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ORIGINAL PAPER

Neuroprotective Mechanism of Hypoxic Post-conditioning Involves HIF1-Associated Regulation of the Pentose Phosphate Pathway in Rat Brain

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Abstract

Post-conditioning is exposure of an injured organism to the same harmful factors but of milder intensity which mobilizes endogenous protective mechanisms. Recently, we have developed a novel noninvasive post-conditioning (PostC) protocol involving three sequential episodes of mild hypobaric hypoxia which exerts pronounced neuroprotective action. In particular, it prevents development of pathological cascades caused by severe hypobaric hypoxia (SH) such as cellular loss, lipid peroxidation, abnormal neuroendocrine responses and behavioural deficit in experimental animals. Development of these post-hypoxic pathological effects has been associated with the delayed reduction of hypoxia-inducible factor 1 (HIF1) regulatory α-subunit levels in rat hippocampus, whereas PostC up-regulated it. The present study has been aimed at experimental examination of the hypothesis that intrinsic mechanisms underlying the neuroprotective and antioxidant effects of PostC involves HIF1-dependent stimulation of the pentose phosphate pathway (PPP). We have observed that SH leads to a decrease of glucose-6-phosphate dehydrogenase (G6PD) activity in the hippocampus and neocortex of rats as well as to a reduction in NADPH and total glutathione levels. This depletion of the antioxidant defense system together with excessive lipid peroxidation during the reoxygenation phase resulted in increased oxidative stress and massive cellular death observed after SH. In contrast, PostC led to normalization of G6PD activity, stabilization of the NADPH and total glutathione levels and thereby resulted in recovery of the cellular redox state and prevention of neuronal death. Our data suggest that stabilization of the antioxidant system via HIF1-associated PPP regulation represents an important neuroprotective mechanism enabled by PostC.

Keywords Severe hypoxia · Hypoxic post-conditioning · HIF1 · Pentose phosphate pathway · Oxidative stress · Neuroprotection

Abbreviations

G6PD	Glucose-6-phosphate dehydrogenase
HIF1	Hypoxia-inducible factor 1
IPostC	Ischemic post-conditioning
PostC	Post-conditioning by mild hypobaric hypoxia
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
SH	Severe hypobaric hypoxia
TUNEL	Terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate (dUTP) nick end labeling

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Introduction

Hypoxia is a condition of reduced oxygen supply which can occur both in physiological and pathological modes. The brain has the highest level of oxygen metabolism that makes it the most vulnerable body organ in response to hypoxia. A pathological decrease of oxygen levels in the brain can

occur as a result of brain vasculature malfunctions, heart diseases (circulatory hypoxia, or ischemia) or anaemias of different etiology [1]. Disclosure of the mechanisms underlying post-hypoxic pathology and endogenous protective processes against the negative consequences of hypoxia and re-oxygenation represents an important research problem and is required for development of effective therapeutic approaches. The endogenous neuroprotective mechanisms can be activated by using different techniques, one of which is a post-conditioning—the phenomenon based on the protective effect of exposures to the same traumatic factors of milder modality following severe hypoxia/ischemia or another kind of injury [2]. Ischemic post-conditioning (IPostC) was first described in the heart and brain as a prospective approach for treatment of hypoxic/ischemic injury [3, 4]. Although there is a large amount of data supporting the neuroprotective effect of IPostC [5–26], its translational potential is doubtful due to its invasiveness and relatively narrow therapeutic time windows. According to the literature, IPostC is effective only when applied within 1–2 h after the ischemic insult.

An effective and noninvasive method of post-conditioning (PostC) has recently been developed in the Laboratory of Regulation of Brain Neuron Function of Pavlov Institute of Physiology [27]. The method involves application of three sequential episodes of mild hypobaric hypoxia as a neuroprotective PostC factor. It has been demonstrated that such hypoxic PostC prevents cellular death caused by severe hypobaric hypoxia (SH) in the hippocampus and neocortex of rats [27, 28], improves the process of rehabilitation after SH and minimizes psycho-emotional stress, including normalization of the endocrine responses and behaviour [27]. In contrast to IPostC, this method has a more potent neuroprotective action even if applied in delayed periods after the severe insult (from 24 h to 3 days). Earlier we have reported the effect of PostC on the cerebral expression of different protective proteins [29, 30], in particular, of the hypoxia-inducible factor-1 (HIF1) which is a key regulator of adaptive reactions to hypoxia at the cellular level. It has been shown that PostC up-regulates HIF1 regulatory subunit (HIF1α) and the HIF1-dependent protective cytokine erythropoietin in the CA1 subfield of the hippocampus and neocortex [30, 31].

According to current knowledge, transcription factor HIF1 plays a pivotal role in the development of post-hypoxic pathology, as well as in protection against it. HIF1 is a heterodimeric protein consisting of HIF1α and HIF1β subunits. The subunits contain a DNA-binding domain, a central PAS (Per-ARNT-Sim) domain responsible for dimerization as well as N- and C-terminal transactivation domains which regulate transcription of target genes [32, 33]. In contrast to HIF1β which is expressed constitutively, HIF1α accumulates only under hypoxic conditions and therefore functions as a regulatory subunit [32]. Under normoxic conditions HIF1α is hydroxylated by prolyl hydroxylase (PHD), and then is ubiquitinylated and undergoes proteasomal degradation. In hypoxia oxygen concentrations are insufficient for PHD activity and HIF1α cannot be recognized by the von Hippel Landau (VHL) E3 ubiquitin ligase complex. From a classical point of view this leads to HIF1α accumulation, dimerization with HIF1β and nuclear translocation resulting in activation of HIF1 target genes. Among transcriptional targets of HIF1 are vascular endothelial growth factor (VEGF), glucose transporter 1, all the enzymes of glycolysis, lactate dehydrogenases and the cytokine erythropoietin [32–39].

