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Silymarin regulates HIF-1α and iNOS expression in the brain and gills of hypoxic-reoxygenated rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: Rainbow trout *Oncorhynchus mykiss* were pre-treated with silymarin (SMN) to investigate its protective effects on hypoxia/reoxygenation (H/R)-induced pathological and oxidative impacts and on the expression of hypoxia-inducible factor 1α (HIF-1α) and inducible nitric oxide synthase (iNOS) at the mRNA level in the brain and gills of trout. Fifty trout were assigned to control (normoxia) or treatment groups (H/R). The treatment trout were grouped into H/R, which received normal saline, and H/R+S 100, H/R+S 400 and H/R+S 800 treatments, which received 100, 400 and 800 mg SMN, respectively, per kg of fish feed each day for 3 d prior to hypoxia. Hypoxia was induced by bubbling N_2 gas into a water bath, resulting in a lower level of oxygen (5 mg l^{-1}). Fish were kept in a hypoxic condition for 3 h followed by 3 h of normoxia (oxygen level at 10.4 mg l^{-1}), and then blood and tissue were sampled. To evaluate the antioxidant status, the total antioxidant capacity (TAC), malondialdehyde (MDA) content, nitric oxide (NO) level and protein carbonylation rate were assessed in the brain and gills. The expression of HIF-1α and iNOS mRNA was examined in the brain and gills using semi-quantitative RT-PCR. SMN lowered the H/R-elevated NO, MDA and carbonylated protein levels, while it enhanced the TAC level. Moreover, SMN regulated the H/R up-regulated level of HIF-1α and iNOS in examined tissues. SMN ameliorated the H/R-induced histopathological injuries in the brain and gills. These results suggest that pre-treatment of trout with SMN might be an applicable method for reducing H/R-induced biochemical, histopathological and transcriptional injuries.

KEY WORDS: Hypoxia · Oxidative status · Reoxygenation · Silymarin · Trout

INTRODUCTION

Aerobic animals, including fish, use oxygen for energy generation via oxidative phosphorylation, which is related to the reduction of oxygen to water by the cytochrome oxidase system (Kitazoe et al. 2011). Oxygen is a vital element in aquatic environments, but oxygen levels may vary widely and are influenced by water temperature, salinity, water flow, photosynthetic activities of algae and the intensity of respiration rates of organisms. Aquatic organisms, including fish, are exposed to different levels of oxygen daily and throughout different seasons. Another factor potentially causing hypoxia in cultured aquatic animals is extended shipping time in insufficiently oxygenated containers.

A large variation in tolerance to changes in oxygen level has been reported: cold-adapted fish, including rainbow trout, are sensitive to hypoxia, while cyprinid species can tolerate very low levels of oxygen (McKenzie et al. 2004, Lushchak & Bagnyukova 2006a,b). During the last decade, it has been established that fish could be a novel model for investigating oxygen-level-mediating alterations.

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Hypoxia and subsequent reoxygenation resulted in a remarkable increase of oxidative stress biomarkers in tissues of the rotan *Perccottus glenii* and *Leporinus elongatus* (Filho et al. 2005, Lushchak et al. 2007). It has been generally accepted that the extra production of pro-oxidants, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), may result in damage to cellular macromolecules, including proteins, lipids and DNA (Dröge 2002, Valko et al. 2007). Therefore, from a farming perspective and particularly in aquatic environments with high variation in oxygen level, the O_2 utilization, ROS generation and antioxidant status are of great importance. To tolerate the various oxygen levels and to minimize hypoxia/reoxygenation (H/R)-induced oxidative injuries, fish species have developed several physiological adaptations, including metabolic rate depression, blood flow rearrangement mainly to the brain and heart and effective methods of energy production (Nilsson & Renshaw 2004).

One of the known mediatory factors in hypoxic animals is the hypoxia-inducible factor 1α (HIF-1α), which is a key transcription factor in mediating different responses of animals and cells to hypoxia (Nikinmaa & Rees 2005). For instance, the crucial role of HIF-1α in hypoxia-induced changes in the gills of crucian carp has been reported. Another oxygen-dependent protein that is induced by HIF-1α is inducible nitric oxide synthase (iNOS) (Palmer et al. 1998). Hypoxia-induced iNOS activity may result in an excessive production of NO and peroxynitrite ($ONOO^-$), which is highly reactive against biomolecules (Curtin et al. 2002).

Hypoxic/reoxygenated animals have developed physiological pathways to minimize injuries. Nevertheless, we hypothesized that implementing alternative and supportive approaches, such as pretreatment with antioxidants, may reduce H/R-induced injuries, especially in aquatic animals, which are frequently exposed to oxygen stress. Previous reports have indicated that the brain and gills are the most susceptible tissues to hypoxia (Olson 2008, Li et al. 2011); therefore, in the present study, these tissues were selected for investigation. Silymarin (SMN) is used as an effective hepatoprotective agent due to its antioxidant, anticancer and anti-inflammatory properties in humans and animals. Its immune stimulating effect has also been reported in rodents (Meeran et al. 2006). To examine our hypothesis, we investigated the protective effect of pretreatment with SMN on H/R-induced oxidative stress, histopathological damage, and the expression of HIF-1α and iNOS mRNA in the brain and gills, of fingerling rainbow trout.

MATERIALS AND METHODS

Chemicals

SMN (S 0292) and 5.5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma. Sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were obtained from Sigma-Aldrich. Thiobarbituric acid, phosphoric acid (85%), dimethyl sulfoxide (DMSO), sodium nitrite and ethanol were purchased from Merck. N-butanol was obtained from Carl Roth. TRI reagent was purchased from Applied Biosystems. All other chemicals were analytical grade commercial products.

Animals

Fifty fingerling rainbow trout (average weight 40 ± 5 g) were obtained from a local trout farm and transported to the Artemia and Aquatic Animals Research Institute (Urmia, Iran) in an oxygenated tank. They were then stocked in a concrete pond containing underground freshwater. The fish were cultured in a flow-through system with a flow rate of 50 l min^{-1}. Dissolved oxygen was maintained above 8 mg l^{-1} using constant aeration and fish were exposed to a natural photoperiod of approximately 12 h light: 12 h dark. Water temperature was 13.5 ± 1°C, and pH 7.30 to 7.50. The fish were fed a standard commercial mycotoxin-free formulated trout diet (Milad-e-Mahabad) throughout the adaptation and experimentation periods.

