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Research Paper

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

Mihael Mišir[1,2], Marija Renić[3], Sanja Novak[2], Martina Mihalj[2], Anita Ćosić[2], Monika Vesel[2] and Ines Drenjančević[2]

[1] *Clinical Hospital Center Osijek, Neurology Clinic, Osijek, Croatia*
[2] *University Josip Juraj Strossmayer Osijek, Faculty of Medicine Osijek, Department of Physiology and Immunology, Laboratory for Circulatory Physiology, Osijek, Croatia*
[3] *Croatian Institute for Brain Research, School of Medicine University of Zagreb, Zagreb, Croatia*

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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 (HBO_2) exposures on the outcome of cerebral tissue injury induced by a transient middle cerebral artery occlusion model in diabetic female rats? Are 20-hydroxyeicosatetreanoic 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 HBO_2 and HET0016 (20-hydroxyeicosatetreanoic acid production inhibitor) and significant mRNA upregulation of epoxyeicosatrienoic acid-producing enzymes (Cyp2J3 and Cyp2C11) in treated groups suggest that HBO_2 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 (HBO_2), 20-hydroxyeicosatetreanoic 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) HBO_2; (3) multiple HBO_2 (HBO_2 immediately and second exposure 12 h after t-MCAO); (4) HET0016 pretreatment (1 mg kg^{-1}, 3 days before t-MCAO) combined with HBO_2 after t-MCAO; (5) HET0016 treatment (1 h before, during and for 6 h after t-MCAO); and (6) HET0016 treatment followed by HBO_2 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 HBO_2- and HET0016-treated rats. Combined treatment with HET0016 and HBO_2 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 HBO_2 group and the HET0016 treatment followed by HBO_2 group, compared with the control group. Expression of endothelial nitric oxide synthase

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was significantly increased after HBO_2 treatments, and expression of epoxide hydrolase 2 was increased in all groups compared with the control group. In diabetic female Sprague–Dawley rats, HBO_2 and HET0016 are highly effective stroke treatments, suggesting the involvement of cytochrome P450 metabolites and the NO pathway in this therapeutic effect.

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Corresponding author I. Drenjančević: University Josip JurajStrossmayer 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 (HBO_2; 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 HBO_2 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 HBO_2 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 HBO_2 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 HBO_2 and the need for delivery of care in a specialized chamber (Bennett *et al.* 2005).

20-Hydroxyeicosatetreanoic 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 epoxyeicosatrienoic 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 HBO_2 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 HBO_2 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 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 HBO_2 treatment after t-MCAO and reperfusion); and experimental groups exposed to: (2) HBO_2 (HBO_2 immediately after t-MCAO); (3) multiple HBO_2 (first HBO_2 immediately, and second 12 h after t-MCAO); (4) HET0016 pretreatment + HBO_2 (single injection of HET0016 I.P. daily, 1 mg kg day^{-1}, for 3 days before t-MCAO, combined with HBO_2 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 + HBO_2 [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 HBO_2 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-Wyheln, 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 periost 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 ± 0.5°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 HBO_2 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 + HBO_2 group (I.P., in a single injection, 1 mg kg^{-1} 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 + HBO_2 (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 1 (Sigma) in order to remove any eventual gDNA, then 1 μg of RNA was transcribed to cDNA using PrimeScript™ RT Reagent Kit (Takara, Shiga, Japan). Quantitative PCR was performed using Absolute qPCR SYBR Low ROX Mix (Thermo Scientific, Wilmington, DE, USA) on CFX96™ 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 phosphoribosyltranspherase 1 (*HPRT1*) was used as the reference gene. Primers were as follows: *Cyp2J3* (forward 5′-CCTTTCT GTTCCTGGCTGATTT-3′, reverse 5′- AGGCCCTGGC GGGTAGT-3′), *Cyp2C11* (forward 5′-CAATCCGCAGT CTGAGTT-3′, reverse 5′-TGCTGAGAATGGCATAAA-3′), *Cyp4A1* (forward 5′-GTTCTACCTGCAAAGGCAA TGG-3′, reverse 5′-TGCCCAAAGAACCAGTGGAA-3′), *Cyp4A3* (forward 5′-TCTCAGGGAGCAAAACACGA-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 Bonferoni *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. 1*A*, 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 HBO_2, ranging from 87 to 113% of baseline (Fig. 1*A* and *B*). Reperfusion was

Table 1. Fasting blood glucose concentrations

	Fasting blood glucose (mmol l^{-1})					
Time point	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	27.26
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	27.93
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	28.56

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. 1*B*). Similar differences were found when HET0016-treated groups were compared with HBO_2-treated groups ($P < 0.05$; Fig. 1*B*).