The present study has been designed to test the hypothesis whether PostC-induced HIF1α enables another down-stream mechanism involving activation of the pentose phosphate pathway (PPP) which acts as a key regulator of antioxidant defense and redox homeostasis of the brain. For this, using our well-established model of hypobaric hypoxia and PostC, we analyzed the effects of SH and PostC on HIF1 dynamics, levels of the activity of the major PPP enzyme glucose-6-phosphate dehydrogenase (G6PD) and other PPP-dependent biochemical parameters.

Materials and Methods

Animals

The experiments were carried out using adult male Wistar rats weighing 220–250 g from the Biocollection of Pavlov Institute of Physiology of RAS. Rats had free access to standard food and water and were kept on 12:12 h dark–light cycle at room temperature with humidity of approximately 60%. All experimental procedures were performed in compliance with the Guidelines for Reporting Animal Research [40] and approved by the Ethical Committee for Use of Animal Subjects in Pavlov Institute of Physiology.

Severe Hypobaric Hypoxia (SH) and Mild Hypobaric Hypoxia Post-conditioning (PostC)

Injurious exposure to severe hypobaric hypoxia (SH) was performed in a hypobaric chamber by reducing and maintaining the inside pressure at 180 Torr (equivalent to 5% of normobaric oxygen) for 3 h as described in detail previously [41]. Some air was blown into the chamber every 20 min to avoid hypercapnia and maintain constant gas composition. A single PostC trial consisted of an exposure to 360 Torr pressure in the chamber (equivalent to 10% of normobaric oxygen) for 2 h. One or three PostC trials spaced at 24 h intervals were applied starting 24 h after SH. This protocol was previously developed and validated as the most efficient

mode of PostC [27]. Control rats were placed in the chamber for the same duration with no hypoxia applied.

The rats were decapitated on the 1st, 2nd and 4th days after SH and on the 1st day after 1 or 3 trials of PostC (Fig. 1). The brain was quickly removed and either fixed for further histological analysis or the hippocampus and neocortex have been dissected and frozen in liquid nitrogen. In each experimental group there were 4–6 rats.

Histology

Tissue samples containing hippocampal and neocortical regions were treated in the molecular fixative FineFix (28 ml Fine Fix + 72 ml 96° ethanol, Milestone, Italy) for 24 h. After that, tissues were dehydrated as described in [42], immersed in paraffin (2 times for 1 h) at 56 °C and sectioned. Coronal sections of the brain with the thickness of 7 μm were then made using a rotation microtome (Reichert, Austria) starting at about − 2.80 mm from the bregma. Sections were mounted onto the poly-L-lysine covered slides, deparaffinized in xylol (2 times for 5 min), rehydrated in alcohols (96° → 96° → 96° → 70° for 5 min in each) and demasked by boiling in citrate buffer solution (pH 6.0).

TUNEL Staining

The number of neurons which underwent apoptotic transformation was assessed in the CA1 hippocampal field and layer II of the neocortex. For this TUNEL staining was performed using a NeuroTACs kit (R&D Systems, United Kingdom) and visualizing DNA fragmentation specific for apoptosis. Briefly, after deparaffinization the sections were incubated with NeuroPore for 60 min at room temperature. After washing they were immersed in Labeling Reaction Mix containing TdT and biotinylated nucleotides for 1 h at 37 °C. The reaction was terminated by the TdT Stop Buffer. After several washes, the sections were incubated with Streptavidin-HRP for 10 min at room temperature. The reaction was visualized with diaminobenzidine. For visualization of all cells, the sections were immersed in Blue Counterstain, then they were air-dried, mounted with cover glass and assayed using an image analysis system. The number of TUNEL-positive cells was counted in the CA1 field of the hippocampus and neocortical layer II, using Videotest Master Morphology software. Four slices were analyzed from each brain with one field of each brain area being measured in each slice. The result were expressed as the ratio of TUNEL-positive cells to the total number of cells.

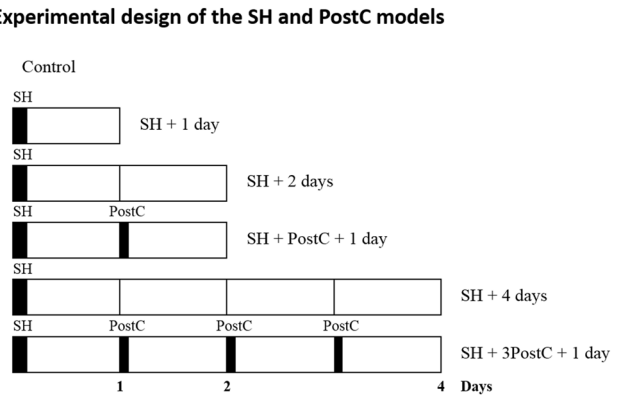

Fig. 1 Experimental design of the study. *SH* severe hypobaric hypoxia; *PostC* postconditioning by mild hypobaric hypoxia

Immunohistochemistry

Protein levels of HIF1α or G6PD were analyzed in the CA1 subfield of the hippocampus and neocortical layer II. The sections were incubated with primary polyclonal antibodies to HIF1α (1:70, sc-10790, Santa-Cruz Biotechnology) or G6PD (1:100, sc-67165, Santa-Cruz Biotechnology) at 4 °C overnight. After several washes, the sections were incubated with biotinylated secondary antibodies (1:200, Vectastain ABC kit, Vector Laboratories) and ABC complex for 30 min each. Diaminobenzidine (DAB substrate kit for peroxidase, Vector Laboratories, USA) was used as a chromogen to visualize the sites expressing HIF1α and G6PD immunoreactivity. The sections were dehydrated, mounted and assayed with an image analysis system consisting of a Jeneval light microscope (Carl Zeiss, Germany), a digital camera Baumer CX05c (Baumer Optronic, Germany) and IBM PC with "VideoTest Master Morphology" software (VideoTest Ltd., Russia). The average optical density normalized to background was analyzed.

Glucose-6-phosphate Dehydrogenase Activity Assay

Activity of G6PD was detected using a colorimetric assay kit (MAK015, Sigma-Aldrich). The hippocampus or neocortex was dissected and rapidly homogenized in ice-cold PBS and centrifuged at 15,000×*g* for 10 min. The G6PD substrate mix (containing glucose-6-phosphate and NAD^+) with the Developer mix from the kit were added to the supernatant for conversion of NAD^+ to NADH, generating an intensely coloured product. The absorbance was measured at 450 nm with a spectrophotometric microplate reader SPECTROstar Nano (BMG Labtech, Germany) at 37 °C for 30 min. The amount of generated NADH was determined using a NADH standard curve, and the G6PD activity was expressed in nmol of NADH generated per min per mg of total protein in the sample.

Measurement of NADPH Levels

The NADPH levels were measured by a colorimetric quantification kit (MAK038, Sigma-Aldrich). The hippocampus or neocortex was dissected and homogenized with an extraction buffer and centrifuged at 10,000×*g* for 10 min to isolate the NADPH/$NADP^+$ containing supernatant. The supernatant then was heated at 60 °C for 30 min to decompose the $NADP^+$, cooled on ice and spun quickly to remove any precipitate. The samples were then incubated with a NADPH Developer for 4 h and the absorbance measured at 450 nm with a spectrophotometric microplate reader SPECTROstar Nano (BMG Labtech, Germany). The amount of NADPH was quantified using a NADPH standard curve and expressed as pmol of NADPH per mg of total protein.

Extraction of the Cytosolic Fraction

The hippocampus or neocortex was homogenized on ice in 0.3 M sucrose, 1 mM EDTA, 0.2 M Tris–HCl (pH 7.4) and centrifuged for 10 min at 1500×*g* and 4 °C. The supernatant was re-centrifuged for 20 min at 9000×*g* and 4 °C [43]. The supernatant obtained was aliquoted and stored at − 80 °C.

Measurement of the Thiol Group and Total Glutathione Contents

For this, 0.4 mg/ml DTNB (5,5′-dithiobis-(2-nitrobenzoic acid), Sigma Ald., USA) in 0.1 M potassium phosphate buffer with 1 mM EDTA (pH 7.4) was added to 25 µl of sample and the optical density was measured at 412 nm using a spectrophotometric microplate reader SPECTROstar Nano (BMG Labtech, Germany) at room temperature. The content of the thiol groups in the samples was calculated using a reduced glutathione standard curve, and the redox status of the thiol groups was expressed as nmol of cysteine per mg of total protein.

For measurement of total glutathione content, 80 µl of cytosolic fraction was diluted with an equal volume of 2 M perchloric acid, incubated for 5 min and centrifuged at 5000×*g* for 5 min. After centrifugation 150 µl of the supernatant was neutralized with 100 µl of 2 M potassium hydroxide. Then 25 µl of the sample was diluted with a reaction mix containing 0.19 mg/ml NADPH (AppliChem., Germany) and 25 ng/ml DTNB in 0.1 M potassium phosphate buffer, 1 mM EDTA (pH 7.4). After that a solution of glutathione reductase (Sigma Ald., USA) was added to the final activity of 6 U/ml. The absorbance of the mixture was measured at 412 nm using a spectrophotometric microplate reader SPECTROstar Nano (BMG Labtech, Germany) at room temperature for 10 min [44]. The amount of reduced glutathione generated during 10 min of incubation was calculated using a reduced glutathione standard curve, and the total glutathione level was expressed as nmol of glutathione per mg of total protein.

Total Protein Content Assay

Protein content in the samples was measured before every experiment by a standard Eppendorf protocol using a Biophotometer plus Eppendorf reader (Eppendorf, Germany) at the Research Centre for Environmental Safety of St. Petersburg State University. For this 20 µl of each sample was diluted to the final volume of 1 ml with distilled water and the optical density was measured according to the standard Eppendorf protocol.

Statistical Analysis

Data analysis was performed using a STATISTICA 7.0 software package (Stat Soft, Inc., USA) with the Mann–Whitney U test applied. The results were considered significant when $p \leq 0.05$. The results were expressed as mean ± standard error of the mean (SEM). The mean and SEM were then recalculated as % of control, which was taken as 100%. The TUNEL data are expressed as the ratio of TUNEL-positive cells to the total number of cells, which was taken as 100%.

Results

PostC Abolished SH-Triggered Apoptosis in Rat Hippocampus and Neocortex

In rats subjected to SH a considerable cellular loss has been observed both in the hippocampus and neocortex 4 days after the insult (Fig. 2). The number of apoptotic cells in the CA1 subfield of the hippocampus was much higher than in layer II of the neocortex (43% and 6% of TUNEL-positive cells, correspondingly). PostC abolished the pro-apoptotic processes caused by SH both in the hippocampus (Fig. 2a, c) and neocortex (Fig. 2b, c).

Effects of PostC on the Hippocampal and Neocortical Expression of HIF1α and G6PD

In the hippocampus SH caused an increase in HIF1α-immunoreactivity up to 377% 1 day after the insult, which then reduced down to 44% (2 days after) and 66% (4 days after) of the controls, respectively (Fig. 3a, c). In the neocortex we observed another dynamic of HIF1α expression (Fig. 3b, d). There was a decrease in HIF1α-immunoreactivity down to 55% 1 day after the exposure followed by a gradual increase up to 79% in 2 days and up to 102% 4 days after SH. Exposure to PostC remarkably modified the dynamics of HIF1α expression after SH. In