Experimental design

Fish were randomly assigned to control (normoxia, n = 10) or treatment groups (H/R). All of the treatment groups (n = 10) were subjected to H/R and classified according to the concentration of SMN that had been added to their diet. The treatment groups included H/R, which received only saline and the solvent of the test compound (<5% of the final volume), and SMN 100, SMN 400 and SMN 800, which received 100, 400 and 800 mg SMN, respectively, per kg of fish feed each day for 3 d prior to exposure to H/R. All procedures were approved by the local ethical committee in accordance with principles of laboratory animal care.

Hypoxia and reoxygenation

Fish in the H/R and treatment groups were exposed to H/R following 3 d of pre-treatment with

normal saline and/or various concentrations of SMN. Hypoxia was induced by the administration of N_2 gas directly into the bath, lowering the oxygen level to 5 mg l^{-1} over 20 min. The oxygen level was monitored throughout the experiment using an oxygen meter (YSI 55). The fish remained in a hypoxic condition for 3 h. The fish were then transferred to normoxic (oxygen level of 10.4 mg l^{-1}) conditions for 3 h and then were subjected to blood and tissue sampling. The 5 mg l^{-1} oxygen level was selected after testing various oxygen levels of 3, 4 and 5 mg l^{-1}; the fingerling trout tolerated 5 mg l^{-1} oxygen for 3 h.

Blood and tissue sampling

For blood and tissue collection, the fish were anesthetized by immersion in eugenol solution (20 mg l^{-1}), and immediately, blood samples were collected from anesthetized trout by puncturing the caudal vein. The blood samples were subjected to serum preparation by centrifugation at 3000 × *g* for 5 min, and the collected serum samples were stored at −20°C for further biochemical analyses. After blood sampling, the fish were dissected to remove the brain and gills. The collected tissue samples were rinsed 3 times with chilled normal saline to remove excess blood and immediately divided into 2 parts. The first part was snap-frozen in liquid nitrogen and stored at −80°C for further molecular analyses, and the second half was kept in 10% formalin for further histopathological examinations.

Alanine aminotransaminase (ALT) serum level

The serum level of ALT was measured using a commercially available standard kit according to the manufacturer's instructions (10-513, Zist Shimi).

Antioxidant status

To evaluate the antioxidant status in the H/R-exposed animals and to determine the protective effect of SMN on H/R-induced alterations in antioxidant status, several biomarkers were assessed.

Total antioxidant capacity (TAC) assay

The total antioxidant capacity of serum was measured in all experimental groups. The assay is based on an assessment of the ferric reduction antioxidant power (FRAP) (Benzie & Strain 1999). Briefly, at low pH, which was achieved by the addition of acetate buffer (300 mM, pH 3.6), reduction of the Fe(III)-TPTZ complex to the ferrous form produces an intensive blue color that can be measured at 593 nm. An aqueous solution of Fe(II) ($FeSO_4.7H_2O$) and appropriate concentrations of freshly prepared ascorbic acid were used as blank and standard solutions, respectively.

Malondialdehyde (MDA) determination

To determine the lipid peroxidation rate in the control and test groups, the MDA content of the brain and gill samples was measured using the thiobarbituric acid (TBA) reaction as described by Niehaus et al. (1968). Briefly, 0.2 to 0.3 g of the samples were homogenized in ice-cooled KCl (150 mM), and the mixture was centrifuged at 3000 × *g* for 10 min; 0.5 ml of the supernatant was mixed with 3 ml of phosphoric acid (1% V/V), and after vortex mixing, 1 ml of 6.7 g l^{-1} TBA was added to the samples. The samples were heated at 100°C for 45 min and then chilled in ice. After an addition of 3 ml of N-butanol, the samples were centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically (Pharmacia Novaspec II, Biochrom) at 532 nm, and the MDA level was calculated according to the simultaneously prepared calibration curve using MDA standards. The amount of MDA was expressed as nmol per mg of protein. The protein content of the samples was assessed following Lowry et al. (1951).

NO measurement

The total NO content of the brain and gill tissue was measured according to the Griess reaction (Green et al. 1982). In the Griess reaction, NO is rapidly converted into the more stable nitrite, and in an acidic environment, nitrite is converted to HNO_2. In reaction with sulfanilamide, HNO_2 forms a diazonium salt, which reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form an azo dye that can be detected by absorbance at a wavelength of 540 nm. The NO content of the examined organs was expressed as nmol per mg of protein in samples.

Protein carbonylation assay

To determine the carbonyl content of the tissue homogenates (brain and gills), the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls was measured (Levine et al. 1994). Briefly, 0.2 to 0.3 g of the samples were homogenized in ice-cooled phosphate buffer (50 mM, pH 6.7, with 1 mM EDTA), and the mixture was centrifuged at 10 000 × *g*

for 10 min at 4°C. For each individual sample (0.2 ml supernatant), a test and a control sample were prepared, and 0.8 ml of DNPH and 2 M HCl solutions were added to the test and control samples, respectively. The samples were kept in the dark at room temperature for 1 h, with vortex mixing every 15 min. After 1 h, 0.5 ml trichloroacetic acid 30% were added in each sample and vortex mixed for 30 s. All samples were centrifuged at 10 000 × *g* for 3 min, the supernatant was discarded, and the precipitate re-suspended for 15 min in 1 ml of (1:1) ethanol/ethyl acetate solution. After centrifugation at 10 000 × *g* for 3 min and discarding the supernatant, the above step was repeated. Following the last wash, the precipitates were dissolved within 0.6 ml guanidine hydrochloride solution (6 M) at 37°C for 15 min. After dissolving the precipitate, the samples were centrifuged at 10 000 × *g* for 3 min, to remove any leftover debris. For each sample, the optical density (OD) was measured against a 6 M guanidine hydrochloride solution at a wavelength of 370 nm.

The carbonyl content was determined as follows:

$$\text{Carbonyl (nmol ml}^{-1}) = [(\text{CA}/0.011\ \text{mM}^{-1})](600\ \mu\text{l}/200\ \mu\text{l}) \quad (1)$$

where CA, which is the corrected absorbance and computed as the average OD for each control sample, was subtracted from the average OD of the test sample at 370 nm. The extinction coefficient for DNPH at 370 nm is 22 000 $M^{-1}cm^{-1}$. To determine the carbonyl content per mg of protein, the protein levels were measured at 280 nm in each sample. The amount of protein was calculated from a bovine serum albumin (BSA) standard curve (0.25 to 2.0 mg ml^{-1}) that was dissolved in guanidine hydrochloride.

