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Research paper

Erythropoietin and caffeine exert similar protective impact against neonatal intermittent hypoxia: Apnea of prematurity and sex dimorphism

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ABSTRACT

Apnea of prematurity (AoP) is associated with severe and repeated episodes of arterial oxygen desaturation (intermittent hypoxia - IH), which in turn increases the number of apneas. So far, there is no data addressing whether IH leads to sex-specific respiratory consequences, neither if drugs targeting AoP are more effective in males or females. We used rat pups for investigating whether IH-mediated increase of apneas is sex-specific. We also tested whether caffeine (treatment of choice of AoP), erythropoietin (Epo - a neuroprotective factor and potent respiratory stimulant), and combination of both (caffeine + Epo) prevent the IH-mediated formation of apneas in a sex-dependent manner. Newborn rats exposed to IH (21% - 10% FIO_2–8 h a day – 10 cycles per hour) during postnatal days (P) 3–10 were used in this work. Animals were administered drug vehicle, Epo, caffeine and Epo + caffeine (daily from P3 to P10) gavage. At P10 the frequency of apneas at rest (as an index of respiratory dysfunction induced by IH), and respiratory parameters were measured by plethysmography. Our results showed that IH significantly increases the number of apneas in male but not in female rat pups. Moreover, caffeine and Epo in males similarly prevented the increase of apneas induced by IH, and the administration of both drugs together did not provide a cumulative beneficial effect. No impact of drugs was evidenced in females. Apart from apneas, IH increased the normoxic basal ventilation (ventilation at rest) of male animals, and treatments did not prevent such alteration. Besides, no IH- nor treatment-mediated modulation of basal ventilation was found in the basal ventilation of female animals. Analysis of the activity of pro- and antioxidative molecules revealed that IH induces oxidative stress in the brainstem of male and female animals and that all tested treatments similarly prevented such oxidative imbalance in pups of both sexes. We concluded that neonatal IH and the treatments tested to prevent its respiratory consequences are sex-specific. The mechanics associated with such prevention are directly linked with the prevention of oxidative stress and the maturation of the brain. These findings are relevant to understanding better the AoP disorder and for proposing Epo as a new therapeutical tool.

1. Introduction

Apnea of prematurity (AoP) occurs in > 85% of infants born with < 34 weeks of gestational age (Gallego and Matrot, 2010). Since the survival of extremely low birth weight preterm infants (gestational age < 28 weeks) has improved tremendously in recent years, the burden of problems associated with AoP is growing (Martin et al., 2011). The recurrent apneic events of AoP are associated with significant, periodic O_2 desaturation resulting in sustained intermittent hypoxia (IH), a deleterious condition that profoundly alters the respiratory control system and remains a troublesome challenge for the neonatologist. IH induces an exaggerated activity of the chemoreceptors that leads to enhanced respiratory instabilities and completes the vicious circle by increasing the number of apneas (Di Fiore et al., 2016a,b). IH affects the maturation and development of the brain, increases morbidity, prolongs the need for hospitalization care and has long-term cognitive and neurodevelopmental outcomes (de Lima et al., 2005; Jobe and Kallapur, 2010). Furthermore, studies at the cellular level show that IH increases the production of reactive oxygen species (ROS), and decreases anti-oxidative defenses (Di Fiore et al., 2016a; Di

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Fiore et al., 2013). To avoid IH exposure, AoP is systematically treated, and caffeine is the most widely used treatment (Di Fiore et al., 2016b). Caffeine is an adenosine receptor antagonist, which increases central respiratory drive, thus reducing the frequency of apneas, without short- or long-term adverse effect (Schmidt et al., 2007). However, caffeine is not completely efficient: in about half of treated infants apnea frequency remains elevated (Erenberg et al., 2000), and aggressive interventions such as mechanical ventilation are needed. Moreover, whether the persistence of apneas and IH are related to a limited effect of caffeine on oxidative stress is yet unknown.

In addition to its classical function as promotor of increased number of red blood cells, erythropoietin (Epo) is naturally secreted by neurons and astrocytes (Marti, 2003), and there is extensive preclinical data showing that in rodents Epo reduces oxidative stress and inflammation following hypoxic-ischemia (Juul and Pet, 2015; Juul et al., 2015; Wang et al., 2014; Millet et al., 2015; Plenge et al., 2012; Qin et al., 2014; Carraway et al., 2010). Clinical trials in preterm infants showed that Epo protects against hypoxic-ischemic encephalopathy (McPherson and Juul, 2010), and ongoing clinical assays are testing the hypothesis that neonatal Epo treatment of preterm infants improves survival and neurodevelopment (Juul et al., 2015). Regarding ventilation at neonatal ages, it was reported that subcutaneous treatment of very preterm neonates (gestational age < 30 wks) with Epo (300 U/kg dose, 3 times/wk) improved erythropoiesis and drastically reduced the need for assisted ventilation and O_2 supplementation (Tempera et al., 2011). In line, studies performed in our team showed that Epo is a potent respiratory stimulant in newborn mice (Ballot et al., 1985; Soliz and Joseph, 2005). Moreover, our results showed that newborn male mice have a lower respiratory response to hypoxia than females and that Epo stimulates breathing under hypoxia to a greater extent in males than in females (Iturri et al., 2016). