The FEBS Journal

FEBS PRESS science publishing by scientists

Ischaemia/reperfusion and permanent ischaemia differentially affect haemoglobin properties – Possible influence of oxidative stress and adaptation to acute hypoxia

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Keywords
fibre-optic interface technology; haemoglobin; hydrogen peroxide; ischaemic stroke; Raman microspectroscopy

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(Received 30 September 2025, revised 24 November 2025, accepted 4 February 2026)

doi:10.1111/febs.70453

Cerebral ischaemia is an acute state characterised by a severe decrease in the supply of oxygen (O_2) to the brain, resulting in the death of neurons and glial cells. Despite multiple studies investigating the processes that lead to cell damage under ischaemic stroke, there is still a lack of information about the changes in blood properties under various phases of transient ischaemia with restoration of blood flow or under prolonged ischaemia. Blood, as a source of O_2, might either contribute to the brain damage owing to the development of oxidative stress, or might prevent cell death by the modulation of O_2 bioavailability to cells. Here, we studied hydrogen peroxide (H_2O_2) generation in mitochondria of neurons *in vivo* using the H_2O_2-sensitive biosensor HyPer7, and blood properties [haemoglobin (Hb) oxygenation and Hb affinity to O_2, estimated with Raman microspectroscopy (RS)] in two experimental rat models: transient 60-min ischaemia induced by the occlusion of the middle cerebral artery (MCAO) followed by 48 h reperfusion, and permanent 48-h MCAO-induced ischaemia. We found that an increase in the amount of H_2O_2 synthesised in neuronal mitochondria under the reperfusion period correlated with a decrease in blood oxygenation level, whereas permanent ischaemia did not affect the amount of oxyhaemoglobin but led to an increase in the affinity of Hb to O_2. We hypothesise that H_2O_2 may initiate processes that lead to increased O_2 penetration to the brain tissue under reperfusion, and that increased Hb affinity to O_2 may be an adaptive reaction of the blood system to acute prolonged ischaemia.

Abbreviations
AAV, adeno-associated viruses; cpYFP, circularly permuted yellow fluorescent protein; dHb, deoxyhaemoglobin; Hb, haemoglobin; hSyn1, human synapsin 1; MCAO, middle cerebral artery occlusion; MTS2, duplicated mitochondrial targeting sequence; oHb, oxyhaemoglobin; ROS, reactive oxygen species; RS, Raman spectroscopy; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase.

Introduction

Cerebral ischaemia is a state of the acute tissues oxygen starvation that occurs not only due to pathological processes like stroke or traumatic brain injury, but also during surgeries. Reperfusion – the restoration of oxygen access to ischaemic tissues – represents the stage with high risk of brain tissue damage due to the intensive production of reactive oxygen species (ROS) in cells [1]. Various approaches have been developed to mitigate injuries caused by ischaemia, aimed at increasing blood flow, improving blood rheology, and enhancing blood oxygen-carrying capacity, including usage of different blood substitutes. So far, a lot of attention has been paid to the study of ROS effect on cells, of the role of mitochondrial ETC to regulate ROS generation, as well as to the development of methodological approaches for the tissue protection during reperfusion (for example, hypoxic preconditioning) [2–5]. However, processes preceding the generation of ROS – the oxygen supply of ischaemic tissues, that depends on properties of erythrocytes, precisely, on haemoglobin's affinity for oxygen – have been studied to a lesser extent. There are various data indicating influence of blood properties on the outcome of ischaemia and reperfusion. Thus, the decrease in Hb's affinity for oxygen was observed for people living upon conditions of chronic hypoxia, for example, under altitude exposure [6]. Recently, various pharmacological substances affecting the affinity of haemoglobin for oxygen have been investigated. Agents that decrease Hb's affinity for oxygen were used to enhance oxygen delivery to tissues under hypoxic conditions, thereby facilitating O_2 release from erythrocytes to brain tissues [7–9]. At the same time, a number of studies demonstrated that under acute cerebral ischaemia, substances that increase Hb's affinity for O_2 or Hb-based O_2 carriers with high oxygen affinity possessed a neuroprotective effect [10–12]. Moreover, Konorova et al. [13] found that Hb via change of its affinity for O_2 may provide adaptive protection against oxidative stress developing under cerebral ischaemia. Thus, at present controversial data exist about the role of haemoglobin in brain damage occurring under cerebral ischaemia and on the optimal strategy for the reducing ischaemia–reperfusion injury via modulation of blood properties. Still it remains unknown how properties of haemoglobin change at different stages of ischaemia and reperfusion.

Here we applied optic fibres for *in vivo* measurement of fluorescence of H_2O_2-selective biosensor HyPer7 in neurons to evaluate H_2O_2 synthesis in neuronal mitochondria, and Raman microspectroscopy for the estimation of blood oxygenation and haemoglobin's affinity for O_2 in two rat models of transient and permanent MCAO-induced ischaemia. We demonstrated that transient ischaemia with reperfusion and permanent ischaemia differently affect properties of erythrocytic haemoglobin. We have found that the increase in the amount of H_2O_2 generated in neuronal mitochondria under reperfusion correlated with the decrease in blood oxygenation level, whereas permanent ischaemia led to the increase in Hb's affinity for O_2.

Results

To assess the state of haemoglobin in the blood during the development of ischaemic brain stroke, we used a model of middle cerebral artery occlusion in rats (spontaneously hypertensive rats, SHR). Fig. 1 schematically demonstrates the design of the experiment. In this work, we modelled two types of pathology. One group of animals underwent cerebral artery occlusion for 1 h, after which the occluder was removed from the lumen of the vessel, thus restoring blood flow in the brain tissues and triggering reperfusion injury. In the second group of rats, we simulated permanent occlusion; for this, the occluder was fixed in the lumen of the artery throughout the entire experiment. All surgical procedures were performed under isoflurane anaesthesia. Blood samples were collected using glass capillaries from the left jugular vein before the onset of ischaemia, in 1 h after ischaemia, and then in 1, 6, 24 and 48 h after reperfusion. For the permanent occlusion model, blood sampling times corresponded to 1, 2, 6, 24 and 48 h of ischaemia. In parallel with the time points of blood sampling, we recorded *in vivo* the redox state of neurons in ischaemic brain areas of animals of both experimental groups. For this purpose, we used a genetically encoded fluorescent biosensor HyPer7 sensitive to H_2O_2 [14]. HyPer7 gene with mitochondrial tag sequence under the neuronal-specific hSyn1 promoter was delivered into brain tissues of rats using adeno-associated viral particles (AAV9) as we described earlier [15]. The mitochondrial version of HyPer7 allows the registration of H_2O_2 regardless of its source of generation (cytosol or mitochondrial matrix), since H_2O_2 transport is facilitated from the cytosol to the mitochondrial matrix, but not vice versa [14]. We performed the injection of viral particles during surgical manipulation using a stereotaxic setup. Based on our previous studies [15,16], we chose the caudate nuclei structure as the injection site in the brain because it is the first to be affected by ischaemic

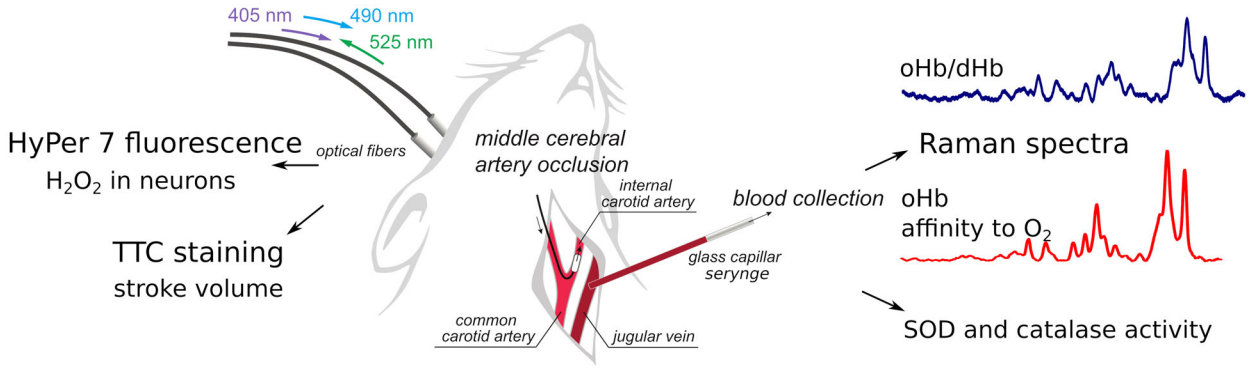

Fig. 1. Design of the experiment. Middle cerebral artery occlusion (MCAO) was used for inducing ischaemia in two experimental groups of rats following 48 h reperfusion in one group and prolonged 48 h ischaemia in another. Blood was taken at certain time points from the jugular vein of falsely operated rats (control), rats with ischaemia–reperfusion and rats with prolonged ischaemia, and then analysed with Raman microspectroscopy (estimation of blood oxygenation and oxyhaemoglobin [oHb] affinity to O_2) or used for the estimation of SOD and catalase activities in erythrocytes. At the same time points, HyPer7 fluorescence from neurons in the striatum was recorded. Estimation of the stroke size was done using 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain slices prepared at the final time point of 48 h.