Histopathological examinations

Tissue samples from the brain and gill tissues, which had previously been stored in 10% buffered formaldehyde, were embedded in paraffin, and 5 to 6 µm sections were cut using a rotary microtome and stained with hematoxylin and eosin. To evaluate the level of damage following exposure to H/R and the protective effect of SMN, indices including vasogenic edema, local congestion, increase of meninges thickness and chromatolysis in Purkinje cells of cerebral tissue, hyperplasia of epithelial and pillar cells, adherence between secondary lamellae and necrosis of the lamellae of the gills were scored numerically. The evaluation criteria were as follows: 0 for no detectable lesion, 1 for mild, 2 for moderate and 3 for severe changes. The pathologist who examined the sections was unaware of the experimental details. For each animal in the test and control groups, at least 3 slides from distinct organs were prepared and scored. The numerical data are presented as the sum of the score of 3 slides for each individual fish, and the deviation among the individuals of each experimental group was computed.

RNA isolation and RT-PCR

To evaluate the effect of H/R and SMN pretreatment on the mRNA level of HIF-1α and iNOS in the brain and gill tissues, total RNA was isolated from pooled sample homogenates for the control and test groups (n = 10) using the standard TRIZOL method (Chomczynski & Sacchi 2006). To avoid genomic DNA contamination, extra care was taken when the colorless aqueous phase was collected after chloroform extraction. The RNA amount was determined spectrophotometrically (260 nm and A260/280 = 1.8 to 2.0), and the samples were stored at −70°C. For RT-PCR, cDNA was synthesized in a 20 µl reaction mixture containing 1 µg RNA, oligo(dT) primer (1 µl), 5 reaction buffer (4 µl), RNAse inhibitor (1 µl), 10 mM dNTP mix (2 µl) and M-MuLV Reverse Transcriptase (1 µl) according to the manufacturer's protocol (Fermentas). The cycling protocol for 20 µl reaction mix was 5 min at 65°C, followed by 60 min at 42°C and 5 min at 70°C to terminate the reaction.

Second strand cDNA synthesis

The reaction was carried out in a total volume of 25 µl containing PCR master mix (12.5 µl), gene specific primers (0.5 µl each) and cDNA as template (1 µl) and nuclease-free water (10.5 µl). PCR conditions were run as follows: general denaturation at 95°C for 3 min in 1 cycle, followed by 40 cycles of 95°C for 20 s, an annealing temperature (61°C for HIF-1α, 65°C for iNOS, and 60°C for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) for 30 s and elongation at 72°C for 1 min, and terminating with 72°C for 5 min. The PCR products were separated on 1.5% agarose gels containing ethidium bromide and visualized using the Gel Doc 2000 system (Bio-Rad). The density of the RT-PCR bands was quantified using the Molecular Analyst software (Bio-Rad) and normalized based on the density of corresponding GAPDH bands. The RT-PCR reaction and subsequent electrophoresis were performed 3 times, and

Table 1. Primer pairs used for PCR. FWD: Forward, REV: Reverse, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, HIF-1α: hypoxia-inducible factor 1α, iNOS: inducible nitric oxide synthase

Name	Gene	PCR product size (bp)	Annealing temperature (°C)	Sequence (5′ to 3′)
GAPDH FWD	GAPDH	556	60	ATG TCA GAC CTC TGT GTT GG
GAPDH REV	GAPDH			TCC TCG ATG CCG AAG TTG TCG
HIF-1α FWD	HIF-1α	378	61	GAC TCA GGC CAC TGT TAT CTA CAA
HIF-1α REV	HIF-1α			CAG GAT GTC AGA GTC GGG GCT GC
iNOS FWD	iNOS	469	65	GTG CGT AAC GTG AAG GAT GGC TC
iNOS REV	iNOS			CGG TAA TCG CAG ACC TTA GGT TTC

the averages of numerical densitometric values along with standard deviation were calculated.

The specific primers for rainbow trout GAPDH (Collet et al. 2003), HIF-1α (GenBank AF304864) and iNOS (Wang et al. 2001) were designed and manufactured by CinnaGen. The primer pairs used and expected size of PCR products are depicted in Table 1.

Statistical analysis

The means and standard deviations of the measured parameters were calculated. The results of 3 independent experiments for each assessment were analyzed using GraphPad Prism software (version 2.01). The comparisons between groups were made by ANOVA followed by Bonferroni post-hoc tests. For comparing the graded degree of pathological findings between groups, the Kruskal-Wallis test was used. $p < 0.05$ was considered significant.

RESULTS

SMN antioxident and anti-inflammatory effects

Exposure to H/R resulted in a significant ($p < 0.01$) decline of TAC compared to the control group, while pre-treatment with SMN at various dose levels increased the TAC significantly ($p < 0.05$). Although SMN treatment at 100 and 400 mg kg^{-1} could recover the H/R-reduced TAC, at the 800 mg kg^{-1} dose, the effect of SMN was not significant (Table 2). The hepatic functional enzyme level in serum was measured in various study groups, and the results revealed that 3 h hypoxia followed with 3 h of reoxygenation resulted in a significant elevation of the serum level of ALT. The pretreatment with SMN at 100 and 400 mg kg^{-1} but not at 800 mg kg^{-1} resulted in a remarkable decline of the ALT serum level (Table 2).

To analyze the antioxidant status following exposure to H/R in trout, the rates of protein oxidation and lipid peroxidation and the NO content of brain and gills were measured. The results showed that all 3 factors were elevated significantly ($p < 0.01$) in the brain and gills of the H/R group. The 3 d pretreatment with various concentrations of SMN resulted in a significant and concentration-dependent reduction of the H/R-elevated NO content, MDA level and rate of protein carbonylation in the brain (Fig. 1A,B,C). SMN was able to reduce the H/R-elevated levels of NO, MDA and carbonylated protein content in the gills of trout. However, the antioxidant effects of SMN in the gills were not concentration dependent; the maximum reduction of the pro-oxidant levels was obtained at 400 mg kg^{-1} of fish feed (Fig. 1D,E,F).

Pathological findings

The histopathological examination of the brain and gills in the control group showed no remarkable pathologic changes (Fig. 2A & 3A). In contrast, trout that were exposed to H/R showed local congestion,

Table 2. *Oncorhynchus mykiss.* Effect (mean ± SD) of pretreatment of trout with 3 levels of silymarin (SMN) on total antioxidant capacity (TAC) and serum level of alanine aminotransaminase (ALT) in experimental groups. Different letters signify statistically distinct groups ($p < 0.05$). H/R: hypoxia/reoxygenation

Groups	TAC (μmol l^{-1})	ALT (U l^{-1})
Control	0.83 ± 0.06^{a}	71.7 ± 18.9^{a}
H/R	0.54 ± 0.03^{b}	160.3 ± 12.8^{b}
H/R+S100	0.77 ± 0.02^{c}	96.9 ± 7.2^{c}
H/R+S400	$0.90 \pm 0.06^{c,d}$	$57.5 \pm 14.3^{c,d}$
H/R+S800	0.60 ± 0.04^{d}	127.0 ± 23.5^{d}

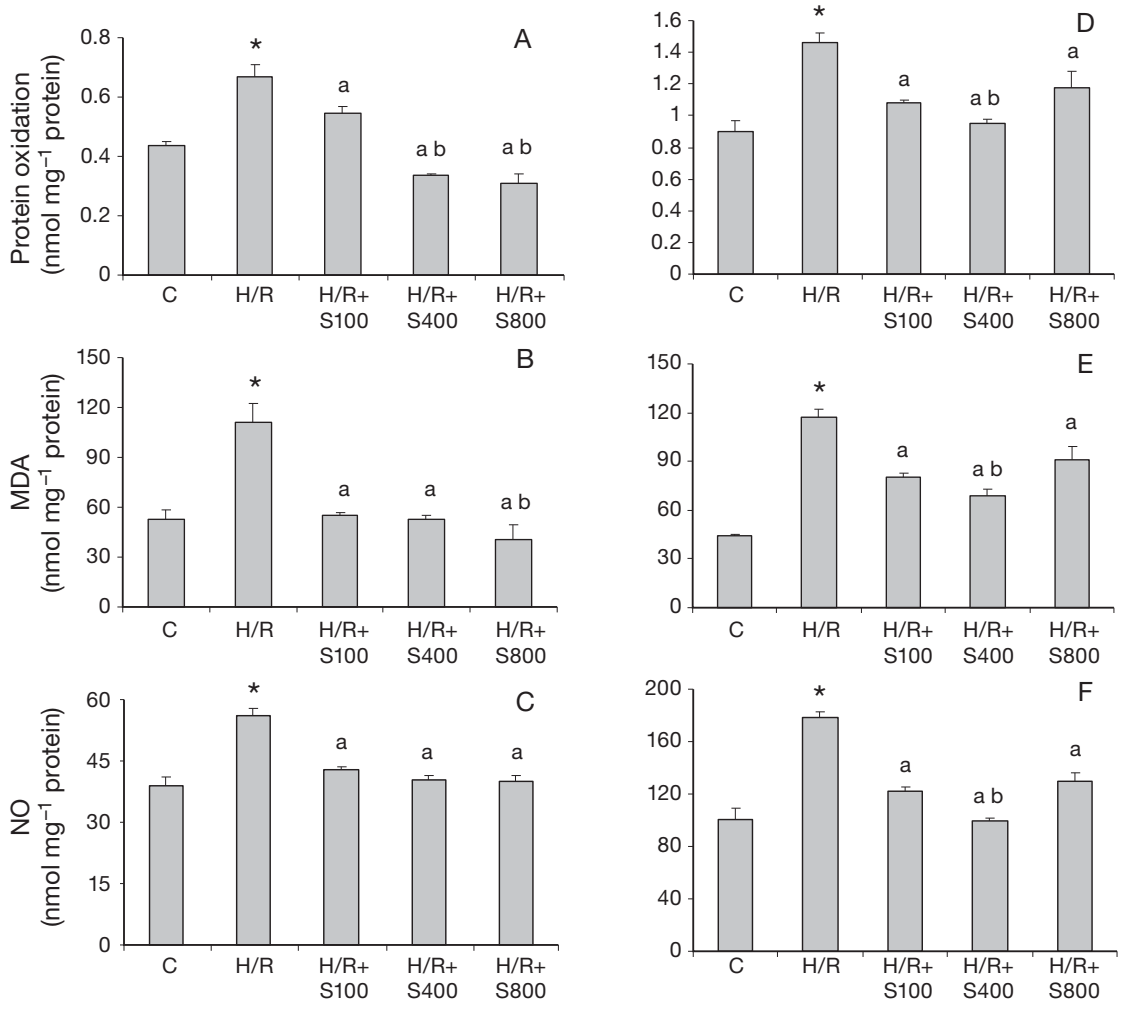

Fig. 1. *Oncorhynchus mykiss*. Diagrams showing the protective effect of silymarin (SMN) on hypoxia/reoxygenation (H/R)-induced oxidative impact in the brain (left) and gills (right) of trout, expressed as levels of protein oxidation, melondialdehyde (MDA) and nitric oxide (NO). H/R: trout exposed to 3 h hypoxia followed by 3 h reoxygenation; H/R+S100, +S400, +S800: animals pretreated with 100, 400, 800 mg kg^{-1} SMN before H/R, respectively. *Significant ($p < 0.01$) increase of all 3 oxidative stress biomarkers in the H/R group compared to the control group. Different lowercase letters represent significant differences among the SMN-treated groups themselves and the H/R group

increase of meningeal thickness and focal aggregation of glial cells (gliosis) in the cerebrum (Fig. 2B). In the same animals, the gills showed hyperplasia of epithelial and pillar cells in secondary lamellae and destruction of superficial cells (Fig. 3B). Interestingly, pre-treatment with SMN reduced the H/R-induced lesions in the brain in a concentration-dependent fashion, as at the highest given concentration, no pathological signs were observed (Fig. 2C,D,E). We found that SMN could strongly protect the gills from H/R-induced damages, but at the highest given concentration (800 mg kg^{-1} feed), severe pathological injuries, such as necrosis of lamellae, were demonstrated (Fig. 3C,D,E). The histopathological lesions are depicted as scored numerical data in Table 3.

