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## Response to hyperoxia is associated with similar ho-1 gene expression level in lungs of aging CBA mice of both sexes



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### ARTICLE INFO

#### Article history:

Received 8 April 2015

Received in revised form

31 August 2015

Accepted 6 September 2015

Available online 10 September 2015

#### Keywords:

Heme oxygenase

Antioxidant enzyme

Mouse

Sex-related

Oxygen

### ABSTRACT

Beside classical antioxidative enzymes, the response to hyperoxia might be mediated via regulation of other systems, such as heme oxygenase (HO). Ho-1 gene expression is found to be upregulated by hyperoxia in all groups of mice, while HO-1 protein isoform was increased only in 4 months old male mice. In steady-state conditions ho-1 and ho-2 gene expression remained unchanged irrespective of sex or age, which was not the case with protein level of both isoforms. This study suggests that in lungs of CBA mice the response to oxidative stress may be mediated through the interaction of other systems such as heme oxygenase, primarily via upregulation of ho-1 gene expression in both sexes. Contrary to our previous study in liver of hyperoxia treated mice, current results might imply that at conventional oxygen conditions lungs of female mice with the emphasis on aging females, are better prepared for oxidative stress conditions through the increase of HO-activity.

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### 1. Introduction

One of the hallmarks of aging is reduced capacity of cellular homeostatic mechanisms that protect the body against variety of oxidative, toxicological and pathological insults [1]. Sohal [2] hypothesized that rates of prooxidant species generation are closely associated with the rate of aging, while Ehrenbrink et al. [3] postulated that alteration in activities of antioxidant enzymes can be sex-specific and related to longevity. Supraphysiological concentrations of oxygen are routinely used to treat several conditions like hypoxemia, acute respiratory failure and acute carbon monoxide poisoning [4]. However, the prolonged administration of oxygen concentration greater than 60% may result in tissue damage in many organs including lungs and lead to both acute and chronic lung injury [5]. Data from numerous animal models both in vivo and in vitro have shown the age-related phenomenon of prolonged hyperoxic tolerance of the newborn compared to the adult animals [6,7]. ROS hyperproduction induces the enzymes involved in direct response to ROS, i.e. catalase or glutathione peroxidase, and the phase II detoxifying enzymes such as heme

oxygenase-1 and thioredoxin generating enzymes. Heme oxygenase (HO) is the rate-limiting enzyme for heme degradation in mammals. To date, two primary isoforms of HO have been identified in human and rodent: the inducible HO-1 and the constitutive HO-2 [8]. HO-1 is a stress response enzyme which is highly induced by variety of agents causing oxidative stress, hypoxia, hyperoxia, proinflammatory cytokines [9] and thus regarded as sensitive and reliable indicator of cellular oxidative stress. In response to oxidative stress, HO-1 induction provides cell protection by promoting the catabolism of pro-oxidant metalloporphyrins to bile pigments (biliverdin and bilirubin) which are considered to have free radical scavenging capabilities [10]. As such, HO acts as a potent antioxidant. In comparison to the constitutively expressed HO-2, it is presumed that HO-1, because of its inducible nature, makes greater contribution to the maintenance of oxidant:antioxidant homeostasis during changes in cellular environments. Lungs have predominance of HO-2, since precipitation with HO-1 antibody reduces HO to 75% of its total activity [11]. In this study we wanted to investigate whether the sex- and age-related differences in response to oxidative stress observed in our previous studies in liver [12] are also present in lungs of hyperoxia treated aging mice of both sexes at the level of ho-1 and ho-2 gene expression, HO-1 and HO-2 protein level and HO activity, respectively.

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## 2. Methods

### 2.1. Animals and experimental design

Male and female CBA/Hr mice aged 1, 4 and 12 months from breeding colony of the Ruder Bošković Institute (Zagreb, Croatia) were used in this study. The animals were maintained under the following laboratory conditions: four to a cage; light on from 06:00 to 18:00;  $22 \pm 2$  °C room temperature; access to food pellets and tap water ad lib and divided in two groups of twelve animals each. Control group received room air at a regular atmospheric pressure of 1 ATA and the other group was subjected to normobaric oxygen (> 95% O<sub>2</sub> for 18 h) in a hyperbaric chamber (Đuro Đaković, Slavonski Brod, Croatia). Both groups (control and experimental) were immediately euthanized after hyperoxia treatment (9 a.m.) at room air at a regular atmospheric pressure of 1 ATA. The experiments were performed in accordance with the current laws of the Republic of Croatia and with the guidelines of European Community Council Directive of November 24, 1986 (86/609/EEC).

### 2.2. Microsomal preparation

Microsomal fractions were prepared as described by Shertzer et al. [13]. Subcellular fractionation was achieved by differential centrifugation and the resulting microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH=7.4), quickly frozen in liquid nitrogen, and stored at  $-80$  °C.

### 2.3. Lipid peroxidation assay

The lipid peroxidation assay (LPO) assay was carried out on lung microsomes from control and mice treated with 100% oxygen using LPO assay kit (Bioxytech<sup>®</sup> LPO-586<sup>™</sup>, OXIS International, Inc. Foster City, CA, USA) according to the protocol. In this study the MDA alone was measured [14].