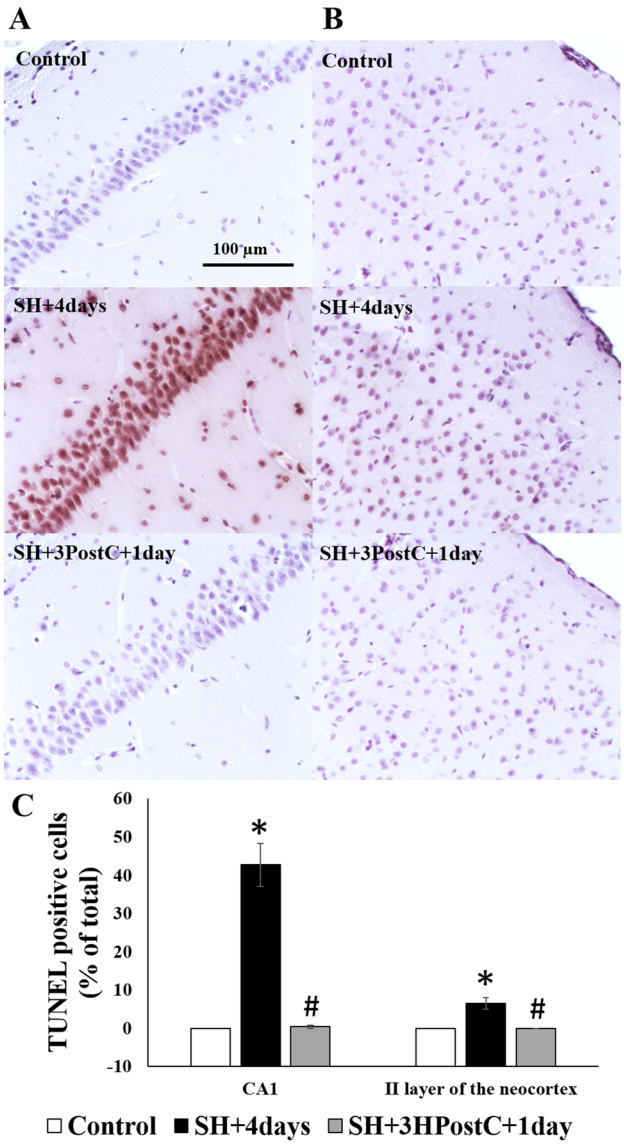

Fig. 2 Effects of SH and SH followed by PostC on the number of TUNEL-positive cells in the CA1 field of the hippocampus (**a, c**) and layer II of the neocortex (**b, c**). **a, b** Representative microphotographs of the hippocampus (**a**) and neocortex (**b**) (×40, scale bar 100 µm); **c** histograms showing the number of TUNEL-positive cells in the hippocampus and neocortex. *Significant differences with the control group; #Significant differences with SH. $p \leq 0.005$ for the hippocampus, $p \leq 0.007$ for the neocortex. n = 4–6

the hippocampus, one episode of PostC decreased HIF1α to the control level whereas three episodes of PostC resulted in further up-regulation of HIF1α expression (up to 293%) (Fig. 3a, c). In contrast to this, PostC did not significantly affect HIF1α expression in the neocortex (Fig. 3b, d).

Expression of the rate-limiting PPP enzyme G6PD in the hippocampus and layer II of the neocortex had a similar dynamics to that of HIF1α (as shown in Fig. 4). The immunoreactivity of the G6DG-positive neurons in the CA1 subfield of the hippocampus decreased down to 84% by the 4th day after SH (Fig. 4a). In the neocortex, on the contrary, the G6DG-immunoreactivty decreased down to 62% 2 days after SH and then returned to the control level 4 days after (Fig. 4b). PostC averted the delayed decrease of G6PD expression after SH in the hippocampus (Fig. 4a) and had no effect in the neocortex (Fig. 4b).

Effect of PostC on G6PD Activity and NADPH Levels

For evaluation of the functional state of PPP in the hippocampus and neocortex we analyzed the activity of G6PD and the levels of NADPH in rats exposed to SH and SH followed by PostC. While SH led to an increase of G6PD activity (up to 135%) in the hippocampus 1 day after SH, 4 days after SH the activity of this enzyme decreased to 65% (Fig. 5a). Levels of NADPH at the same time were down to 38, 34 and 52% 1, 2 and 4 days after SH, respectively (Fig. 5c). Three trials of PostC restored G6PD activity up to 114% (Fig. 5a) and normalized NADPH levels (Fig. 5c).

Although SH generally demonstrated no notable effect on G6PD activity in the neocortex (Fig. 5b), there was a slight decrease in G6PD activity (down to 74%) 2 days after SH. This could be explained by the delayed HIF1α underexpression in the neocortex at the same time period. Levels of NADPH in the neocortex were depleted down to 46% and 76% 1 and 2 days after SH, and returned to the control values 4 days after SH (Fig. 5d). PostC prevented down-regulation of G6PD activity (Fig. 5b) and normalized NADPH levels already after the first trial (Fig. 5d).

Effect of PostC on SH-Induced Oxidative Stress and Glutathione Levels

To analyze the effects of SH and PostC on cytosolic redox status, we compared the ratio of the thiol groups to the total protein amount. Despite the relative simplicity of the measurement, it helps to estimate the level of reduced cysteine residues in proteins, peptides and glutathione. Moreover, cysteine residues are molecular sensors of cellular redox status and their level corresponds to the changes in the cytosolic redox homeostasis.