SMN regulatory effect on HIF-1α and iNOS expression

The expression of HIF-1α and iNOS in the brain and gills at mRNA level was examined through densitometric analyses for both genes in the examined tissues after normalization to the corresponding GAPDH bands, and the results indicate that although HIF-1α was expressed in the brain of intact trout, iNOS expression was not detectable. Both genes were expressed in the gills of the control trout. Both genes were up-regulated following exposure to hypoxia and reoxygenation in the brain and gills (Fig. 4A,B). The pretreatment with SMN regulated the expression of HIF-1α in a dose-dependent man-

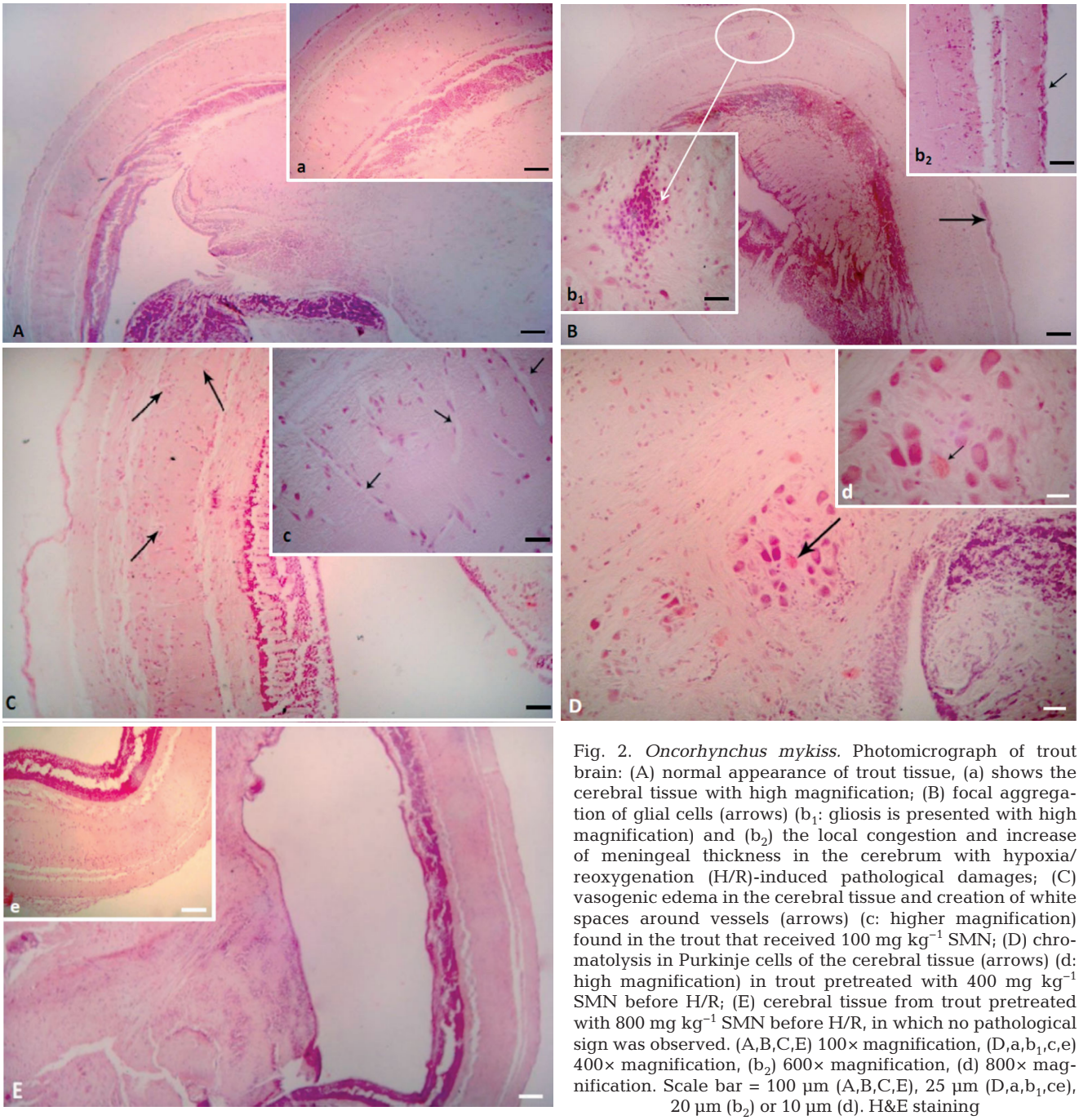

Fig. 2. *Oncorhynchus mykiss.* Photomicrograph of trout brain: (A) normal appearance of trout tissue, (a) shows the cerebral tissue with high magnification; (B) focal aggregation of glial cells (arrows) (b_1: gliosis is presented with high magnification) and (b_2) the local congestion and increase of meningeal thickness in the cerebrum with hypoxia/reoxygenation (H/R)-induced pathological damages; (C) vasogenic edema in the cerebral tissue and creation of white spaces around vessels (arrows) (c: higher magnification) found in the trout that received 100 mg kg^{-1} SMN; (D) chromatolysis in Purkinje cells of the cerebral tissue (arrows) (d: high magnification) in trout pretreated with 400 mg kg^{-1} SMN before H/R; (E) cerebral tissue from trout pretreated with 800 mg kg^{-1} SMN before H/R, in which no pathological sign was observed. (A,B,C,E) 100× magnification, (D,a,b_1,c,e) 400× magnification, (b_2) 600× magnification, (d) 800× magnification. Scale bar = 100 µm (A,B,C,E), 25 µm (D,a,b_1,ce), 20 µm (b_2) or 10 µm (d). H&E staining

ner in the brain but not in the gills. SMN at 800 mg kg^{-1} did not regulate the H/R-induced expression of HIF-1α in the gills. The iNOS expression in the brain of all SMN-pretreated groups was completely silenced, while in the gills, the H/R-induced iNOS expression was down-regulated at the studied doses. The down-regulation of iNOS in the SMN-pretreated groups was not dose-dependent (Fig. 4C,D).

DISCUSSION

The present study showed that pretreatment with SMN was able to reduce the H/R-induced oxidative/nitrosative stress in the brain and gills of trout. The up-regulated mRNA levels of HIF-1α and iNOS were also a result of the SMN pretreatment.

Experimentally produced or environmentally occurring hypoxia may cause various damage to differ-