Hyperbaric O_2 treatment alone reduces brain infarct volume after t-MCAO. Cortical infarct size and the total infarct size were significantly reduced in rats treated with HBO_2 compared with the control group (Fig. 2*B* and *C*); however, the subcortical infarct size was not reduced by HBO_2 (single HBO_2 $P = 0.966$, multiple HBO_2 $P = 0.078$; Fig. 2*A*). There was no significant difference in infarct volumes between the groups treated with different HBO_2 protocols (Fig. 2). Hyperbaric oxygenation reduced the total infarct volume by 44.9 ± 7.41% in the HBO_2 group and by 47.6 ± 12.07% in the multiple HBO_2 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 ± 3.34%, cortical infarct volume by 71.6 ± 4.74% and subcortical infarct volume by 44.1 ± 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 HBO_2 treatment (Fig. 2*B* and *D*).

Treatment or pretreatment with HET0016 combined with HBO_2 provides no significant additive effect in reducing brain infarct size compared with exclusive HET0016 treatment. Hyperbaric oxygenation in addition to pretreatment (HET0016 pretreatment + HBO_2 group) or treatment (acute perioperative administration) with HET0016 (HET0016 treatment + HBO_2) 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 HBO_2 treatment (Fig. 2). Multiple HBO_2 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. 3*C*). In contrast, mRNA of *Cyp2J3* was significantly increased in all groups compared with the control group and in HBO_2 and multiple HBO_2 groups compared with the HET0016 pretreatment + HBO_2 group (Fig. 3*B*). *Cyp2C11* mRNA was significantly increased in multiple HBO_2 and HET0016 pretreatment + HBO_2 groups compared with the control group (Fig. 3*A*). The relative expression of *eNOS* was significantly increased in the HBO_2 group compared with all other groups, and in the HET0016 treatment + HBO_2 group compared with the control, HET0016 pretreatment + HBO_2 and HET0016 treatment groups, and in the multiple HBO_2 group compared with the control group (Fig. 4*B*). Expression of *EPHX2* was significantly increased in all groups compared with the control group, and in HET0016 pretreatment + HBO_2 group compared with the HET0016 treatment + HBO_2 group (Fig. 4*A*).

Discussion

The most important findings of the present study are as follows: (i) if administered shortly after the stroke, HBO_2 (used as a single exposure or multiple exposures to HBO_2) 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 HBO_2 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 HBO_2 or HET0016 treatment, suggesting that EETs contributed to the reduction of brain infarct volume.

To our knowledge, these are the first experiments using HBO_2 and HET0016 in *in vivo* experiments on diabetic