As shown in Fig. 6a, PPP deficit induced by SH in the hippocampus was accompanied by a long-term oxidative shift in the cellular redox status. On days 1, 2 and 4 after SH, levels of the thiol groups were reduced down to 75, 42 and 57% of the controls, respectively. This was accompanied by a substantial decline in the total glutathione levels (down to 14, 15 and 11% 1, 2 and 4 days after SH, respectively) (Fig. 6c). It provided further evidence that SH induced persistent oxidative stress in the hippocampus. PostC was able normalize the levels of the thiol groups (Fig. 6a) and caused

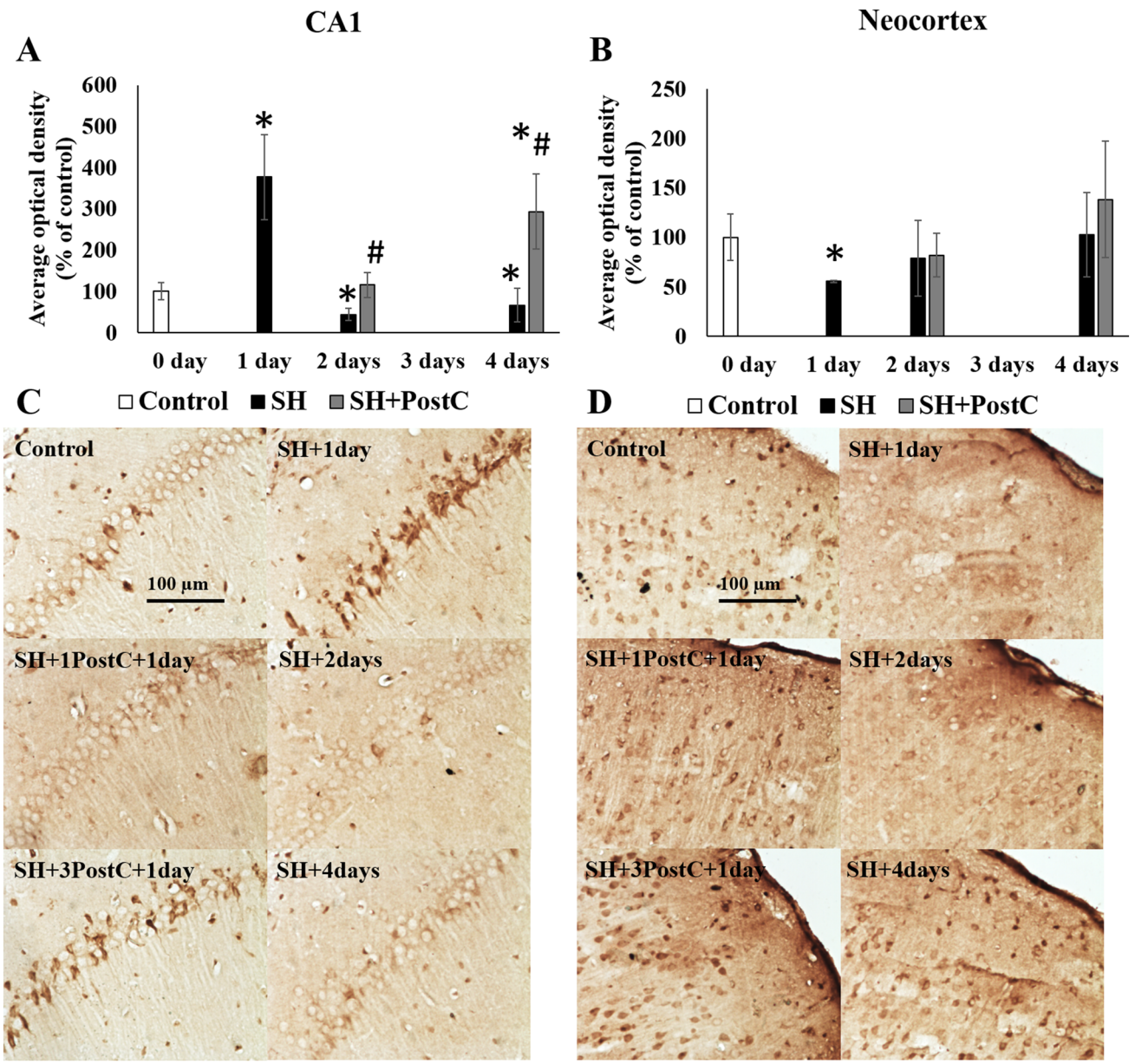

Fig. 3 Effects of SH and SH with PostC on HIF1α protein expression in the CA1 field of the hippocampus (**a, c**) and layer II of the neocortex (**b, d**). Histograms showing the average optical density of the immunopositive cells in the hippocampus (**a**) and layer II of the neocortex (**b**); **c, d** representative microphotographs of the hippocampus (**c**) and the neocortex (**d**) (×40, scale bar 100 μm). *Significant differences with the control group; #significant differences with SH. $p \leq 0.02$ for the hippocampus. $p \leq 0.05$ for the neocortex. n = 4–6

an increase of total glutathione content, which, however, did not reach the control values (Fig. 6c).

In contrast to the hippocampus, oxidative stress in the neocortex was weaker and observed only 1 day after SH. Content of the thiol groups at this time point was down to 71% and the amount of total glutathione was down to 81% of the control values (Fig. 6b, d). On days 2 and 4 after SH there were no significant differences in these two parameters compared to the controls. PostC had no significant effect on the levels of thiol groups in the neocortex (96 and 83% of the control values after one and three trials, respectively) (Fig. 6b), and total glutathione level was only slightly reduced after the third trial of PostC (83% of the control level) (Fig. 6d).

Discussion

Hypoxia is one of the prevalent damaging factors in different traumatic and endogenous brain pathologies. Severe hypoxia suppresses brain neuroplasticity, causes memory

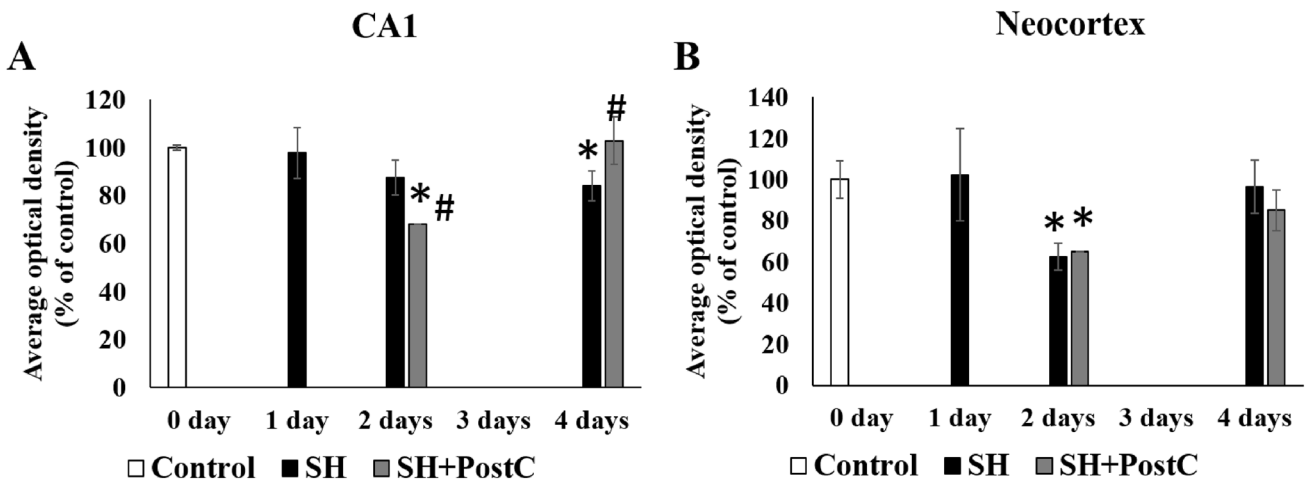

Fig. 4 Effects of SH and SH with PostC on G6PD protein expression in the hippocampus and neocortex. Histograms showing the average optical density of the immunopositive cells in the hippocampus (**a**) and neocortex (**b**). *Significant differences with control group; #significant differences with SH. $p \leq 0.04$ for the hippocampus, $p \leq 0.05$ for the neocortex. n = 4–6

and behavioural deficits and damages the most vulnerable brain structures, e.g. hippocampus [45]. The acute forms of hypoxic stress (e.g. ischemic stroke) represent one of the most frequent causes of mortality comparable to cardiovascular and neurological diseases, as well as cancer [46]. In contrast, mild hypoxia has a protective effect which was utilized in different protocols of hypoxic training and post-hypoxic rehabilitation. The most commonly applied techniques include hypoxic interval training and ischemic and hypoxic pre- and post-conditioning [3]. Post-conditioning is aimed at improvement of the quality of life in patients surviving acute ischemic stroke and include early [47], delayed [48] and distant ischemic post-conditioning [49] as well as normobaric [50–52] and hypobaric post-conditioning [3, 53]. In respect to clinical implementation, normobaric and hypobaric post-conditioning is currently being considered as the most beneficial and providing the highest translational potential. Their noninvasive and physiologically-friendly techniques with relatively wide therapeutic windows (hours and days after acute hypoxic insult) have low risks and practically no side-effects [53].

The present study was aimed at clarification of the neuroprotective mechanisms activated by our original PostC protocol using mild hypobaric hypoxic exposures. As demonstrated by the TUNEL method PostC can protect cells of the vulnerable brain structures against the harmful effects of SH [27, 28, 41, 54]. Taking into account that HIF1 is a major pro-adaptive factor to hypoxia and master-regulator of energy metabolism [55], in our experimental paradigm we focused on its role in the mechanisms of cerebral hypoxic damage and tolerance. As reported earlier, SH results in a delayed down-regulation of HIF1α in rat hippocampus and its short-term suppression in the neocortex. Down-regulation of HIF1α after SH might be linked to the up-stream mechanism that determines massive neuronal loss in the hippocampus and less prominent cell death in the neocortex observed 7 days after SH [27]. In contrast, exposure of SH-injured rats to sequential episodes of PostC decreases the apoptotic rate and correlates with the delayed up-regulation of HIF1α in the CA1 subfield of the hippocampus. In layer II of the neocortex where the increase in the number of apoptotic (TUNEL-positive) cells after SH is less pronounced than in the hippocampus, HIF1α suppression is observed only on day 1 after the insult and not in the later periods. In this connection, it is important to note that HIF1α levels in the neocortex in later periods after SH are not affected by PostC. These data are in strong agreement with the current theory on the involvement of HIF1 in pre- and post-conditioning in the brain and heart [5, 52, 56] and with the fact that HIF1 inhibition blocks the neuroprotective effect of normobaric post-conditioning in rats exposed to transient 10-min global ischemia [52].

There are reports that HIF1-dependent resistance of tumour cells to radiotherapy involves modulation of glucose metabolism via transcriptional regulation of PPP [57]. This fact led us to the hypothesis that the same mechanism might contribute to the HIF1-mediated neuroprotective action of PostC. The main characteristic of PPP in the brain is that its enzymes [glucose-6-phosphate dehydrogenase (G6PD)] and 6-phosphogluconate dehydrogenase are the only producers of NADPH [58] which, in turn, is involved in macromolecule biosynthesis, antioxidant defense and redox homeostasis [59, 60]. Because NADPH is essential for reduction of such antioxidants as glutathione and thioredoxins [59, 61–63], PPP is considered as a key regulator of antioxidant defense and redox homeostasis of the brain. Both antioxidant