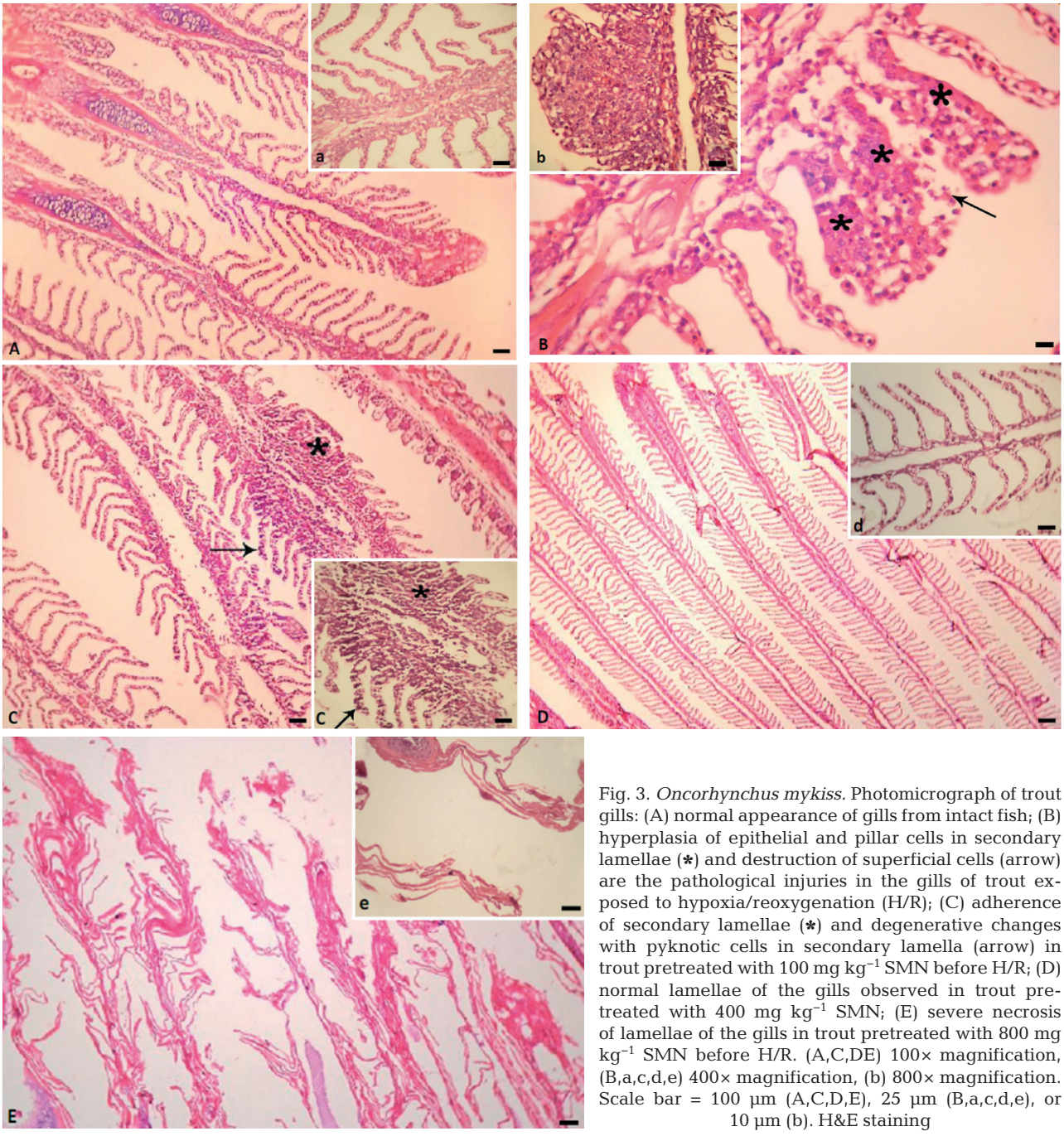

Fig. 3. *Oncorhynchus mykiss.* Photomicrograph of trout gills: (A) normal appearance of gills from intact fish; (B) hyperplasia of epithelial and pillar cells in secondary lamellae (*) and destruction of superficial cells (arrow) are the pathological injuries in the gills of trout exposed to hypoxia/reoxygenation (H/R); (C) adherence of secondary lamellae (*) and degenerative changes with pyknotic cells in secondary lamella (arrow) in trout pretreated with 100 mg kg^{-1} SMN before H/R; (D) normal lamellae of the gills observed in trout pretreated with 400 mg kg^{-1} SMN; (E) severe necrosis of lamellae of the gills in trout pretreated with 800 mg kg^{-1} SMN before H/R. (A,C,DE) 100× magnification, (B,a,c,d,e) 400× magnification, (b) 800× magnification. Scale bar = 100 µm (A,C,D,E), 25 µm (B,a,c,d,e), or 10 µm (b). H&E staining

ent organs. As a prerequisite of the present study, we established the tolerance level of hypoxia for fingerling trout at 5 ppm oxygen for 3 h (data not shown). This finding is in accordance with previous studies which also reported 5 ppm as the lowest level of oxygen that trout can tolerate (Piper et al. 1982).

To show the H/R-induced impact and to evaluate the potential protective effect of SMN, 2 tissues were selected, the brain and gills, because both tissues are the most sensitive and vulnerable organs to oxygen changes and oxidative stress-induced damages (Zhang et al. 2009). Also, it has been shown that the responses of fish to hypoxia are initiated by chemoreceptors, which are located in the first arch of the gills (Coolidge et al. 2008).

Generally, cultured trout are introduced to freshly prepared water with a high level of oxygen. There are increasing data indicating that reoxygenation itself may cause severe cellular and molecular injuries. As it is the excessive production of reactive

Table 3. *Oncorhynchus mykiss.* Pathological findings (mean ± SE) in the brain and gills of trout. Values in the same column with different superscripts differ significantly ($p < 0.05$). H/R: trout exposed to 3 h hypoxia followed by 3 h reoxygenation; H/R+S100, +S400, +S800: animals pretreated with 100, 400, 800 mg kg^{-1} SMN before H/R, respectively

Groups	Brain		
	Vasogenic edema	Chromatolysis	Increase of meningeal thickness
Control	0.0[a]	0.7 ± 0.6[a]	0.0[a]
H/R	7.0 ± 1.3[b]	5.0 ± 0.7[b]	8.0 ± 1.6[b]
H/R+S100	5.2 ± 1.2[b]	4.0 ± 1.3[b]	3.6 ± 0.3[c]
H/R+S400	4.0 ±1.5[c]	4.0 ± 0.3[b]	3.0 ± 0.4[b]
H/R+S800	0.0[d]	0.0[c]	0.0[c]
	Gills		
	Hyperplasia in epithelial cells	Adherence of secondary lamellae	Pyknotic cells in secondary lamellae
Control	0.0[a]	0.0 [a]	0.0[a]
H/R	8.0 ± 1.7[b]	7.0 ± 1.2[b]	6.0 ± 1.8[b]
H/R+S100	5.0 ± 0.9[c]	7.0 ± 1.3[b]	4.0 ± 1.3[b]
H/R+S400	1.0 ± 0.3[d]	1.0 ± 0.6[c]	0.0[c]
H/R+S800	5.0 ± 1.2[c]	8.0 ± 1.8[b]	6.0 ± 0.9[b]

oxygen species (ROS) and reactive nitrogen species (RNS) that play a key role in H/R-induced damages (Chuanyu & Jackson 2002), it was necessary to identify any changes in the antioxidant status of trout that were exposed to H/R. Indeed, our results for the H/R group showed a significant increase in ROS and RNS production and consequently ROS/RNS-induced damages, which were characterized by lipid and protein peroxidation or by a reduction in TAC. The reduction in TAC was later confirmed by a remarkable increase in the NO content of brain and gills. These findings confirmed previous reports that H/R results in oxidative stress and demonstrated that in H/R-induced injuries, NO production may also play a key role in lipid and protein oxidation in trout.

There are reports indicating that NO formation from nitrite is elevated in mammals during hypoxia (Lundgreen et al. 2008). Once the NO production due to hypoxia is elevated and the hypoxia is followed by reoxygenation, the extra NO will rapidly react with O_2 and yield the potent oxidant peroxynitrite anion ($ONOO^-$), which can ultimately act as an OH free radical (Beckman et al. 1990). Therefore, the elevated NO content in the examined tissues of the trout exposed to H/R may contribute to the reduction in TAC and the marked enhancement of lipid peroxidation and protein oxidation. In a rodent model, it has been shown that a high concentration of NO inhibits the cellular respiratory chain complexes, probably by nitrosylation and oxidation of protein thiol (Brown 1999).

The second part of the present study was performed to highlight any changes in the mRNA levels of the HIF-1α and iNOS genes in the H/R-exposed trout. Although the expression of the 2 genes varied between the studied organs in normoxic trout, the exposure of fingerling trout to 3 h of hypoxia and 3 h of reoxygenation resulted in remarkable up-regulation of both genes in both organs. It has been shown in previous studies that the main transcriptional regulator of hypoxia is HIF-1α, and in normoxic situations, an oxygen-dependent hydroxylation of proline residues stimulates protein degradation and ultimately inhibits the accumulation of HIF-1α (Ivan et al. 2001). Our findings support the previous *in vitro* studies as the HIF-1α protein accumulated in trout hepatocytes that were exposed to oxygen stress, and the highest amount of protein was measured 2 h after hypoxia. The accumulation of HIF-1α protein was explained by up-regulation of oxygen-sensitive prolyl hydroxylases during hypoxia (Stroka et al. 2001, Aprelikova et al. 2004, Rissanen et al. 2006).

The present study showed that in addition to HIF-1α, iNOS was also up-regulated in the examined tissues under the H/R conditions. There are reports indicating that iNOS is another oxygen-dependent gene that is regulated by the HIF-1α transcription factor during hypoxia; however, in the present study, we did not study the iNOS regulation through HIF-1α in the H/R exposed trout. It seems there should be a strong relationship between the H/R-induced up-regulation of HIF-1α and the iNOS expression, which was mediated by the elevation of NO production in the examined organs. In agreement with our hypothesized pathway in the H/R-exposed trout, there are supporting reports indicating biphasic relationships among hypoxia, NO concentration and HIF-1α protein accumulation. In cell culture models, it has been shown that in the early phase of hypoxia (the first 2 h), the level of NO concentration increases and leads to inhibition of prolyl hydroxylases and HIF-1α accumulation. In the late phase (after 2 h), however, as the levels of newly synthesized prolyl hydroxylases increase, an antagonistic effect of hypoxia and NO on HIF-1α stabilization occurs (Berchner-Pfannschmidt et al. 2007).