damage during the standard MCAO protocol. Immediately after the injection of the AAV suspension, we implanted an optical fibre into the same brain coordinate, which was fixed to the skull using a ceramic adapter. The adapter serves to protect the implanted fibre while the animal is awake, and to connect the optical cable leading to our custom optical setup. Such fibre-optic neurointerface technology enables *in vivo* measurements of the ratiometric signal of yellow fluorescent protein-based sensors in deep layers of brain tissues in laboratory rodents [15,17]. We use the right hemisphere of the brain as a reference in such experiments and perform virus injection and optical fibre implantation at the same coordinates. Here, as well as in our previous studies, we revealed that ratiometric HyPer7 signal (F_{490}/F_{405}) did not increase significantly during 60-min MCAO induced ischaemia, but increased gradually over the course of 48 h during the reperfusion period [15,16,18] (Fig. 2). A significant increase by several times in the fluorescent signal of HyPer7 was observed only the following day after 60-min arterial occlusion. For the damaged tissues of the left hemisphere of rats with ischaemia–reperfusion, HyPer 7 signal was significantly elevated relative to that in the healthy right hemisphere (Fig. 2A). In the permanent ischaemia model, we obtained results different from the 60-min ischaemia/reperfusion model. Thus, the average HyPer7 signal in the damaged tissues also increased the following day after MCAO, but the ratiometric values for the group of animals with permanent ischaemia were highly variable compared to the group with ischaemia–reperfusion (Fig. 2B). In particular, in three animals with permanent artery occlusion, the signal changed 3–4 times, while in another three animals in the same group, the fluorescence changes were virtually indistinguishable from the initial values and the signals in the right hemisphere. This result indicates variability in H_2O_2 production in mitochondria of neurons from the ischaemic brain area, demonstrating absence of the increase in H_2O_2 amount or significant generation of H_2O_2. Severe left-sided stroke was confirmed in all animals in the experiment. However, all animals with permanent 48-h MCAO-induced ischaemia, irrespective of HyPer 7 signal, demonstrated a greater stroke volume compared to animals with 60-min MCAO-induced ischaemia and following 48-h reperfusion (Fig. 2C,D). Thus, in this series of experiments, the signal of redox sensor HyPer7 did not correlate with the type of brain tissue damage and its final volume.

To estimate changes in blood properties occurring under transient ischaemia following reperfusion or under prolonged permanent ischaemia, we collected blood from the left jugular vein which is known to mostly carry venous blood from the left hemisphere, and applied Raman microspectroscopy to estimate blood oxygenation and haemoglobin's affinity for O_2 (Figs 3 and 4). Raman spectra recorded from sealed glass capillaries with venous blood demonstrated a set of peaks corresponding to vibrations of haem molecules in oxyhaemoglobin (oHb) and deoxyhaemoglobin (dHb) in blood erythrocytes with the same oxygenation as in the left jugular vein at the moment of the blood collection (Fig. 3). Most of the intensive peaks in the Raman spectra of blood represent vibrations of haem pyrrols and methine bridges sensitive to the