### 2.4. Assay for antioxidant enzyme activities

Glutathione peroxidase (GPx) activity was assayed spectrophotometrically at 340 nm based on the method of Paglia and Valentine [15]. Catalase (CAT) activity was determined spectrophotometrically at 240 nm and expressed as U/mg Hb [16].

### 2.5. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from pool of four individual mouse lungs in each group using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed according to the manufacturer's instructions. Real-time PCR analysis was carried out on the ABI 7300 sequence detection system using the comparative CT ( $\Delta\Delta\text{CT}$ ) method described in Taqman<sup>®</sup> Gene Expression Assays Protocol (Applied Biosystems, Foster City, CA) to quantify relative P450 mRNA expression of Ho-1 and Ho-2 in lungs of CBA mice. Primer length and Assay ID used for the analysis are shown in Table 1. Data are presented as the fold-change in gene expression normalized to endogenous reference gene (Gapdh) and relative to the untreated control. Relative gene expression of each gene was calculated by using the Relative Expression Software Tool (REST<sup>®</sup>) to calculate result significance [17].

### 2.6. Heme oxygenase activity

Heme oxygenase activity was assessed measuring bilirubin formation after chloroform extraction [18]. Briefly, lung supernatant (400  $\mu\text{l}$ ) was added to a reaction mixture in the 1 ml final

**Table 1**

Primers used for quantitative real-time PCR analysis.

Gene	Assay ID	Product size (bp)
ho-1	Mm00516007_m1	92
ho-2	Mm00468922_m1	62
gapdh	Mm99999915_g1	107

reaction volume. The reaction was incubated for 1 h at 37 °C in the dark and terminated by the addition of 1 mL chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm with use of a quartz cuvette (extinction coefficient, 40 mM<sup>-1</sup> cm<sup>-1</sup> for bilirubin).

### 2.7. Determination of protein concentration

Protein concentration in the tissue samples (mg/g) was estimated by the method as described in previous report [19] using bovine serum albumin as standard.

### 2.8. Western blot analysis

Eighty micrograms of post-mitochondrial supernatant from each sample (three pools, each of four mouse lungs) were subjected to 12.5% SDS-PAGE according to the method previously described [20]. Western blotting was performed using the primary polyclonal rabbit anti-mouse HO-1 or HO-2 antibody (Abcam, Cambridge, UK), following donkey anti-rabbit IgG, horseradish peroxidase-conjugated, secondary antibody (Amersham Biosciences Inc., USA). Anti-ERK-2 (C-14, Santa Cruz Biotechnology, Inc., USA), was used as a loading control [12]. The chemiluminescence signals were detected with the Alliance 4.7 Imaging System (UVITEC, Cambridge, UK).

### 2.9. Statistical analysis

Statistical analyses of data were performed using R v2.15.3 (CRAN, <http://cran.r-project.org>) and RStudio for Windows, v0.97 (<http://www.rstudio.com/>). Before all analyses samples were tested for normality of distribution using Shapiro–Wilk test. Since the samples followed normal distributions, parametric tests were performed: one-way ANOVA, followed by Tukey's post-hoc tests for multiple comparisons of the groups. For the determination of significance between two groups, Student's two tailed *t*-test was performed. For all tests significance level was set at  $p < 0.05$ .

## 3. Results

### 3.1. The effect of hyperoxia on the level of MDA, Gpx and CAT activity

As measured by the level of MDA and antioxidant enzymes Gpx and CAT activity, no signs of oxidative stress in the lungs of hyperoxia treated animals were observed in males or in females of any age, compared to their corresponding control group, calculated by Student's two tailed *t*-test (Table 2.). The same was demonstrated on both mRNA and protein level (data not shown).

### 3.2. HO-1 and HO-2 pattern in lungs of CBA/H mice

As revealed with one-way ANOVA and Tukey's post-hoc test, aging did not affect steady-state gene expression of Ho-1 or Ho-2 in lungs neither in male nor in female mice ( $F_{2,7} = 1.320$ ,  $p = 0.334$ ) (Fig. 1). On the contrary, protein level of HO-1 and HO-2 in lungs of aging mice showed dependence upon age (for HO-1 one-way