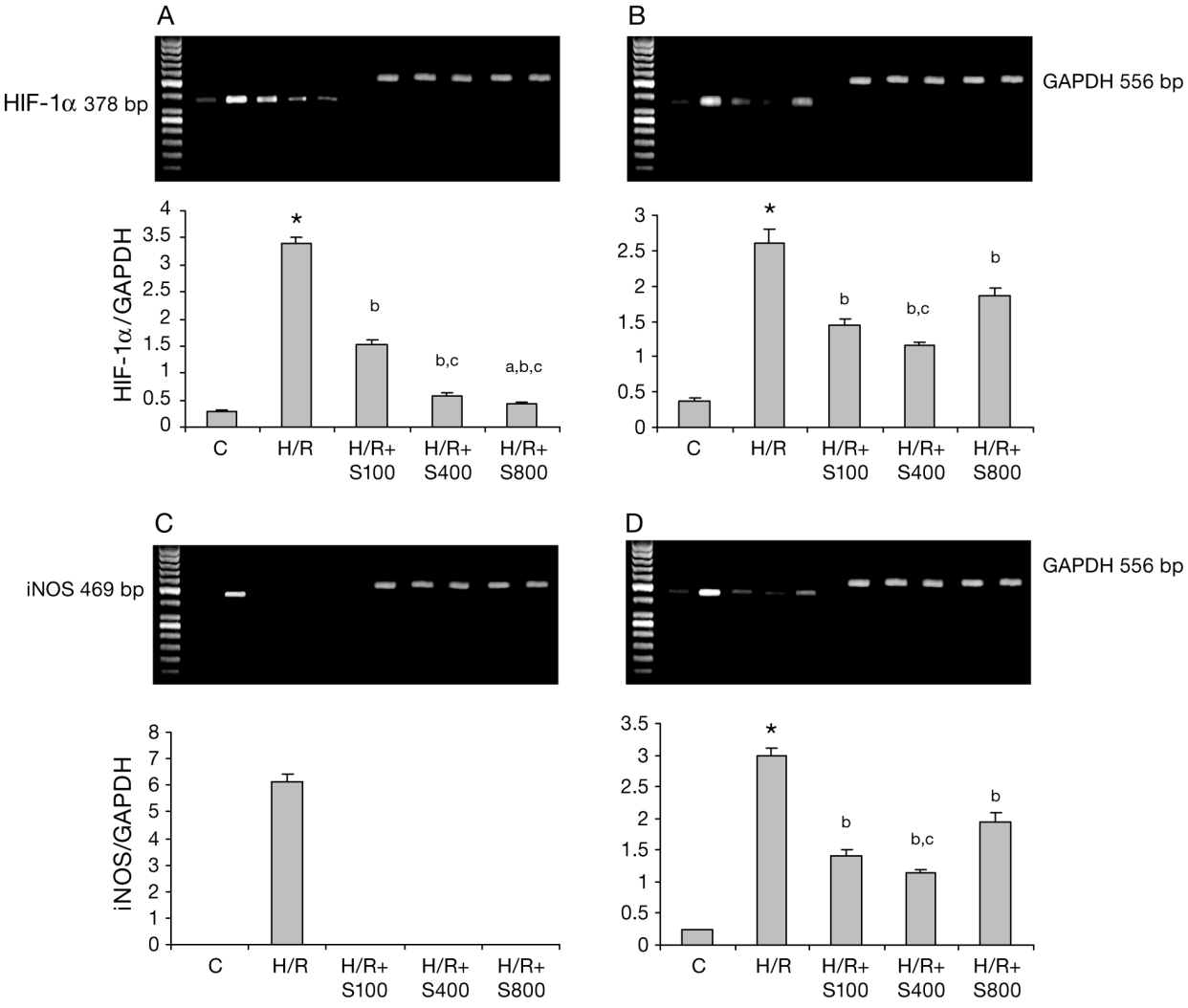

Fig. 4. *Oncorhynchus mykiss.* Effect of SMN on (A) HIF-1α expression in the brain, (B) HIF-1α expression in the gills, (C) iNOS expression in the brain and (D) iNOS expression in the gills; upper panels: expressed level of both genes along with the corresponding GAPDH gene in the brain and gills on an agarose gel; lower panel: mRNA levels of either gene normalized to the GAPDH mRNA expression level from corresponding animals. Results were expressed as integrated density values (IDV) of HIF-1α and iNOS mRNA levels. C: control; H/R: trout exposed to 3 h hypoxia followed by 3 h reoxygenation; H/R+S100, +S400, +S800: trout pretreated with 100, 400, 800 mg kg^{-1} SMN before H/R, respectively. *Significant ($p < 0.01$) difference between the control and H/R groups. Different lowercase letters represent significant differences between the H/R and SMN-treated groups and among the SMN-treated groups themselves

Our data showed a clear up-regulation of iNOS in the examined organs after the H/R period. As in hypoxic situations, both genes were up-regulated; therefore, it may be concluded that another important factor in hypoxia and the HIF-1α and NO concentration complex should be iNOS. In H/R-exposed trout, following an up-regulation of HIF-1α and iNOS, the iNOS-derived NO level is elevated and in turn leads to sustained HIF-1α levels. The key role of iNOS-derived NO levels in the regulation of HIF-1α has been reported in microvascular endothelial cells (Natarajan et al. 2005). Moreover, up-regulation of iNOS after 30 min of hypoxia followed by 2 h of reoxygenation in rat lungs has also been demonstrated (Rus et al. 2010).

Another interesting finding of the present study is that 3 d of pretreatment with SMN before the H/R treatment resulted in a remarkable protective effect both on H/R-induced biochemical changes and on the expression of HIF-1α and iNOS in the examined tissues.

Antioxidant effects of SMN in various forms, such as reducing the lipid peroxidation in the brain of rats (Galhardi et al. 2009), reversing the sepsis-decreased

tissue glutathione and total antioxidant capacity in the lungs and brain of rats (Toklu et al. 2008), enhancing the acetaminophen-reduced superoxide dismutase activity and ascorbic acid level in the brain of rats (Nencini et al. 2007) and lowering the lipopolysaccharide-induced nitrite in mesencephalic mixed neuron-glia cultures, have been shown (Wang et al. 2002). However, there is scarce data to show the antioxidant effect of SMN in aquatic animals. Therefore, our findings extended the knowledge of the antioxidant properties of SMN in trout for protection from H/R-induced oxidative damages. Previous studies reported that SMN treatments resulted in a significant suppression of iNOS expression in the liver, kidney and heart of pyridine-exposed Syrian hamsters (Tunca et al. 2009). Therefore, SMN acts both transcriptionally and post-transcriptionally, as we showed in the present study. The antioxidant effect of SMN is mainly attributable to its antiradical and ROS scavenging capabilities (Kiruthiga et al. 2007, Shaker et al. 2010). Moreover, other mechanisms, such as increasing the pro-oxidant-reduced mitochondrial membrane potential and inhibition of the sepsis-elevated cytokines (IL-1β and PGE_2) production, can also explain the SMN-induced antioxidant effects (Kang et al. 2004).

Although the protective effects of SMN on the brain were dose dependent, the TAC and all measured oxidative/nitrosative markers in the gills at the highest given dose of SMN did not follow the dose-dependency. The pro-oxidant effect of flavonoids at high concentrations, as reported in the present study, may be related to auto-oxidation and increased ROS generation. The cytotoxic effect of baicalein as a known antioxidant flavonoid at higher doses on cardiomyocytes has been demonstrated (Woo et al. 2005). The obtained pro-oxidant properties of SMN at the highest given dose, which were characterized by a significant increase of NO and MDA levels in the gills of trout, may be explained by various dose-dependent effects of SMN. SMN exerted various effects in different organs at the highest given dose levels. Although there are no available data regarding the pharmacokinetics of SMN in trout, these different effects may be associated with differing amounts of distributed SMN into specific organs. Pharmacokinetic analyses in mice indicated different amounts of recovered silibinin, an active compound of SMN, following oral administration in different organs of mice, supporting our hypothesis of uneven distribution (Zhao & Agarwal 1999).

Our histopathological findings in the brain support the biochemical and molecular findings. The cerebral edema observed in the H/R group may be related to vascular injuries and released vasoactive metabolites, such as arachidonic acid and lactic acids (Grace 1994). Thus, in aquatic animals, including trout, both hypoxia and reoxygenation may result in cerebral injuries, as are reported in the current study. A possible explanation for the increase of free radicals and NO content of the H/R-treated brain may be a massive influx of Ca^{2+} and activation of NO synthesis, which may occur after H/R-induced damage to cell membranes (Gilgun-Sherki et al. 2002). Interestingly, the SMN pretreatment resulted in the same kind of protective effects that we observed in the biochemical and molecular analyses, and the appropriate pretreatment concentration for the H/R-induced damages in the gills is lower (400 mg kg^{-1} feed) than that needed for the brain injuries (800 mg kg^{-1} feed). The reason for this diversity may relate to the concentrations of SMN that reach the proposed tissue. Because in principle SMN does not dissolve in water, its major absorption via the gill surfaces cannot be the reason for the obtained pathological damages in the gills. Our careful daily surveillance showed no reduction in food intake, suggesting that even at high doses of SMN, the fish feed did not become unpalatable. Therefore, the high concentration of SMN should be provided to the gills mainly via systemic blood circulation, which may consequently result in a pro-oxidant effect.

In conclusion, we report for the first time the protective effects of SMN on H/R-induced biochemical, histopathological and transcriptional alterations in trout. The protective effect of SMN may be explained by its antioxidant properties, which reflect its capability to reduce the H/R-elevated NO concentration, MDA formation and protein oxidation. SMN might regulate HIF-1α expression through iNOS-derived NO concentrations. These findings could be applicable in future pharmacokinetics studies and may help to reduce H/R-induced damages in trout.

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