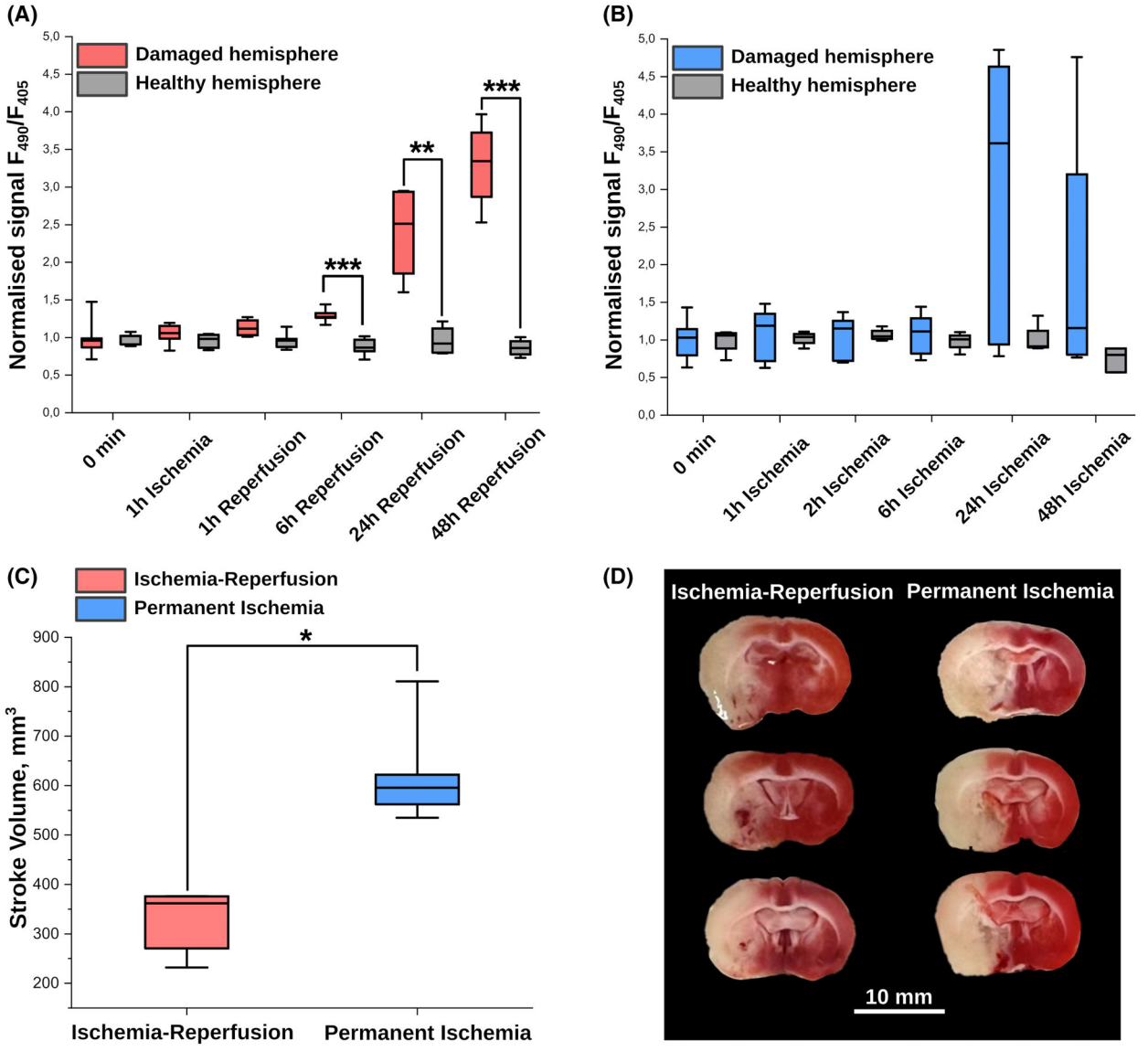

Fig. 2. Generation of H_2O_2 in neuronal mitochondria and the stroke volume under transient 60-min ischaemia induced by middle cerebral artery occlusion (MCAO) followed by 48-h of reperfusion and under permanent 48-h MCAO-induced ischaemia. (A, B) Estimation of H_2O_2 amount in mitochondrial matrix of neurons before MCAO surgery (0 min) and after MCAO-surgery in left damaged hemisphere (red or blue boxes) and right healthy hemisphere (gray boxes) in 1 h of MCAO-induced ischaemia and in 1, 6, 24 and 48 h of reperfusion that followed (A) or in 1, 2, 6, 24 and 48 h of permanent MCAO-induced ischaemia (B). *Y* axes – normalised HyPer7 signal F490/F405 indicated the amount of generated H_2O_2; *X* axes – time points of HyPer7 fluorescence measurements. (C) Stroke volume estimated after 48 h of reperfusion of rats with ischaemia/reperfusion (red box) and after 48 h of permanent ischaemia (blue box). On Figs A–C, data are shown as box-and-whiskers plots, where horizontal box borders correspond to 25% and 75% quartiles, horizontal lines inside the boxes show median values, and vertical whiskers indicate minimal and maximal values. Figs A, B: **, ***$P < 0.01$, 0.001, respectively (unpaired *t*-test, $n = 7$ (ischaemia–reperfusion group), $n = 9$ (permanent ischaemia), comparison of healthy and damaged hemispheres at certain time points); Fig. C: *$P < 0.05$. (Mann–Whitney test, $n = 7$ (ischaemia–reperfusion group), $n = 9$ (permanent ischaemia)). (D) Photographs of typical 2,3,5-triphenyltetrazolium chloride-stained brain slices of three randomly chosen rats after 48 h of reperfusion followed 1 h MCAO-induced ischaemia (left column) and of three randomly chosen rats after 48 h of permanent MCAO-induced ischaemia. White areas show the areas with the stroke. Horizontal scale bar corresponds to 10 mm.

presence/absence of O_2 molecules bound to the Fe^{2+} atom (Fig. 3A,B). The main interest represents pairs of peaks, corresponding to the same haem group vibrations, but with different sensitivity to the haemoglobin oxygenation state. Thus, both peaks at 1357 and 1375 cm^{-1} correspond to symmetric pyrrol

half-ring vibrations (so-called 'haem breathing') [19–21], though the peak at a 1375 cm^{-1} peak corresponds to oHb and the peak at 1357 cm^{-1} is the signature of dHb [22]. Four other higher frequency peaks at 1547, 1585, 1604 and 1638 cm^{-1} correspond to methine bridges, whose vibrations depend both on the presence of bound O_2 and on haem conformation (probability of planar or dome—deformed out-of-plane—conformations) (Fig. 3C,D) [19,20,23,24], where intensive peaks at 1585 and 1638 cm^{-1} are assigned to oHb, while peaks at 1547 and 1604 cm^{-1} – to dHb [19–21,23,25]. A pair of peaks at 1547/1585 cm^{-1} also depend on vibrations of C_aC_b bonds and are less sensitive to the haem planar/dome conformation, than the pair of peaks at 1604/1638 cm^{-1} [19–21,23,25]. The Raman spectra of blood also demonstrate O_2-unsensitive peaks with the maxima positions at 1127, 1170 and 1338 cm^{-1}, corresponding to vibrations of haem side radicals -CH_3, asymmetric pyrrole vibrations and vibrations of all haem bonds, respectively (Fig. 3C,D). Considering O_2-sensitivity of certain Raman peaks it is possible to measure semi-quantitative blood oxygenation. Here we used a ratio of intensities of peaks at 1375 and 1357 cm^{-1} (I_{1375}/I_{1357}) to estimate the relative amount of oHb compared to dHb in analysed samples of the jugular venous blood of rats under control conditions, transient ischaemia/reperfusion and under permanent ischaemia. We have observed the most pronounced changes in blood oxygenation of the jugular vein on the reperfusion period of rats that were subjected to 60-min MCAO-induced ischaemia. Thus, the reperfusion period is characterised by the steady decrease in the I_{1375}/I_{1357} ratio (Fig. 3G) that can be seen on Raman spectra, as the decrease in the relative intensity of the oHb peak at 1375 cm^{-1} with the increase in the intensity of dHb peaking at 1357 cm^{-1} (Fig. 3D). Observed changes correspond to the decrease in the blood oxygenation, and the most pronounced blood deoxygenation was observed on the 48th hour of reperfusion after 60-min MCAO-induced ischaemia (Fig. 3D,G). Unexpectedly, we did not observe a decrease in the blood oxygenation of rats with permanent 48-h MCAO-induced ischaemia (Fig. 3E,H). On the contrary, we have found a slight increase in blood oxygenation at the 24th hour of MCAO-induced ischaemia (Fig. 3H). Falsely operated-on animals did not demonstrate changes in blood oxygenation, which indicates the absence of the effect of surgery and of the blood collection on the relative amount of oHb/dHb (Fig. 3C,F).

As mentioned, Raman peaks at 1547/1585 cm^{-1} and especially at 1604/1638 cm^{-1} depend not only on O_2 binding to haem Fe^{2+}, but also on the haem conformation – planar or dome (so-called out-of-plane deformation) [20,24]. It is known that planar haem conformation corresponds to the state with high affinity to O_2, whereas Hb with low affinity for O_2 possesses non-planar dome haem (with out-of-plane deformation) [26,27] (Fig. 4A,B). To avoid possible influence of O_2-binding on the intensity of Raman peaks sensitive to haem conformation, we prepared fully oxygenated samples of 100-times diluted blood collected from the jugular vein. Fig. 4A demonstrates a typical Raman spectrum of fully oxygenated erythrocytes from diluted blood of randomly chosen control rats. It can be seen that the spectrum represents intensive typical peaks of oHb at 1375, 1585 and 1838 cm^{-1}, indicating absence of dHb. In this case any change in relative intensity of peaks, sensitive to haem conformation, can be attributed to the changes in the probability of planar/deformed haem conformations and therefore to the change in Hb's affinity for O_2 (Fig. 4B). Here we used intensity of the peak at 1638 cm^{-1}, normalised on the intensity of the peak at 1375 cm^{-1} (I_{1638}/I_{1375}) as the estimation of the probability of planar haem conformation in oHb with high affinity for O_2 (Fig. 4C–E). Such an approach—normalisation of the Raman peak intensity at 1638 cm^{-1} on the intensity of the conformation-insensitive peak at 1371–1375 cm^{-1}—was already used for the estimation of the probability of the planar conformation of the haem *c* in cytochrome *c* and its ability to accept electrons that is known to depend on haem *c* conformation [28–30]. The conditions of fully oxygenated erythrocytes intensity of the peak at 1375 cm^{-1} depends on the total amount of Hb only and therefore can be used as the normalisation factor [20,31].

We have found that oHb's affinity for O_2 significantly increases within the 48 h of permanent MCAO-induced ischaemia compared to oHb O_2 affinity before MCAO-surgery and to other time points of ischaemia (Fig. 4E). Importantly, we also observed a significant increase in oHb's affinity for O_2 in peripheral blood collected from the tail vein compared to 6 h of permanent MCAO-induced ischaemia with the tendency to an increase in O_2 affinity compared to the state before MCAO-surgery. This result indicates that the change in Hb affinity to O_2 is an intrinsic change occurring in Hb of erythrocytes, irrespective of the place of blood collection. We should note that we did not find any change in oHb's affinity for O_2 in rats with the transient 60-min MCAO-induced ischaemia followed by reperfusion (Fig. 4D). oHb's affinity for O_2 was also not affected by the procedure of the surgery or blood collection manipulations (Fig. 4C).

Remarkably, we observed certain correlations between some stroke characteristics and blood properties (Fig. 5A–C). Thus, for all experimental rats with transient 60-min MCAO induced ischaemia followed by reperfusion we observed a negative correlation between blood oxygenation in the jugular vein and H_2O_2 generation in the mitochondria of neurons in the course of reperfusion (Fig. 5A). We also observed a negative correlation between these parameters for the same experimental group of rats inside each time point of reperfusion, for example, on the 48th hour of reperfusion (Fig. 5B), indicating the relationship between the H_2O_2 amount in neuronal mitochondria (and possibly H_2O_2-dependent processes) with local O_2 release from erythrocytes in the brain and especially in the stroke region. We did not observe any correlation between normalised HyPer7 signal and I_{1375}/I_{1357} parameter in the blood of rats with permanent 48-h MCAO-induced ischaemia or in the control group of falsely operated rats. We also didn't find any correlation between the normalised HyPer7 signal or I_{1375}/I_{1357} ratio and the stroke volume in all

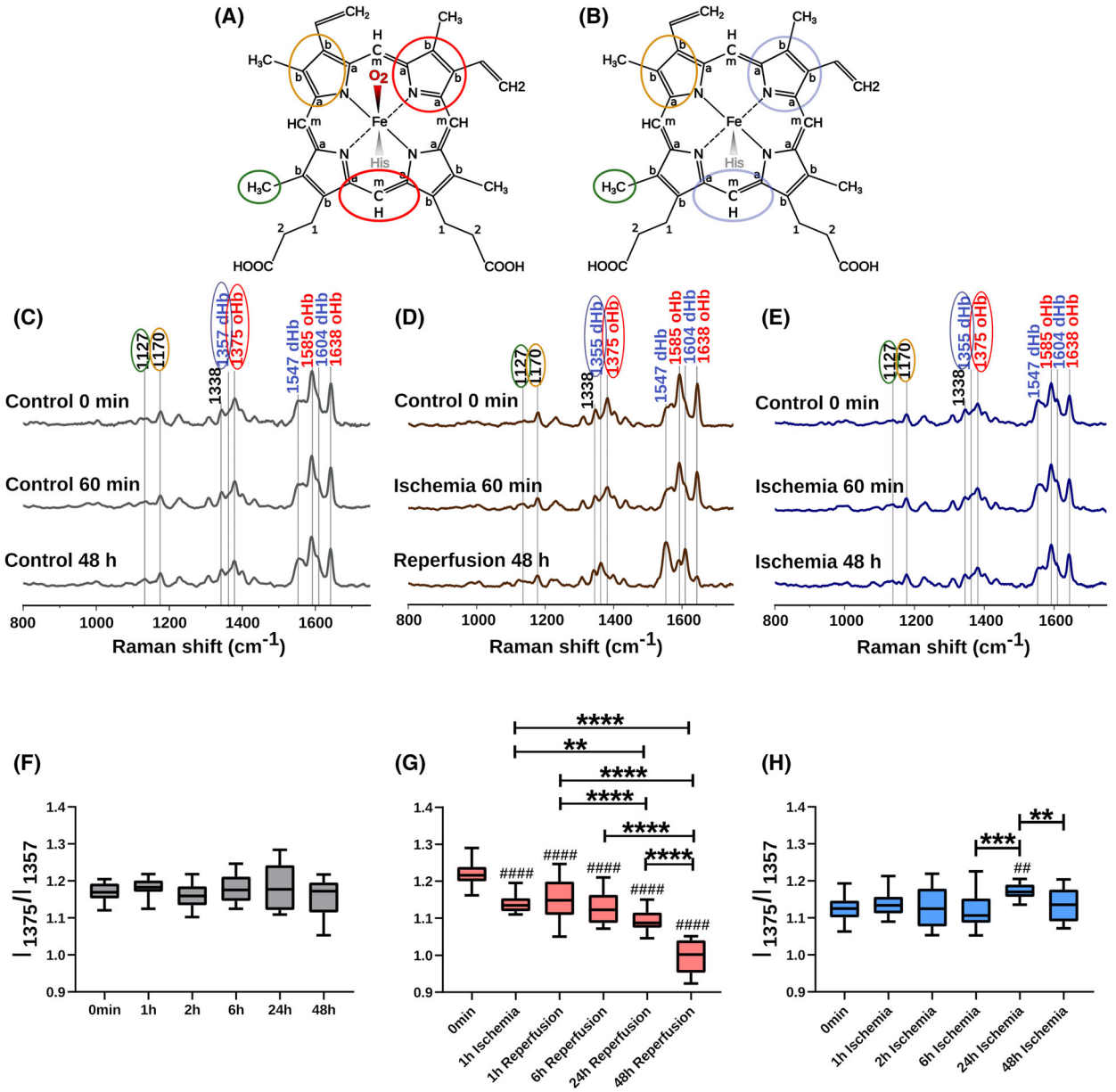

Fig. 3. Oxygenation of blood collected from the left jugular vein of rats with transient 60-min ischaemia induced by middle cerebral artery occlusion (MCAO) followed by 48-h reperfusion and permanent 48-h MCAO-induced ischaemia. (A, B) Structural formulas of haem *b* molecules with bound O_2 in oxyhaemoglobin and without O_2 in deoxyhaemoglobin, respectively. Circles and ellipses indicate groups of atoms whose vibrations are responsible for the most intensive peaks in the Raman spectra of blood (C–E). (C–E) Typical Raman spectra of jugular vein blood from three rats: (C) a control falsely operated rat before surgery (0 min), in 60 min and 48 h after the surgery (spectra from the top to the bottom, respectively); (D) an experimental rat before MCAO surgery (0 min), in 60 min after MCAO, and in 48 h of reperfusion after 60 min of MCAO-induced ischaemia (spectra from the top to the bottom, respectively); (E) an experimental rat before MCAO surgery (0 min) and in 60 min and 48 h of ischaemia following MCAO (spectra from the top to the bottom, respectively). Vertical dotted lines and numbers above them show maximum positions of main peaks in blood Raman spectra. Blue and red colours show maxima positions of Raman peaks sensitive to vibrations of haem atoms in deoxy- and oxyhaemoglobin, respectively. Blue and red ellipses around numbers 1357 and 1375 indicate peaks used for the semiquantitative estimation of blood oxygenation. *X*-axes on Figs C–E – Raman shift, cm^{-1}. For better representation, Raman peaks are shifted vertically. (F–H) Oxygenation of blood samples from left jugular vein of a control group of falsely operated rats (F), rats with transient 60-min middle cerebral artery occlusion following a 48-h reperfusion period (G), and of rats with 48-h middle cerebral artery occlusion without a reperfusion period (H). *Y*-axes on Figs F–H – Ratio of peaks' intensities at 1375 and 1357 cm^{-1} (I_{1375}/I_{1357}) corresponding to the semiquantitative estimation of blood oxygenation. *X*-axes on Figs F–H – time points of blood samples collection. Data are shown as box-and-whiskers plot, where horizontal boxes' borders correspond to 25% and 75% quartiles, horizontal lines inside the boxes show median values, and vertical whiskers indicate minimal and maximal values. #signs show *P* value for the comparison of the certain time point with initial 0 min time point. *signs indicate *P* values for the comparison of all other time points between each other. **, $^{\#\#}P < 0.01$, ***$P < 0.001$, ****, $^{\#\#\#\#}P < 0.0001$ (mixed effect analysis, Sidak's multiple comparison test, $n = 4$, $N = 30$ (ischaemia–reperfusion group), $n = 8$, $N = 40$ (permanent ischaemia)).

experimental rat groups, indicating an absence of the stroke volume dependence on the amount of H_2O_2 generated in mitochondria of neurons and on the local blood oxygenation. However, we observed some tendency to the negative correlation between oHb's affinity for O_2 and the stroke volume in rats with permanent 48-h MCAO-induced ischaemia (Fig. 5C).

In order to find whether the observed changes in Hb properties in erythrocytes of experimental rats occur due to the change in the overall redox status of erythrocytes under transient ischaemia with reperfusion or under permanent ischaemia, we evaluated activities of superoxide dismutase (SOD) and catalase in erythrocytes from the venous blood collected from the left jugular vein (Fig. 5D,E, respectively). We have found that only the catalase activity increases at the 24th and possibly 48th hour of the permanent 48-h MCAO-induced ischaemia (Fig. 5E). All other parameters did not change at other permanent ischaemia time points, nor at any of the reperfusion time points or at any time points after the false surgery. These results demonstrate the absence of oHb autooxidation with the formation of metHb and superoxide anion-radical. The observed increase in the catalase activity could be due to the diffusion of H_2O_2 into erythrocytes from the blood plasma.

Discussion

In spite of the numerous studies of stroke development in animal models and various types of medical research on humans it is still unclear how blood properties change in the course of short-term ischaemia and reperfusion or under prolonged ischaemia. It is natural to assume that oxygenation of Hb and its ability to bind and to release oxygen (Hb affinity for O_2) may play an important role in the development of ischaemia, recovery of the brain cells after ischaemia and, at the same time, in O_2-related processes of oxidative damage of cells in the course of reperfusion. With it, specific blood parameters could potentially be used for the detection of ischaemia development and for the prognosis of recovery after a stroke. Currently, many hypotheses of the cell damage under ischaemia and reperfusion refer to the theory of oxidative stress with a special emphasis on the role of the H_2O_2 in ischaemia–reperfusion-induced pathological processes and in the development of brain lesions [1]. In the current study we investigated H_2O_2 generation in mitochondria of neurons *in vivo*, blood oxygenation and Hb affinity to O_2 in two experimental models of ischaemia: transient 60-min MCAO-induced ischaemia followed by 48 h reperfusion and permanent 48-h MCAO-induced ischaemia. We have found that during the late reperfusion period (24th and 48th hour) following 60-min MCAO-induced ischaemia, a sharp increase in H_2O_2 generation occurred in the mitochondria of neurons in the caudate nuclei, whereas in neuronal mitochondria in rats with permanent hypoxia we observed a high heterogeneity in the amount of synthesised H_2O_2. We attribute this dissimilarity to different processes relating to the oxidative stress and to the type of cell death occurring under transient ischaemia–reperfusion and permanent ischaemia [32,33]. Remarkably, we also observed different changes in blood properties under transient ischaemia–reperfusion and

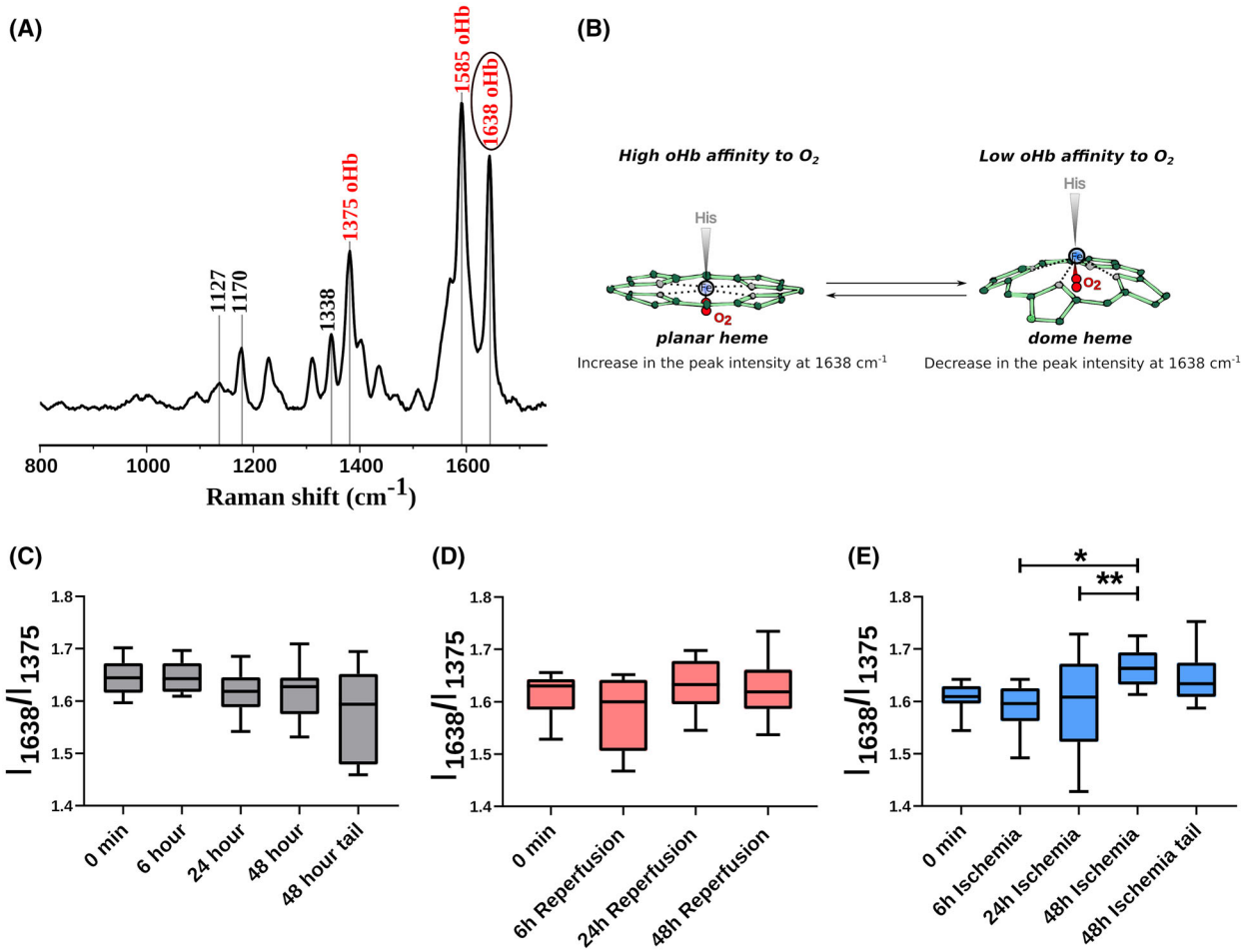

Fig. 4. Oxyhaemoglobin (oHb) affinity for oxygen in erythrocytes from left jugular vein of rats with transient 60-min ischaemia induced by middle cerebral artery occlusion (MCAO) followed by 48-h reperfusion and permanent 48-h MCAO-induced ischaemia. (A) Typical Raman spectrum of fully oxygenated erythrocytes recorded from 100-times diluted blood taken from left jugular vein of the control rat. Numbers above main peaks show their maxima positions, red-coloured numbers show maxima positions of characteristic oHb peaks, oval indicates maximum position of the peak originating from methine bridges' vibrations and sensitive to the planar/dome haem conformation. (B) Schemes of planar and dome (out-of-plane deformed) haems in oHb. In the planar haem Fe^{2+} atom is located in the plane of pyrrole rings and therefore O_2 molecule binds tighter to it, than in deformed dome haem, in which Fe^{2+} atom is shifted towards globin His residue. Raman peak intensity at 1638 cm^{-1} increases under planar haem conformation and decreases under dome haem conformation. (C–E) Raman spectroscopy estimation of oHb's affinity for O_2 in erythrocytes from the jugular vein of falsely operated rats before false surgery (0 min), in 6, 24 and 48 h after the false surgery (C); of experimental rats with transient 60-min MCAO-induced ischaemia before MCAO surgery (0 min), in 6, 24 and 48 h of reperfusion following 60-min MCAO-induced ischaemia (D); of experimental rats with permanent MCAO-induced ischaemia before MCAO-surgery (0 min), in 6, 24 and 48 h of ischaemia (E). To compare changes in oHb's affinity for O_2 in venous blood from the left jugular vein and peripheral vein we collected blood from the tail vein at 48th hour of ischaemia (E). *Y*-axes: ratio of peak intensities at 1638 and 1375 cm^{-1} (I_{1638}/I_{1375}) corresponding to the probability of planar haem conformation in oHb. An increase in the I_{1638}/I_{1375} ratio corresponds to the increase in oHb's affinity for O_2. Data are shown as box-and-whiskers plot, where horizontal boxes' borders correspond to 25% and 75% quartiles, horizontal lines inside the boxes show median, and vertical whiskers indicate minimal and maximal values. #signs show *P* value for the comparison of the certain time point with initial 0 min time point. *signs indicate *P* values for the comparison of all other time points between each other. *$P < 0.05$, **$P < 0.01$(mixed effect analysis, Sidak's multiple comparison test, $n = 4$, $N = 30$ (ischaemia–reperfusion group), $n = 8$, $N = 40$ (permanent ischaemia)).

permanent ischaemia. We have thus found that blood oxygenation significantly decreases during the reperfusion period, negatively correlating with the amount of H_2O_2 generated in neurons. We assume that, from neurons and astrocytes (that are known to generate H_2O_2 in mitochondria during an earlier period of reperfusion compared to neurons [18]), H_2O_2 can diffuse into the endothelial cells of capillaries, causing

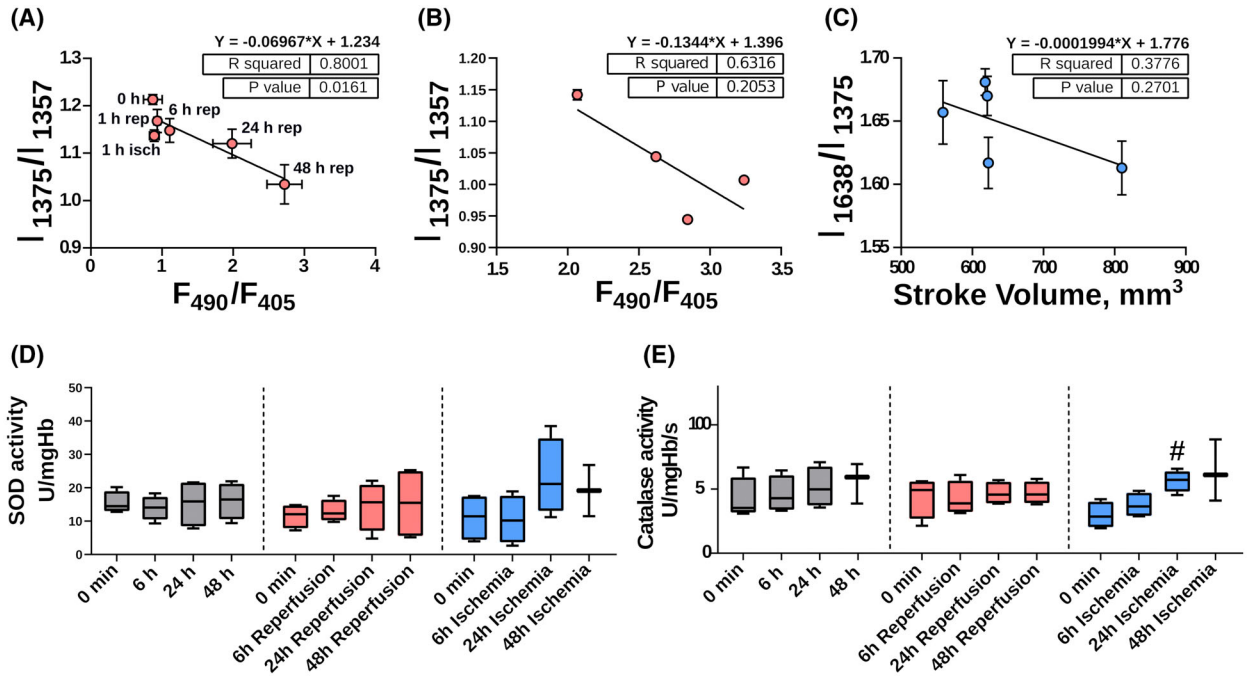

Fig. 5. Redox status of erythrocytes and correlation of haemoglobin oxygenation and affinity for O_2 with estimated stroke parameters. (A) Correlation of the oxygenation of venous blood in the left jugular vein with amount of H_2O_2 generated in neuronal mitochondria at all studied time points of rats with transient ischaemia induced by middle cerebral artery occlusion (MCAO) followed by reperfusion. Amount of studied rats used for the correlation plot: $n = 4$, total amount of Raman spectra recorded at each time point was 30; (B) correlation of blood oxygenation in left jugular vein of rats on 48th hour of reperfusion after transient 60-min MCAO-induced ischaemia with following reperfusion. Amount of studied rats used for the correlation plot: $n = 4$, *Y* axes on Figs A, B – I_{1375}/I_{1357} ratio, X axes on Figs A, B – ratiometric HyPer7 signal (F_{490}/F_{405}). (C) Correlation of oxyhaemoglobin (oHb) affinity to O_2 with the stroke volume in rats with permanent 48-h MCAO-induced ischaemia. Amount of studied rats used for the correlation plot was: $n = 5$, total amount of Raman spectra recorded for the blood sample of each rat was 5. *Y*-axis – I_{1638}/I_{1375} ratio, *X*-axis – Stroke volume, mm^3. All data on Figs A–C are shown as mean ± SE, where SE values are calculated for I_{1638}/I_{1375} ratio (Figs A–C) and normalised HyPer7 signal (F_{490}/F_{405}) (Figs A, B) or stroke volume, mm^3 (Fig. C). (D, E) Superoxide dismutase (SOD) and catalase activities in erythrocytes of all studied groups before false or MCAO-surgery (0 min), on 6th, 24th, 48th hour after false surgery (control group, gray bars); on 6th, 24th, 48th hour of reperfusion after transient 60-min MCAO-induced ischaemia (red bars); on 6th, 24th, 48th hour of permanent MCAO-induced ischaemia (blue bars). *Y* axes – (D) SOD activity, U/mgHb; (E) catalase activity, U/mgHb/s, *X*-axes – time points of the blood collection. Data are shown as box-and-whiskers plot, where horizontal boxes' borders correspond to 25% and 75% quartiles, horizontal lines inside the boxes show median values, and vertical whiskers indicate minimal and maximal values (mixed effect analysis, Sidak's multiple comparison test, $n = 4$ (ischaemia–reperfusion group), $n = 8$ (permanent ischaemia)).

damage [2] and thus increasing their penetration for O_2. In its turn, this will facilitate O_2 diffusion from erythrocytes to brain tissues, decreasing blood oxygenation. Enhanced tissue supply with O_2 may provide the mitochondrial respiratory chain with the O_2 necessary for the increased need in ATP in the recovery period, but may also result in the appearance of additional ROS. However, we did not observe a correlation of the stroke volume with the decrease in blood oxygenation or with H_2O_2 amount in mitochondrial neurons, so we believe that increased O_2 supply of the brain tissue under reperfusion does not lead to the development of pathological processes. On the contrary, under permanent ischaemia we did not observe changes in blood oxygenation, but we found an increase in oHb's affinity for O_2 that was independent of components of blood plasma. In addition, we demonstrated the tendency to a negative correlation between oHb's affinity for O_2 and the stroke volume. Remarkably, various authors demonstrated the decrease in the stroke volume and the increase in the survival rate in animals after ischaemia with artificially increased Hb affinity to O_2 [10,34]. So, the increase in the Hb affinity to O_2 may be regarded as an adaptation to acute prolonged brain ischaemia. We assume that this change in Hb may be due to some allosteric conformational modification of Hb subunits and possibly due to nitric oxide binding to the Cys-residue of

the globin beta-subunit leading to the stabilisation of Hb R-conformation and to the increase in the probability of planar haem conformation [27]. All observed changes in Hb properties under transient ischaemia–reperfusion and under permanent ischaemia were not accompanied by the development of oxidative stress in erythrocytes. Thus, we did not observe changes in SOD activity or the appearance of oxidised Hb (methaemoglobin). Only under 48 h of permanent ischaemia did we find an increase in the catalase activity that could be explained by the penetration of H_2O_2 from the blood plasma into erythrocytes. The source of this H_2O_2 could be immune cells in the blood stream or cells of blood vessel walls. We assume that neurons or astrocytes do not contribute to the blood H_2O_2, since we did not observe a change in the catalase activity in erythrocytes under transient ischaemia and reperfusion.

In the present study, we chose spontaneously hypertensive rats as a model organism. Indeed, hypertension is a major risk factor for ischaemic stroke. SHR exhibit specific cerebrovascular characteristics, such as increased wall thickness of arteries and reduced vessel diameter, increased collateral vessel resistance and impaired vasodilation. These features impede blood flow, which probably explains the sensitivity of SHR tissues to ischaemic damage [35]. SHRs also have a number of other unique physiological characteristics, including increased heart rate [36], heightened activity of the sympathetic nervous system [37], hypoperfusion of several brain areas including the brainstem, particularly the area responsible for regulating blood pressure which is thought to be one of the reasons for their developing hypertension [38]. Previous studies on SHRs demonstrated lower gene expression and reduced Mn-SOD activity in their brain [39]. Similarly, stroke-prone spontaneously hypertensive rats (SHRSP, a line derived from SHR) also have decreased SOD activity specifically within a key blood-pressure-control centre, leading to increased oxidative stress and presumably contributing to increased blood pressure [40]. It is important to consider that any physiological and biochemical characteristics of a specific model organism can directly influence the researchers' results. It would be beneficial to estimate the correlations of redox events we found not only in other rat strains, but also in species that are used in ischaemia models.

To conclude, we demonstrated for the first time the difference in changes of Hb properties in the blood of SHR subjected to transient ischaemia/reperfusion and to permanent ischaemia. Decreased blood oxygenation under reperfusion may be regarded as a sign ROS-dependent damage of capillaries, whereas the increase in Hb affinity to O_2 under permanent ischaemia may be among adaptive reactions developing under acute prolonged ischaemia. Further studies may help to elucidate the role of erythrocytes in the development of brain lesions under reperfusion or in the adaptation to chronic ischaemia.

Materials and methods

Animal husbandry and ethical statement

Founder SHR rats (spontaneously hypertensive rats – SHR/NCrl strain) purchased from Charles River Laboratories, Wilmington, MA, USA, were maintained and bred at the animal facility of the Institute of Bioorganic Chemistry at the Russian Academy of Sciences (IBCh RAS), and their offspring were used in the experiment. Animals were housed under a 12-h light–dark cycle with *ad libitum* access to food and water. Experiments were conducted using 4–6-month-old male and female rats. The work was carried out under the supervision of the IBCh IACUC and in accordance with the Regulations of the Ministry of Health of the Russian Federation. All procedures were approved by IBCh IACUC protocol No. 354 from 12.09.2022.

Injection of AAV particles and implantation of optical fibres into the rat brain

The genetic construct hSyn1-HyPer7-MTS2 was delivered to rat brain tissues using adeno-associated virus serotype 9 (AAV9, 8×10^{12} VG·mL^{-1}). The neuron-specific promoter hSyn1 [41] and the MTS2 tag (duplicated mitochondrial targeting sequence) [42] were used to localise the HyPer7 biosensor to the mitochondrial matrix of neurons. The procedures were performed as previously described [15,16]. Here we give a brief overview of the procedures. All manipulations were carried out on anaesthetised animals. Isoflurane (2% Miralek) was used for anaesthesia, while analgesia in the surgical field was obtained with 0.25% bupivacaine (s.c.). Animals were operated on using a stereotaxic framework (Kent Scientific, Torrington, CT, USA). The skull surface was cleared of soft tissue, after which four bilateral craniotomies were made: two 0.4 mm holes at the injection sites and two 1 mm holes in the parietal bone for consequent placement of anchoring screws (Stoelting, Wood Dale, IL, USA). A 1 μL volume of viral particles suspension was then injected at the rate of 200 nL·min^{-1} through the smaller holes into caudate nuclei using a 33-gauge needle coupled to a 5 μL Hamilton syringe (AP = −0.9; ML = ± 4; 5.5 mm below the skull surface). Optical fibres, which had been pre-fixed in ceramic ferrules, were immediately inserted into the holes. The ferrules were fixed to the surface of the skull with light-curing dental composite (VladMiVa, Moscow, Russian Federation) and then sealed

with acrylic dental cement (Yamahachi, Gamagori, Japan). Postoperative analgesia was maintained with the administration of ketoprofen (5 $mg \cdot kg^{-1}$, s.c.) for the next two days. The subsequent experiment was carried out after 3–4 weeks.

Stroke induction and *in vivo* imaging of HyPer7

In vivo monitoring of HyPer7 fluorescence in the rat striatum during an ischaemic stroke was carried out using implanted optical fibre probes. The ferrules of these probes were connected to a custom fibre-optic interface that provided dual-wavelength excitation (405/490 nm) and enabled simultaneous fluorescence detection. A detailed description of the optical circuit system characteristics is provided in our previous articles [15–18]. The stroke was induced through a widely established procedure – occlusion of the middle cerebral artery (MCAO) [43]. In this section, we outline the essential aspects of our experimental setup. Induction and maintenance of anaesthesia and analgesia in animals are similar to those described in the previous section. Occlusion was performed using silicone-coated MCAO sutures (Doccol, Sharon, MA, USA) with a tip length of 0.5 cm and a tip diameter of 0.35 or 0.37 mm. Rats were divided into three groups: one that underwent classic MCAO surgery; one that received permanent occlusion until the end of the experiment; and one that underwent sham surgery and served as a control group. During the procedure, rats were connected to the fibre-optic framework only at predefined key time points, when HyPer7 fluorescence signals from both healthy and ischaemic hemispheres were recorded and averaged over two-minute intervals. These time points were: immediately before the start of the operation (baseline), 1 h (immediately before the reperfusion), 2, 6, 12, 24 and 48 h after occlusion of the middle cerebral artery. The fluorescent signal in the striatum of the healthy hemisphere was used as a control. Immediately after the final measurement, the animals were euthanised using deep anaesthesia with 1-chloro-2,2,2-trifluoroethyl-difluoromethyl ether. The brain was extracted from each animal and sliced into two-millimeter coronal sections. They were then stained in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 10 min at 37 °C to define the ischaemic area.

Blood collection

During the experiment, blood was collected from the external jugular vein of rats using either borosilicate glass capillaries (10–20 μL of the collected blood volume) or insulin syringes (100 μL of the collected blood volume) without exposure to the air. The capillary was pre-stretched using a puller (Sutter), washed with sodium heparin (5000 $IU \cdot mL^{-1}$), and connected to a 50-μL Hamilton syringe via a glass needle compression fitting (Hamilton). After the blood collection, capillaries were sealed with the glass-proofed plastic to avoid contact with air and artificial changes in blood oxygenation. Glass capillaries were used for the estimation of blood oxygenation. Syringes were also washed with sodium heparin (5000 $IU \cdot mL^{-1}$) and contained 1 μL of heparin to avoid blood coagulation. Blood in syringes was used for the estimation of Hb affinity to O_2 and for the estimation of SOD and catalase activities in erythrocytes. To compensate for fluid loss, the animals were injected subcutaneously with 2 mL of saline solution (Solopharm, Moscow, Russian Federation). To compare changes in Hb properties from the jugular vein and the peripheral vessels we also collected blood from the tail vein of a control group of falsely operated rats and of rats with 48 h of MCAO-induced ischaemia.

Estimation of blood oxygenation and Hb affinity to O_2 with Raman spectroscopy

Monitoring of blood oxygenation with Raman microspectroscopy

To estimate the oxygenation state of venous blood collected from the left jugular vein of rats at different time points of 60-min MCAO-induced ischaemia and following the reperfusion period or of permanent 48-h MCAO-induced ischaemia we used Raman microspectroscopy. Blood was collected from the left jugular vein with pulled glass capillaries, avoiding contact with air during the blood collection and afterwards during Raman spectra registration. Blood samples were collected at the following time points for the three studied rat groups: (a) 0 min (before surgery), 1, 2, 6, 24 and 48 h after false surgery for the control group of falsely operated rats; (b) 0 min (before MCAO surgery), 1 h of MCAO-induced ischaemia, 1, 6, 24 and 48 h of reperfusion followed by 1 h of MCAO-induced ischaemia for the experimental group of rats with ischaemia/reperfusion; (c) 0 min (before MCAO surgery), 1, 2, 6, 24 and 48 h of MCAO-induced ischaemia for the experimental group of rats with permanent ischaemia. Experiments were performed using confocal Raman microspectrometer InVia Qontor (Renishaw, New Mills, UK) with 532 nm laser excitation. Immediately after blood collection, the capillary was placed on the scanning table of the Upright Leica FSM microscope connected to the Raman spectrometer. A FSM microscope with an objective 20× NA 0.4 was used to focus the laser light inside the capillary. The diameter of the excitation/registration spot was app. 800 nm with the laser power less than 0.1 mW per registration spot. For each capillary, Raman spectra were recorded from 5 to 7 different spots. Raman spectrum accumulation time was 30 s.

Analysis of Raman spectra for the semi-quantitative estimation of the oxygenation of venous blood in capillaries was done in several steps. The baseline was corrected using linear approximation of the peak-free regions of

1250–1280 cm^{-1} and 1445–1455 cm^{-1} with open-source software PYRAMAN, available at https://github.com/abrazhe/pyraman. After that we defined intensities of peaks at 1357 and 1375 cm^{-1} and calculated intensities ratio I_{1375}/I_{1357}. Since peaks at 1357 and 1375 cm^{-1} correspond to haem atoms' vibrations in dHb and oHb, respectively, and depend only on the absence/presence of O_2 bound on the Fe^{2+} atom, the intensity ratio I_{1375}/I_{1357} corresponds to the relative amount of oHb vs dHb [18–21,25,44–46]. Here we should mention that Raman cross-sections of haem *b* excited with 532 nm differ for dHb and oHb, and therefore change in the I_{1375}/I_{1357} ratio corresponds to changes in blood oxygenation semi-quantitatively.

Estimation of haemoglobin's affinity for oxygen with Raman microspectroscopy

To estimate oHb's affinity for O_2— the oHb property that defines the ability of oHb to release O_2 in tissues' capillaries —we obtained blood samples with fully oxygenated haemoglobin. For this purpose blood samples were collected from the left jugular vein with the 100 μL insulin syringe at the following time points: (a) 0 min (before false surgery), 6th, 24th and 48th hour after false surgery for the control rat group; (b) 0 min (before MCAO surgery), on 6th, 24th and 48th hour of reperfusion after 60-min MCAO-induced surgery for the ischaemia/reperfusion group; (c) 0 min (before MCAO surgery), on 6th, 24th and 48th hour of ischaemia after MCAO surgery for the permanent ischaemic rat group. Each blood sample was diluted 100 times by Allen buffer (145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM glucose and 1 mM $CaCl_2$, pH 7.4). Afterwards a small volume of 100 μL of diluted blood was placed on the glass bottom of a Petri dish and left for an hour at 4 °C temperature until complete oxygenation of haemoglobin in erythrocytes. Then the Petri dish was placed on the scanning table of an FSM microscope of a confocal Raman microspectrometer (Renishaw, UK) and 532 nm laser light was focused via 20 × objective (NA 0.4) on the erythrocyte layer. Laser power per registration spot with the diameter of 800 nm was no less than 0.1 mW, spectrum accumulation time was 60 s. Oxygenation of erythrocytes was monitored by highly specific O_2-dependent Raman peaks of Hb: blood samples were considered to be fully oxygenated when the intensive peak at 1375 cm^{-1} corresponding to the pyrrol rings vibration in oHb was observed and no Raman peak of dHb at 1357 cm^{-1} was found on the Raman spectra of erythrocytes.

To estimate oHb's affinity for O_2 we performed the baseline substruction in each Raman spectrum of oxygenated diluted blood with the open-source software PYRAMAN, available at https://github.com/abrazhe/pyraman (accessed on 24th of November, 2025). The approach used for the baseline substruction was described in our previous paper [47]. Briefly, the baseline interpolation was done using a cubic spline whose set of knots, number and *x*-coordinates were selected manually outside any Raman peaks in the spectra. The number and *x*-coordinates of the knots were fixed for all spectra in the study once they were chosen. *Y*-coordinates of the knots were defined separately for each spectrum as five-point neighbourhood averages of spectrum intensities around the user-specified *x*-position of the knot. We choose the parameters for baseline substruction after the processing of at least 50 spectra of oxygenated blood samples. After the baseline substruction, the intensities of Raman peaks with maxima at 1375 and 1638 cm^{-1} were defined and the ratio of intensities of these peaks (I_{1638}/I_{1375}) was calculated. The Raman peak at 1638 cm^{-1} originates from vibrations of haem methine bridges that depends on the Fe atom redox state, binding of the sixth ligand (e.g. O_2) and, most importantly, on haem planar conformation and its out-of-plane distortion [19–21,23,25,45,46]. Under conditions of 100% Hb oxygenation, any change in the intensities' ratio I_{1638}/I_{1375} corresponds to change in the probability of the planar haem conformation that defines Hb affinity to O_2 [20,31].

Determination of haemoglobin concentration, superoxide dismutase activity, catalase activity and amount of nonprotein thiols in erythrocytes

Blood samples were collected with the syringe from the left jugular vein of all rat groups at time points specified in the previous section. After taking 10 μL of blood for the estimation of oHb's affinity for O_2, the rest of the blood samples (90 μL) were centrifuged at 1500 g, 10 min at room temperature, supernatant was removed and the remaining sediments of erythrocytes were frozen at -70 °C. On the day of the measurements of total Hb concentration, catalase and SOD activity in all erythrocyte samples were defrosted and used in the study.

Estimation of haemoglobin concentration

The total haemoglobin concentration was measured by using a Kit for determination of haemoglobin in blood (Gemichrom Agat, Balashikha, Russia). The method is based on the transformation of haemoglobin forms into hemichrome ones by sodium dodecyl sulfate, followed by measurement of absorption at 540 nm.

Estimation of superoxide dismutase (SOD) activity

The erythrocyte SOD activity was estimated by inhibition of the adrenaline self-oxidation at 25 °C [48]. In an alkaline environment, adrenaline is spontaneously oxidised by a superoxide anion radical, forming a coloured product, adrenochrome, for which the accumulation is recorded spectrophotometrically at 485 nm. Since superoxide anion

radical is also the substrate for the catalytic reaction of SOD that decomposes it to H_2O_2 and O_2, the increase in SOD activity prevents adrenaline autooxidation and decreases adrenochrome formation. One unit of SOD activity was defined as the amount of SOD required to cause 50% inhibition of the oxidation of the adrenaline. The SOD activity was expressed as units per mg of haemoglobin (Units/mg Hb).

Estimation of erythrocyte catalase activity

The measurement of catalase activity in the hemolysates was determined by using a Catalase (CAT) Assay Kit Cat. No: BC013 (ELK Biotechnology, STE12 Sugar Land, TX, USA). The principle of assay is that ammonium molybdate can stop the H_2O_2 decomposing reaction catalysed by catalase immediately, and residual H_2O_2 can react with ammonium molybdate to produce a yellowish complex. CAT activity was assessed by measuring OD value at 405 nm. The calculation of the catalase enzyme activity of the sample was done by using the catalase activity equation according to the manual of the kit. 1 μM H_2O_2 decomposing per mg haemoglobin per second is considered as one activity unit (U).

Animal cohorts

The total number of animals used in the experiment was 23 rats of both sexes. All the animals had approximately the same age and weight, and a good health status. The distribution into three groups (ischaemia/reperfusion, control (falsely operated rats), permanent ischaemia) occurred randomly. In experiments with H_2O_2 measurements and estimation of the stroke volume, the number of animals in each studied group was: seven animals in both control and ischaemia/reperfusion groups, and nine animals in the permanent ischaemia group (the number of animals was increased due to higher mortality risks). In experiments on the estimation of blood oxygenation, oxyhaemoglobin affinity to O_2 and measurements of SOD and catalase activities, the number of animals were: four rats in the control and ischaemia/reperfusion groups and eight rats in the permanent ischaemia group.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.0.1). All data are shown as box-whiskers plots where horizontal boxes' borders correspond to 25% and 75% quartiles, horizontal lines inside the boxes show median, and vertical whiskers indicate minimal and maximal values. The normality of data distribution was assessed with the Shapiro–Wilk test. Unpaired *t*-test was used to analyse Hyper7 signal in damaged and healthy hemispheres in the ischaemia/reperfusion group. For comparison, infarct volumes between ischaemia/reperfusion and permanent groups, and Hyper7 signal in damaged and healthy hemispheres in the permanent group, used the Mann-Whitney test. To compare blood oxygenation, Hb affinity to O_2 and activities of SOD and catalase at different time points of ischaemia/reperfusion, permanent ischaemia or in the control group, we used mixed effect analysis, Sidak's multiple comparison test. Statistical significance was set at $P < 0.05$.

Acknowledgements

The research was funded by the Ministry of Science and Higher Education of the Russian Federation (grant agreement No 075-15-2024-530).

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

DSB, MAK, VAK and AEN performed surgical procedures; KVV and AMKarhov injected adeno-associated viruses and implanted optical fibres, prepared animals for experiments; MAS and IVF maintained the functioning of fibre-optic neurointerface; AAMoshchenko prepared adeno-associated viral particles; ZVB and NAB performed experiments on estimation of Hb properties with Raman spectroscopy; ZVB, KIM, EYP and NAB analysed Raman spectra; VAO maintained the functioning of Raman microspectrometer; ZVB, AMKassem and AAB estimated activities of SOD and catalase; DSB and NAB conceived the study; DSB designed experiments with optic photometry; GVM and NAB designed experiments with RS study of Hb properties; VVB, VAM, AAMakarov conducted consultations, provided resources, funding acquisition; ZVB, MAK, VAK, DSB and NAB prepared figures; ZVB, AAB, EYP, DSB and NAB prepared the draft of the manuscript and DSB and NAB finalised the manuscript.

Data availability statement

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Dr. Dmitry S. Bilan (d.s.bilan@ibch.ru). Original photometry and RS data sets reported in this paper will be shared by the corresponding authors, Dr. Dmitry S. Bilan (d.s.bilan@ibch.ru) and Dr. Nadezda A. Brazhe (nadezda.brazhe@biophys.msu.ru), upon request.

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