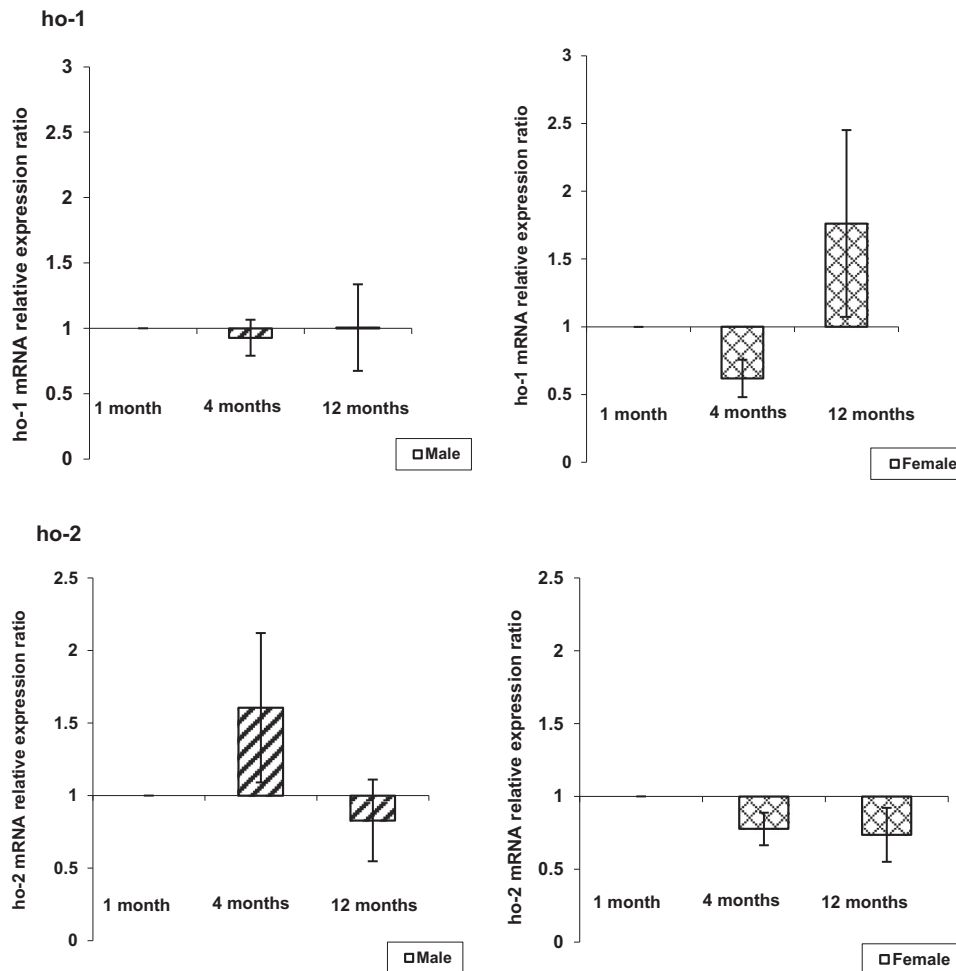
**Table 2**

The effect of hyperoxia on oxidative/antioxidative parameters in lungs of ageing male and female mice. For comparison between each treatment and control group, Student's two tailed *t*-test was used. Data are presented as mean  $\pm$  SD from 3 pools of mice (each pool containing lungs of 4 mice) per each sex and each treatment.