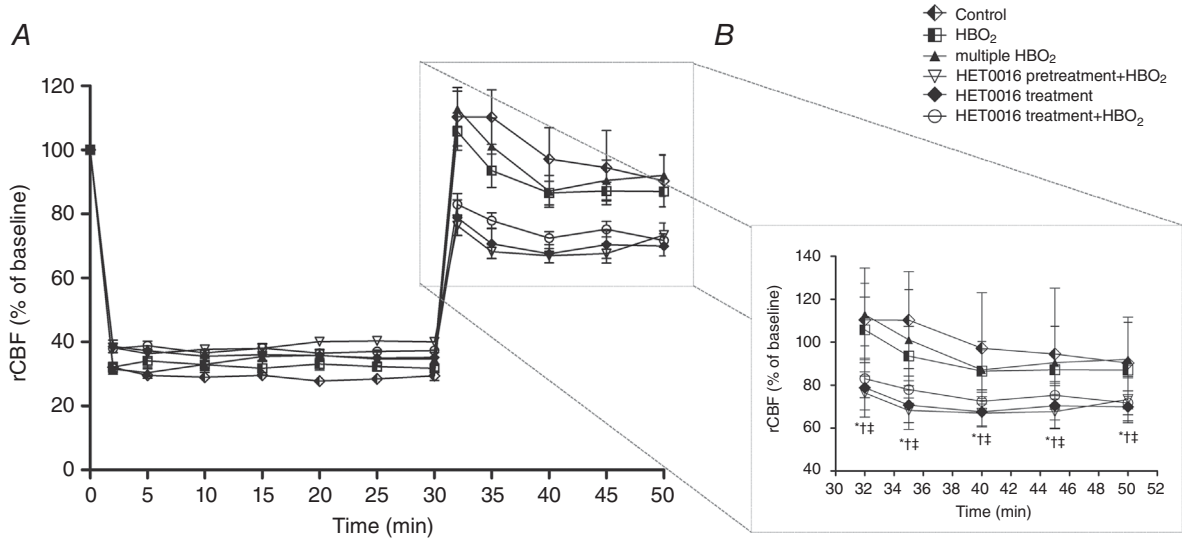

Figure 1. Changes in regional cerebral blood flow (rCBF) before, during and after transitory middle cerebral artery occlusion (t-MCAO) in all study groups
Groups are as follows: (1) control, t-MCAO for 30 min; (2) HBO_2, t-MCAO for 30 min followed by one hyperbaric oxygen treatment (HBO_2); (3) multiple HBO_2, t-MCAO for 30 min followed by two HBO_2 (immediately after t-MCAO and after 12 h); (4) HET0016 pretreatment + HBO_2, 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 HBO_2; (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 + HBO_2, t-MCAO for 30 min in rats treated with HET0016 (administered in the same manner as in the previous group) followed by one HBO_2. 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 + HBO_2 group. $^\dagger P < 0.05$ for HET0016 treatment. $^\ddagger P < 0.05$ for HET0016 treatment + HBO_2 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 HBO_2 reduces stroke volume after t-MCAO. In the t-MCAO model, ischaemia produces

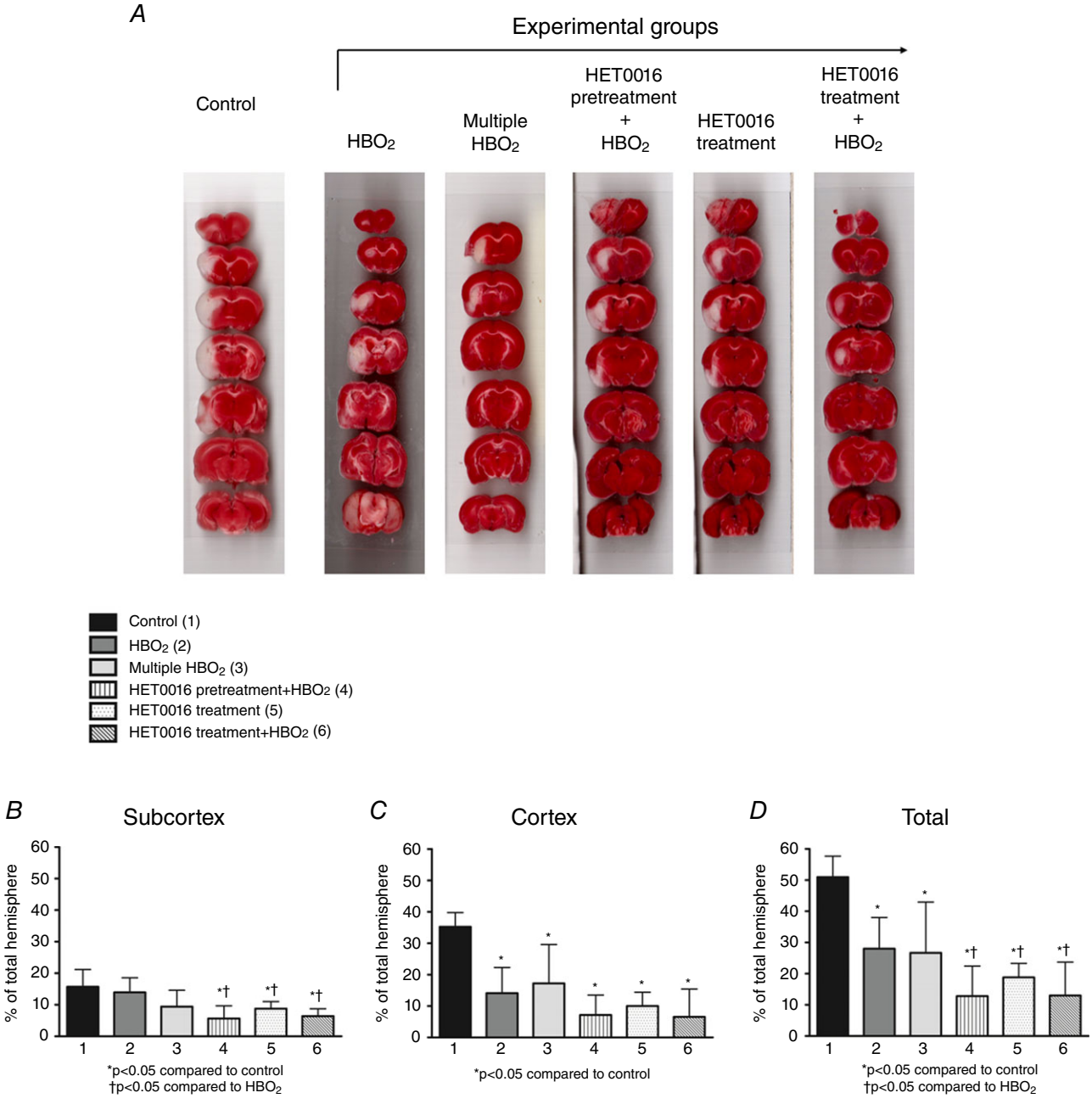

Figure 2. Representative brain slices (*A*) and infarct sizes of different experimental groups [subcortical (*B*), cortical (*C*) and the total brain infarct volume (*D*)] 24 h after transitory middle cerebral artery occlusion (t-MCAO) in all study groups
Groups are the same as in Fig. 1. The infarct size is expressed as a percentage of the total hemisphere size (volume). $^{*}P < 0.05$ compared with control group. $^{\dagger}P < 0.05$ compared with HBO_2 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

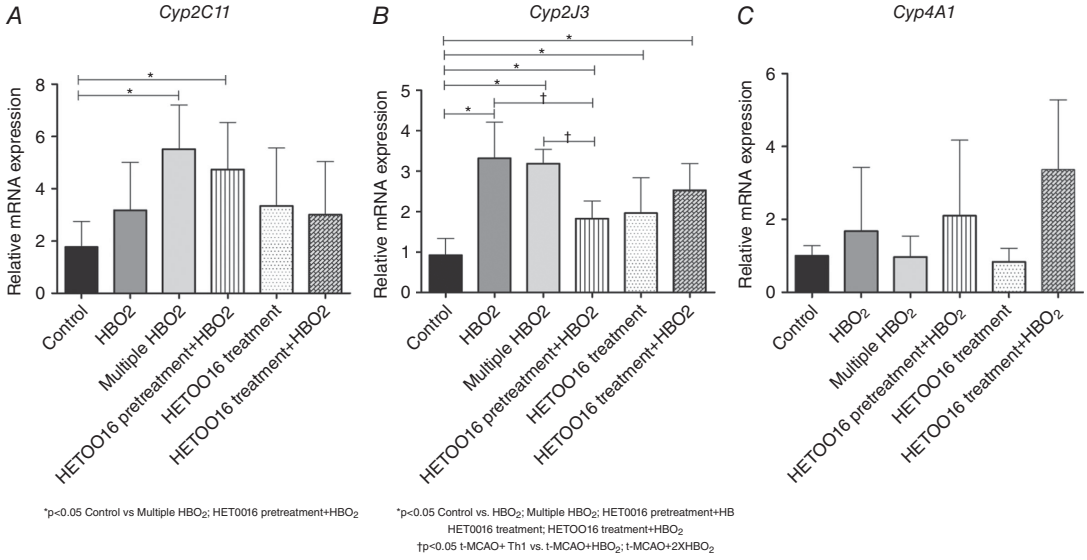

Figure 3. Relative mRNA expression levels of *Cyp2C11* (*A*), *Cyp2J3* (*B*) and *Cyp4A1* (*C*) genes in brain tissue
Groups are the same as in Fig. 1. Data are presented as means + SD, and the level of significance was determined at $P < 0.05$ ($n = 5$).

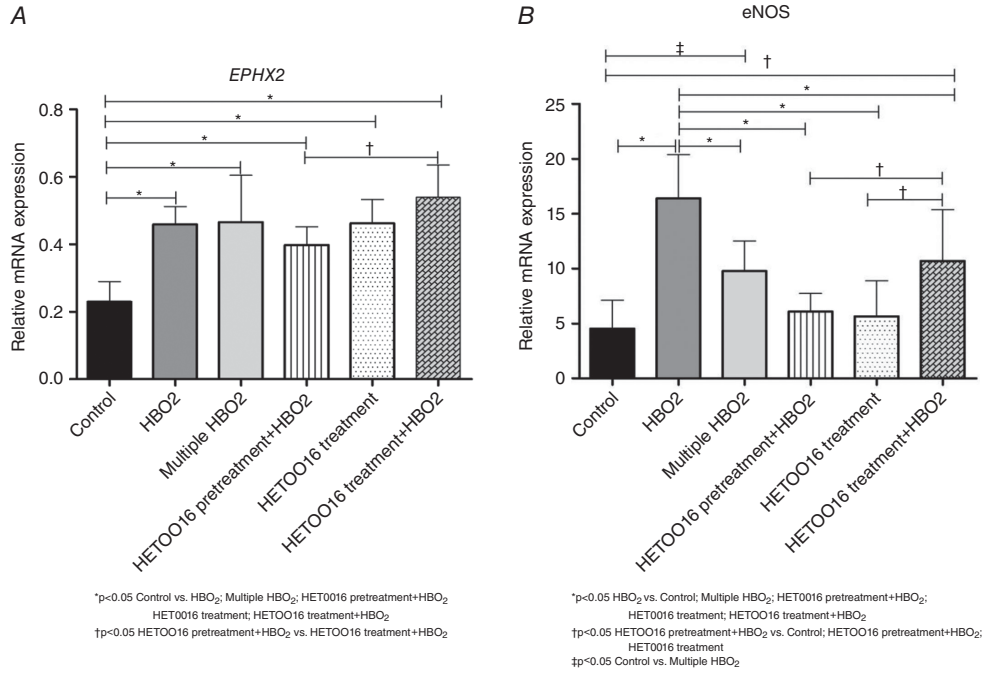

Figure 4. Relative mRNA expression levels of *EPHX2* (*A*) and *eNOS* (*B*) genes in brain tissue
Groups are the same as in Fig. 1. Data are presented as means + SD, and the level of significance was determined at $P < 0.05$ ($n = 5$).

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, HBO_2 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 HBO_2 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 HBO_2 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 HBO_2 were unsuccessful because of the lack of recognition of the vulnerability of neurons, prolonged ischaemia and the use of HBO_2 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 HBO_2, but also differences between combinations of HBO_2 with HET0016 pretreatment and HBO_2 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 HBO_2 reduces oxidative stress (Rossignol *et al.* 2007), one may speculate that HBO_2 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 HBO_2 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. 4*B*).

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 + HBO_2 group compared with the HET0016 pretreatment + HBO_2 group (Fig. 4*A*). 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 HBO_2, could be the future of stroke treatment.

Conclusion

The present study indicates that in female Sprague-Dawley diabetic rats, HBO_2 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 HBO_2, was equally effective, but contrary to our expectations, did not show superiority to single and multiple HBO_2 treatments. The results suggest that both inhibition of 20-HETE and HBO_2 treatment are promising, equally effective, new therapeutic options in stroke complicated with diabetes treatment.

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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.

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