2836.
- Toung TK, Hurn PD, Traystman RJ & Sieber FE (2000). Estrogen decreases infarct size after temporary focal ischemia in a genetic model of type 1 diabetes mellitus. *Stroke* **31**, 2701–2706.
- Unfirer S, Kibel A & Drenjancevic-Peric I (2008). The effect of hyperbaric oxygen therapy on blood vessel function in diabetes mellitus. *Med Hypotheses* **71**, 776–780.
- Wang GH, Zhang XG, Jiang ZL, Li X, Peng LL, Li YC & Wang Y (2010). Neuroprotective effects of hyperbaric oxygen treatment on traumatic brain injury in the rat. *J Neurotrauma* **27**, 1733–1743.
- Xu Y, Ji R, Wei R, Yin B, He F & Luo B (2016). The efficacy of hyperbaric oxygen therapy on middle cerebral artery occlusion in animal studies: a meta-analysis. *PLoS ONE* **11**, e0148324.
- Yin D & Zhang JH (2005). Delayed and multiple hyperbaric oxygen treatments expand therapeutic window in rat focal cerebral ischemic model. *Neurocrit Care* **2**, 206–211.
- Zhang JH (2007). Editorial: hyperbaric oxygen in neurological diseases. *Neurol Res* **29**, 113–115.
- Zhang PB, Liu Y, Li J, Chen XL, Tian YF, Sun JJ & Liu JX (2004). Effects of ketamine-midazolam anesthesia on focal cerebral ischemic injury in rats. *Di Yi Jun Yi Da Xue Xue Bao* **12**, 1337–1341.
- Zhao L, Xu J, Wang Q, Qian Z, Feng W, Yin X & Fang Y (2015). Protective effect of rhGLP-1 (7–36) on brain ischemia/reperfusion damage in diabetic rats. *Brain Res* **1602**, 153–159.
- Zuloaga KL, Zhang W, Roesse NE & Alkayed NJ (2015). Soluble epoxide hydrolase gene deletion improves blood flow and reduces infarct size after cerebral ischemia in reproductively senescent female mice. *Front Pharmacol* **5**, 290.

Additional information

Competing interests

None declared.

Author contributions

M. Mišir, M.R. and I.D. designed the study. A.Ć. and M.V. performed the experiments. M. Mišir, M.R., S.N., M. Martina, A.Ć., M.V. and I.D. analysed and interpreted data and drafted the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This study was supported by a grant from the Ministarstvo Obrazovanja, Znanosti i Sporta (Ministry of Science, Education and Sports; no. 219-2160133-2034) and Faculty of Medicine Osijek VIF-2016 grant (principal investigator, I.D.).