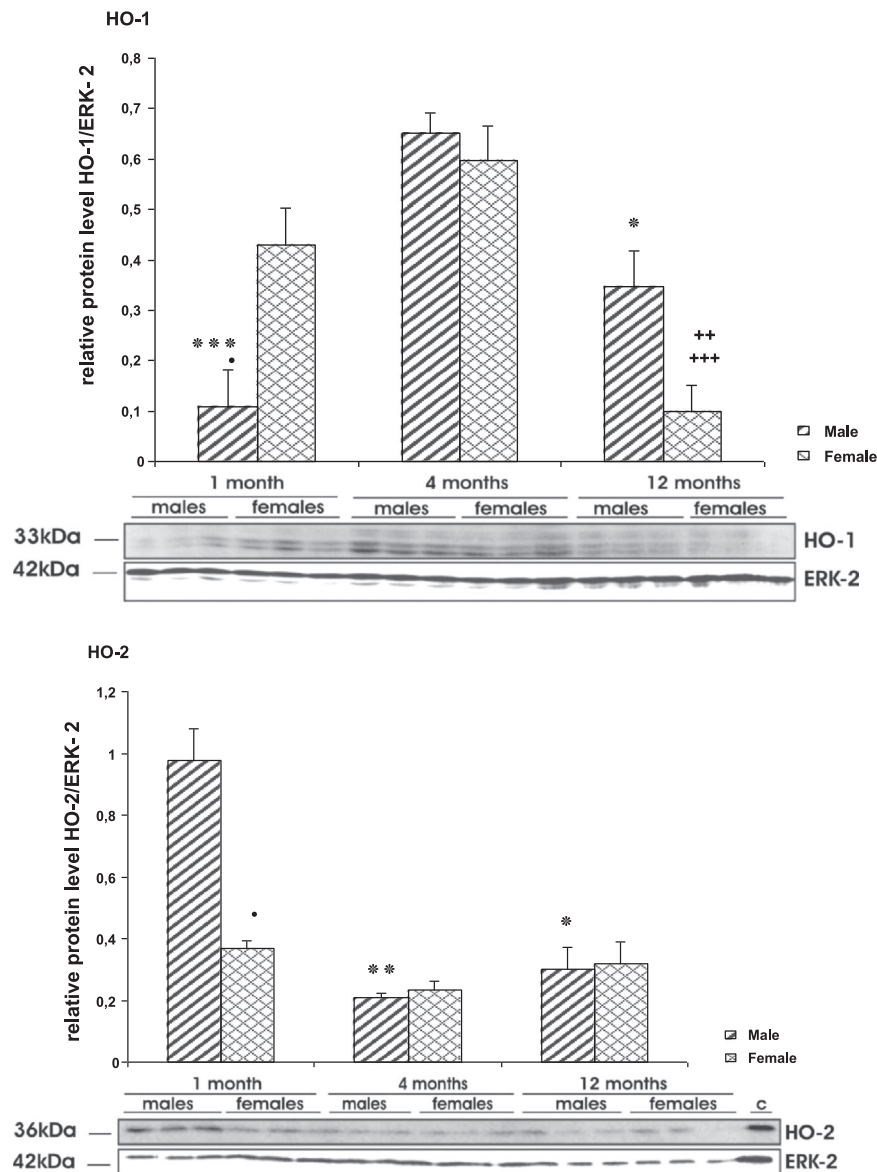
	1 Month		4 Months		12 Months	
	Control	Hyperoxia	Control	Hyperoxia	Control	Hyperoxia
<b>Male</b>						
MDA (nmol/mg protein)	5.77 $\pm$ 1.09	2.61 $\pm$ 0.41	2.66 $\pm$ 0.17	2.87 $\pm$ 0.11	3.99 $\pm$ 0.32	3.67 $\pm$ 0.16
GPX (U/mg protein)	1.10 $\pm$ 0.11	0.77 $\pm$ 0.06	2.77 $\pm$ 0.03	2.90 $\pm$ 0.17	1.95 $\pm$ 0.06	1.81 $\pm$ 0.06
CAT (U/mg protein)	12.1 $\pm$ 1.15	9.97 $\pm$ 1.04	16.2 $\pm$ 3.30	16.7 $\pm$ 3.14	17.5 $\pm$ 2.26	18.1 $\pm$ 2.93
<b>Female</b>						
MDA (nmol/mg protein)	4.78 $\pm$ 0.52	3.47 $\pm$ 0.19	3.96 $\pm$ 0.80	3.45 $\pm$ 0.34	3.86 $\pm$ 0.41	4.00 $\pm$ 0.11
GPX (U/mg protein)	1.31 $\pm$ 0.08	1.14 $\pm$ 0.10	3.25 $\pm$ 0.36	3.40 $\pm$ 0.04	2.50 $\pm$ 0.13	1.96 $\pm$ 0.07
CAT (U/mg protein)	13.6 $\pm$ 4.05	10.5 $\pm$ 0.99	17.9 $\pm$ 0.89	18.2 $\pm$ 1.90	16.0 $\pm$ 0.64	19.5 $\pm$ 3.30

ANOVA  $F_{5,17}=13.441$ ,  $p < 0.001$ ; for HO-2 one-way ANOVA  $F_{5,16}=19.954$ ,  $p < 0.001$ ) and sex (for comparisons between males and females for each age group Student's two tailed *t*-test was performed) (Fig. 2). In males, HO-1 protein level was significantly higher in 4 months old than in 1 and 12 months old mice ( $p=0.001$  and  $p < 0.05$ , respectively). The level of HO-1 protein in female mice did not differ in 1 and 4 months old mice, but declined in 12 months compared to 4 months old ( $p=0.002$ ) and 1 month old mice ( $p=0.031$ ). The difference in HO-1 protein level between male and female mice was observed only in lungs of

1 month old mice ( $p=0.037$ ). Aging had no influence on HO-2 protein level in lungs of female mice. Contrary to this, HO-2 protein level in male mice was significantly higher in 1 month old compared to 4 ( $p=0.011$ ) and 12 months old mice ( $p=0.027$ ). Only at 1 month of age, HO-2 protein level was different in lungs of both sexes ( $p < 0.05$ ). As revealed with one-way ANOVA with Tukey's post-hoc test ( $F_{2,6}=4.256$ ,  $p < 0.05$ ) and demonstrated on Fig. 3. HO activity was significantly higher at 4 months compared to 1 month of control male mice ( $p=0.032$ ). In females we observed significant increase in HO activity with age ( $F_{2,6}=6.824$ ,  $p < 0.05$ )



**Fig. 1.** Real-time PCR analysis of ho-1 and ho-2 mRNA expression in lungs of 1, 4 and 12 months old control male and female mice. Data are mean  $\pm$  SEM of 3 pools of 4 mice per each sex and each treatment. The relative fold change compared to 1 month (defined as 1) was calculated using the  $2^{-\Delta\Delta CT}$  method as described in Materials and methods.