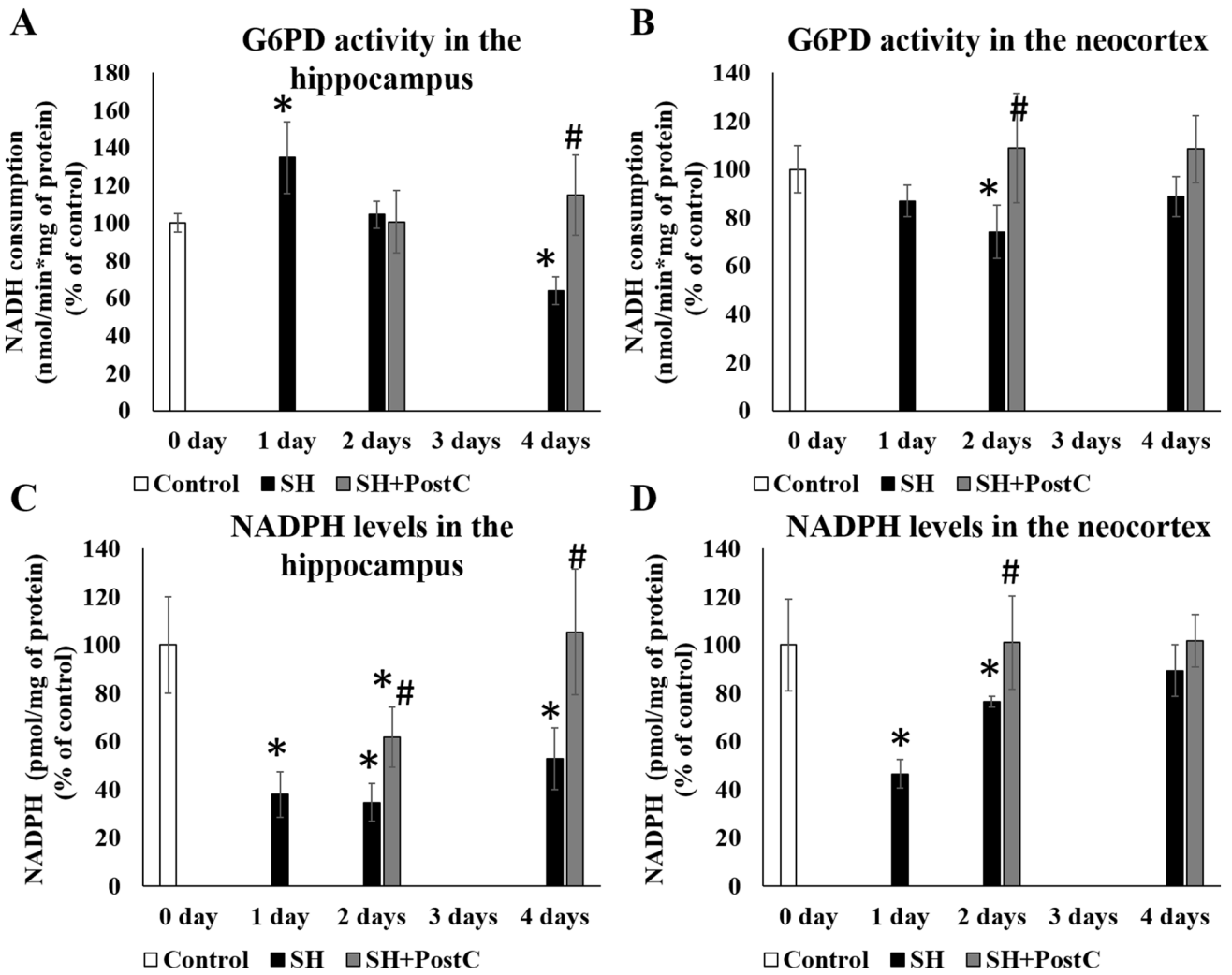

Fig. 5 Effects of SH and SH with PostC on the G6PD activity (**a, b**) and the NADPH levels (**c, d**) in the hippocampus (**a, c**) and neocortex (**b, d**). *Significant differences with control group; #significant differences with SH. **a, b** $p \leq 0.05$ for the hippocampus, $p \leq 0.05$ for the neocortex. **c, d** $p \leq 0.04$ for the hippocampus, $p \leq 0.05$ for the neocortex. n = 4–6

defense and redox homeostasis are affected by reactive oxygen species (ROS). The excessive ROS formation during reoxygenation leads to necrotic and apoptotic cellular death and can contribute to the development of neurodegeneration [64]. As the antioxidant defense effectiveness depends on the level of NADPH, any alterations of the PPP enzyme activity can influence ROS-induced cellular death.

The neuroprotective role of PPP has been well-documented in several ischemic models. In particular, PPP plays a crucial role in prevention of oxidative stress caused by oxygen-glucose deprivation in vitro [65]. NADPH is shown to be a prospective compound in the early treatment of post-hypoxic states [66]. The present study for the first time demonstrates a direct link between the levels and activity of G6DG (the key enzyme of PPP), the product of its reaction NADPH and the expression of HIF1α in rat hippocampus and neocortex after SH and PostC. In the CA1 subfield of the hippocampus SH causes a delayed down-regulation of HIF1α which is accompanied by a reduction in the protein levels and activity of G6PD as well as in NADPH levels. The decrease of the NADPH levels is accompanied by an oxidative shift of the redox status and a decline of the glutathione levels. PostC normalizes HIF1α expression in the hippocampus and leads to a steady increase of the G6DG levels and activity. Under these conditions the levels of NADPH are stabilized at the control values and prevent oxidative shift of the redox status. PostC also gradually restores the total glutathione levels. Since it is generally accepted that intensive ROS formation and oxidative stress play a major role in post-ischemic/hypoxic pathogenesis [67–71], it is possible to suggest that the neuroprotective effect of PostC can be at least partially implemented through the activation of cellular antioxidant defense via maintaining normal PPP functions.

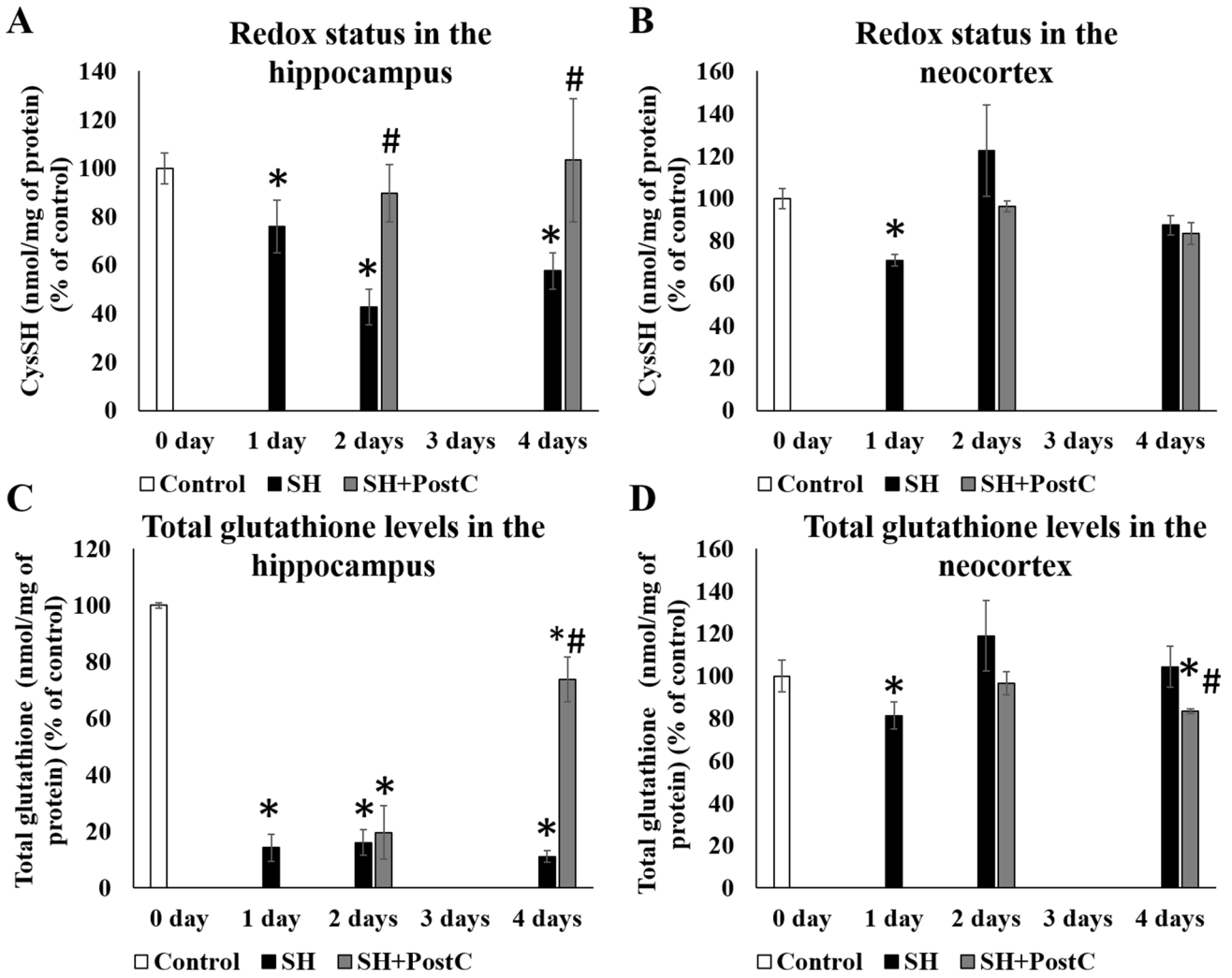

Fig. 6 Effects of SH and SH with PostC on the content of thiol groups (**a, b**) and total glutathione (**c, d**) in the hippocampus (**a, c**) and neocortex (**b, d**). *Significant differences with control group; #significant differences with SH. **a, b** $p \leq 0.008$ for the hippocampus, $p \leq 0.03$ for the neocortex. **c, d** $p \leq 0.0004$ for the hippocampus, $p \leq 0.05$ for the neocortex. $n = 4–6$

Together with this major finding outlined above, the present study also confirms higher tolerance of the neocortex to hypoxia in comparison to the hippocampus. According to our data, in the neocortex SH causes only a short-term decrease of HIF1α expression followed by its fast recovery, normalization of the G6PD levels and activity, as well as of NADPH levels. These effects correlate with a lower number of apoptotic cells in the neocortex compared to hippocampus. Because of that, the antiapoptotic effect of PostC in the neocortex is less pronounced compared to the hippocampus and does not considerably change all studied parameters (levels of HIF1α and G6PD expression, content of the thiol groups and total glutathione levels) or enhances restoration to the control levels of G6PD activity and NADPH.

Another interesting implication of this study and applied PostC paradigm is the link between HIF1, PPP activity and neurodegeneration. Previously we have shown that three trials of mild hypoxia in our PostC paradigm up-regulates ADAM17 which possesses α-secretase activity [54] and shifts the amyloid protein precursor metabolism towards the non-amyloidogenic pathway [72]. Present data also imply that the anti-amyloidogenic properties of the mild hypobaric hypoxia might involve HIF1-dependent modifications of PPP. A very recent report by Kang et al. [73] supports this hypothesis demonstrating involvement of HIF1 and PPP activity in neurodegenerative processes underlying Parkinson's disease, but this molecular aspect of Alzheimer's disease pathology needs further studies.

In conclusion, the results of the present study suggest a possible causal relationship between HIF1 and G6PD protein levels and activity transcription although transcriptional regulation of the G6PD gene by HIF1 still has to be

demonstrated [57]. The data also demonstrate an essential role of PPP activation in the neuroprotective effect of PostC via enhancement of the antioxidant defense system.

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Author Contributions OV was responsible for conducting the study and experimental design, performed experiments and wrote the paper. KS performed the experiments, contributed to experimental design and data analysis. ET and TG contributed to the experiments. AL analyzed the data statistically. OG, NE, and ER contributed to implementation of the study, data analysis and preparation of the paper. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval Animal experiments were carried out according to domestic regulations and the European Community Council Directive of 24 November 1986 (86/609/EEC) [40]. Experimental protocols were approved by the local ethical committee.

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