**Fig. 2.** Western blot analysis of HO-1 and HO-2 protein level in lungs of 1, 4 and 12 months old control male and female mice. Data are mean  $\pm$  SEM of 3 pools of 4 mice per each sex and each treatment. Protein intensities are expressed relative to ERK-2 content. C-positive control for HO-2 protein. For males HO-1: \*\*\*1 month old vs 4 months old,  $p=0.001$ ; \*12 months old vs 1 month old,  $p<0.05$ . For females HO-1: +++12 months old vs 4 months old,  $p=0.002$ ; +++12 months old vs 1 month old,  $p=0.031$ ; •1 month old males vs 1 month old females,  $p=0.037$ ; For males HO-2: \*\*4 months old vs 1 month old,  $p=0.011$ ; \*12 months old vs 1 month old,  $p=0.027$ ; \*1 month old females vs 1 month old males,  $p<0.05$ .

HO activity remained unchanged between 1 and 4 months of age, but was upregulated at 12 months of age in comparison to 1 months old mice ( $p=0.011$ ). At 4 months of age lungs of control male mice had higher HO activity than lungs of control female mice (Student's two tailed  $t$ -test:  $p<0.05$ ).

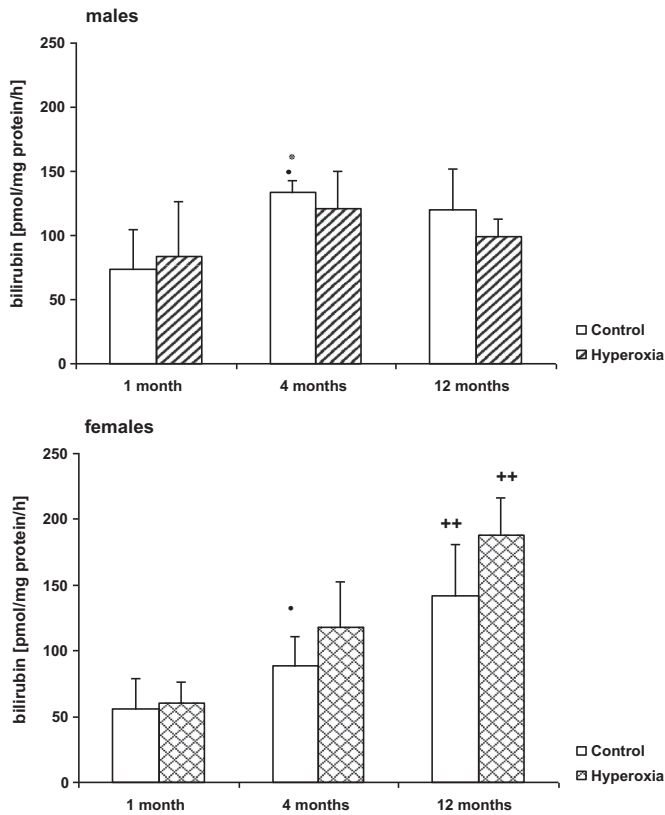
### 3.3. HO-1 and HO-2 status in lungs of hyperoxia treated CBA/H mice

Hyperoxia upregulated Ho-1 gene expression in all groups of animals examined, as revealed with Student's two-tailed  $t$ -test ( $p=0.001$  for each group compared to control). Ho-2 gene expression remained unchanged after treatment except in 12 months old males, where it showed significant downregulation, compared to 1 month old control group ( $p=0.001$ ) (Fig. 4). The results of relative HO-1 protein expression between control and hyperoxia in 1, 4 and 12 months old males and females are demonstrated on

Fig. 5. Hyperoxia significantly increased HO-1 protein level in 4 months old male mice, compared to control group ( $p=0.01$ ). HO-1 protein was absent from both control and hyperoxia-treated 12 months old mice of both sexes. The results of relative HO-2 protein expression between control and hyperoxia in 1, 4 and 12 months old males and females are demonstrated on Fig. 6. HO-2 protein was downregulated in hyperoxia-treated 12 months old female mice ( $p<0.05$ ). HO activity in lungs remained unchanged upon hyperoxia in any age group of males and females (Fig. 3).

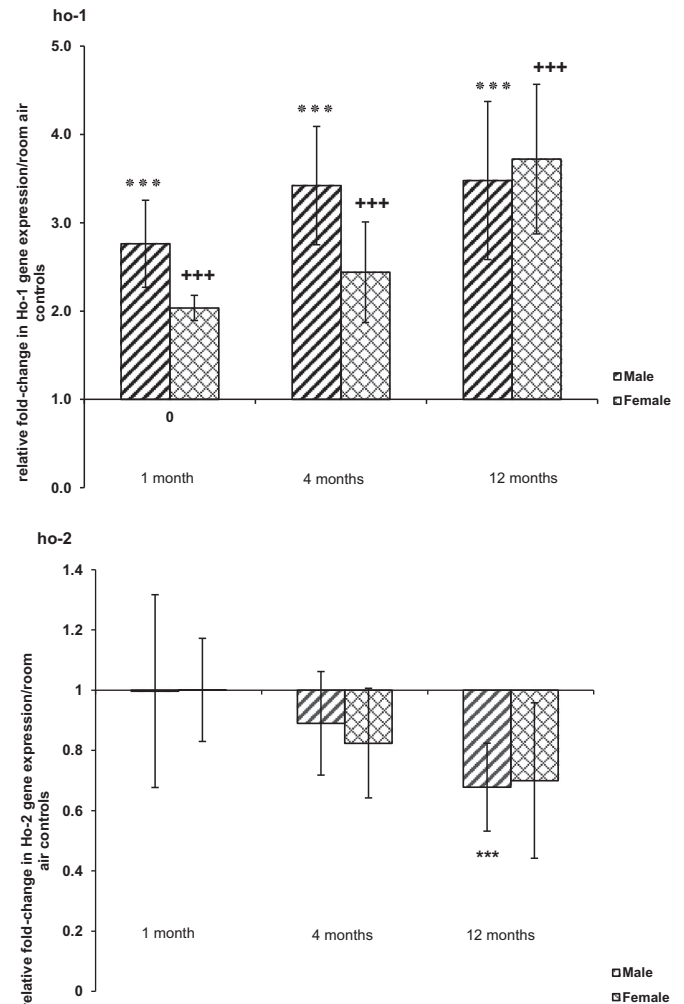
## 4. Discussion

Contrary to our previous study [12] where hyperoxia induced oxidative stress only in male liver while females were protected by



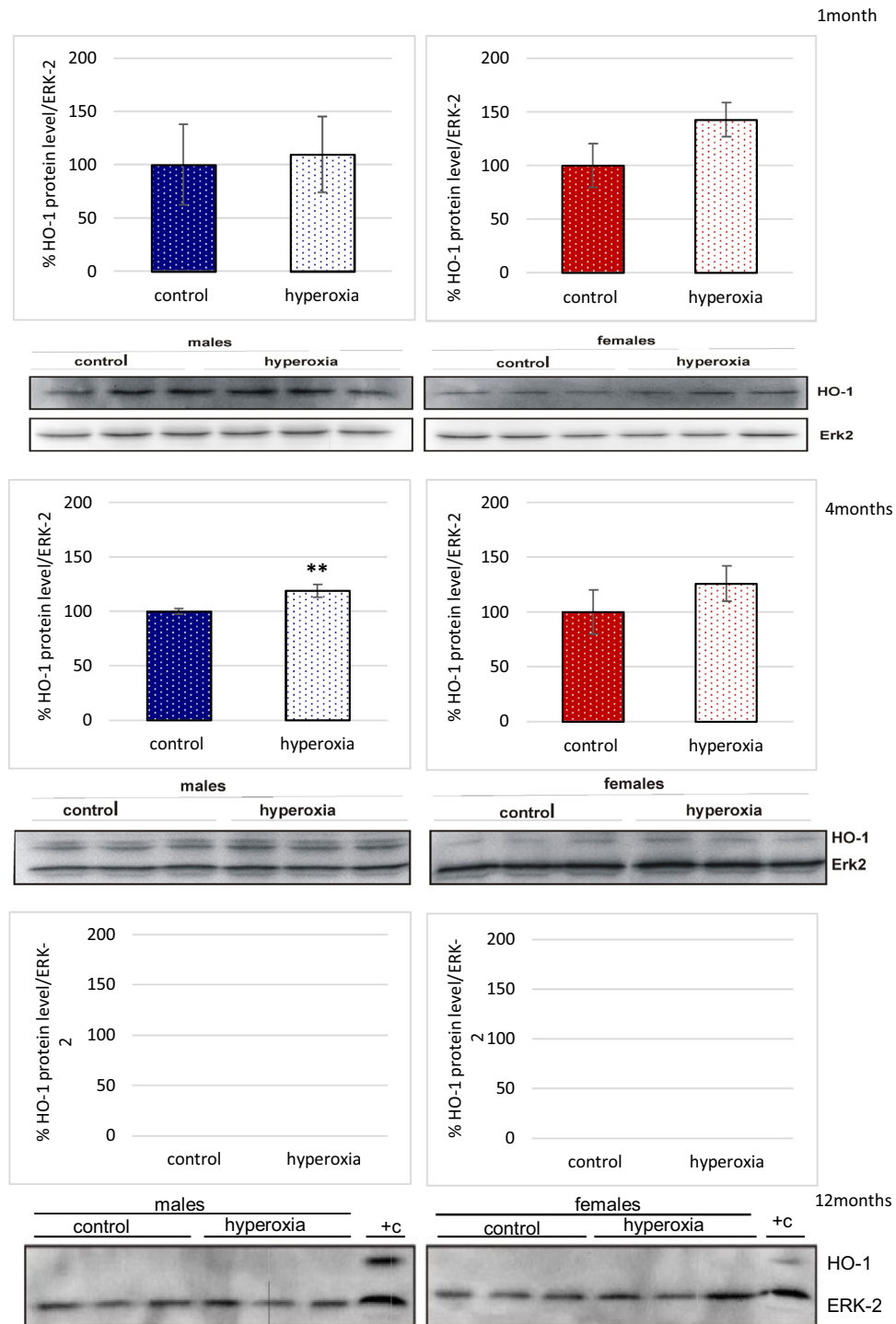
**Fig. 3.** Lung HO activity of 1, 4 and 12 months old control and hyperoxia-treated mice of both sexes. HO activity was expressed as pmol/mg protein/hr of bilirubin. Data are mean  $\pm$  SEM of 3 pools of 4 mice per each sex and each treatment. For males: \*control 4 months old vs. control 1 month old,  $p=0.032$ ; For females: ++control 12 months old vs control 1 month old,  $p=0.011$ . For males vs. females: \*4 months old control males vs 4 months old control females.

overexpression of CYP4A14, in this study no change of enzymes involved in direct response to ROS such as SOD, CAT or Gpx were noticed in lungs of hyperoxia treated mice, irrespective of age or sex. However, antioxidant defences that counteract free radical excess besides the enzymes involved in direct response to ROS, i.e. superoxide dismutase, catalase or glutathione peroxidase include phase II detoxifying enzymes such as heme oxygenase-1, glutathione and thioredoxin generating enzymes. While data about enzymes involved in direct response to ROS are abundant, data on HO enzymes, specially concerning gender are still insufficient. The results of our study have shown that in control animals gene expression of ho-1 and ho-2 is neither sex- nor age-related. Our results of basal levels of ho-1 gene expression with age are comparable with those of Ito et al. [21], who noticed no significant difference in the expression of ho-1 with age in lungs of male mouse. Other authors, depending on the organs and species examined, have noticed age-related decrease in ho-1 expression in brain [22] or age-dependent increase in steady state of ho-1 in rat liver [23]. According to Dennery et al. [24] the overall lung heme oxygenase expression in both sexes was highest prenatally and lowest in adulthood, but in his study the oldest mice were 2 months of age. Our study is the first report demonstrating that basal levels of ho-1 gene expression in mouse lungs are unaffected by age and sex as opposed to protein levels of both isoforms, which appeared to be dependent upon both variables. HO-1 protein level has a trend to be at its maximum at 4 months in both sexes and declines with age. Ito et al. [21] noticed that HO-1 protein levels were also lower in older mice. The diminished HO-1 protein level in aged mice observed in our samples could be partially explained by the fact that aging lung might have morpho-



**Fig. 4.** Real-time PCR analysis of ho-1 and ho-2 mRNA expression in lungs of 1, 4 and 12 months old male and female mice after hyperoxia treatment. Data are mean  $\pm$  SEM of 3 pools of 4 mice per each sex and each treatment. The relative fold change compared to control mice at room air (defined as 1) was calculated using the  $2^{-\Delta\Delta CT}$  method as described in Materials and methods. For males ho-1: \*\*\*control vs hyperoxia  $p=0.001$ ; For females ho-1: +++control vs hyperoxia  $p=0.001$ . For males ho-2: \*\*\*control vs hyperoxia  $p < 0.001$ .

structural modifications, including progressive fibrosis, that lead to functional decline, the result of which (among other factors) might be altered/low or even undetectable level of HO-1 protein [25]. Moreover, protein synthesis is shown to be inhibited under hyperoxia conditions, as shown by [26]. Literature data have also shown that oxidative stress conditions such as heat shock, could influence transcriptional efficacy of mRNAs, as explained by Serra and Zapata [27]. Contrary to HO-1, our study revealed that HO-2 protein level is practically unchanged during aging except in 1 month old male mice where HO-2 protein level was at its maximum. At 1 month of age mice demonstrated sex-related level of HO isoforms; with significantly higher HO-1 protein in females and HO-2 in male mice. The most interesting phenomenon noticed in our study seems to be the fact that HO activity was unaltered during aging in males, while in females aging upregulated HO activity. The observed activity at older age seems to be the result of HO-2 rather than HO-1 protein expression. Thus, higher HO activity in aging females, which might be mainly related to HO-2 seems to be important for protection of oxidative stress in aging female mice. This is in accordance with numerous reports of old females being more efficiently protected from oxidative stress than old males [28;29]. Basal HO expression not only depends of



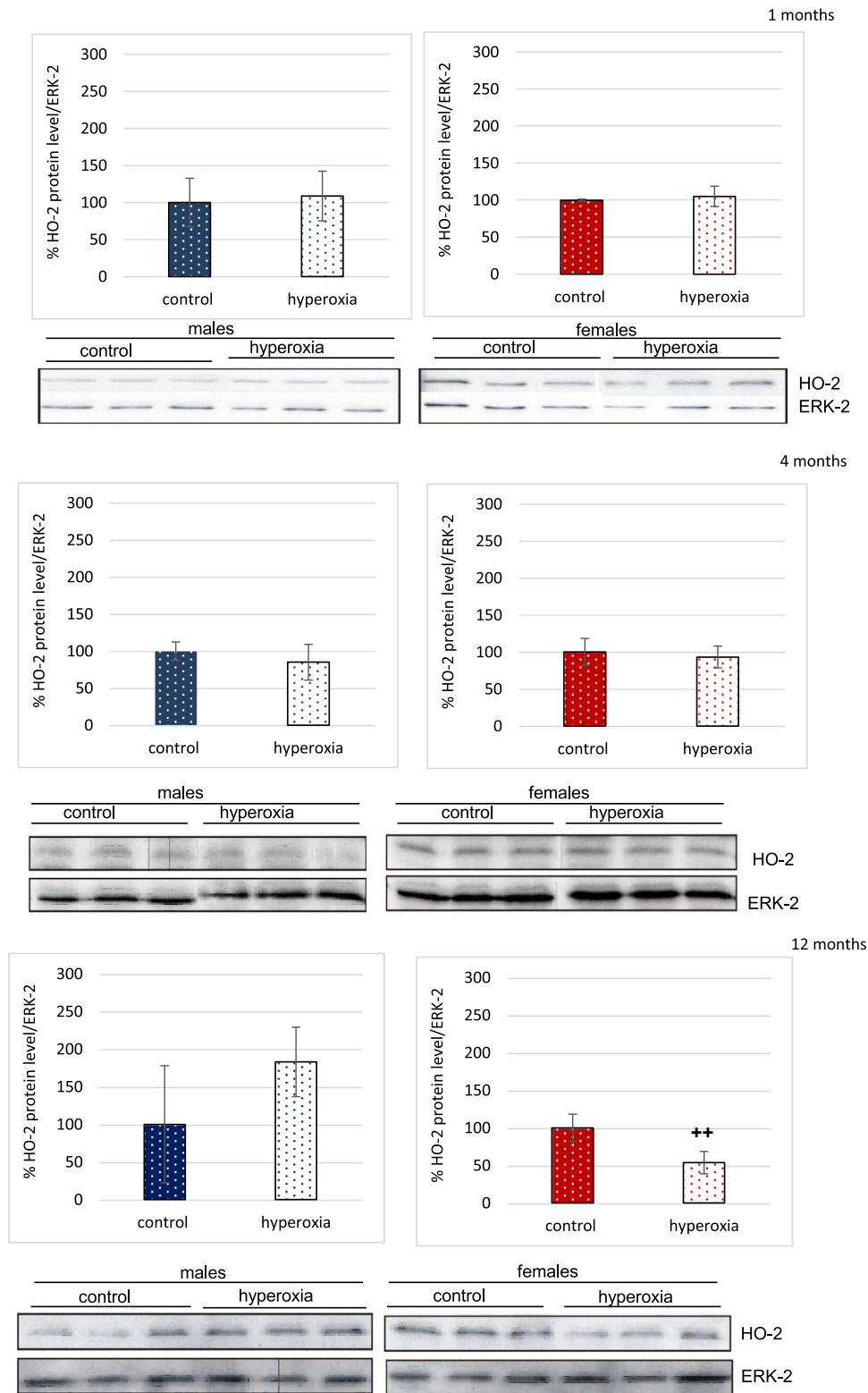
**Fig. 5.** Western blot analysis of HO-1 protein level in lungs of 1, 4 and 12 months old male and female mice after hyperoxia treatment. Protein intensities are expressed as percents and relative to ERK-2 content. Spleen (+C) is shown as positive control for HO-1 in 12 months old mice. For males: \*\*HO-1 control vs hyperoxia treatment in 4 months old mice,  $p=0.01$ .

species and organs, but the response of HO to inducers varies to a great extent in different organs and is often age dependent. As determined by direct parameters of oxidative stress such as MDA, CAT or Gpx, no effect of hyperoxia was observed in any group examined. However, this mild oxidative stress upregulated HO-1 gene expression and the induction were equal in all groups. This is in accordance with the fact that HO-1 is primarily regulated at the transcriptional level as demonstrated by Morse et al. [30]. The results of our study present an example that in the absence of classical antioxidative enzyme induction, the response to oxidative

stress may be, depending on the organ examined, mediated via regulation and the interaction of other systems such as heme oxygenase primarily via upregulation of ho-1 gene expression in both sexes.

## 5. Conclusions

In this study we found that in steady state conditions hemoxygenase-1 and hemoxygenase-2 isoforms are affected by age and sex



**Fig. 6.** Western blot analysis of HO-2 protein level in lungs of 1, 4 and 12 months old male and female mice after hyperoxia treatment. Protein intensities are expressed as percents and relative to ERK-2 content. For females: <sup>++</sup>HO-2 control vs hyperoxia treatment in 12 months old mice,  $p < 0.05$ .

only on protein level. Ho-1 gene expression was induced upon hyperoxia treatment in all age groups, as opposed to ho-2 which showed male-specific downregulation in 12 months old group of animals. Higher HO activity in control aging females compared to their youngest counterparts demonstrates better protection of old females *per se*. However, no sex-related differences in HO activity was observed under hyperoxia conditions in this age group. In the

absence of classical antioxidative enzyme induction, sex-related response to oxidative stress may be mediated via regulation and interaction of other important systems such as hemoxygenase.

#### Declaration of Interest statement

No conflicts of interest declared.

## Acknowledgments

The authors thank Iva Pešun-Međimorec for her excellent technical assistance. The research is funded by Croatian Ministry of Science, Education and Sports, Grant no. 0982464-1647.

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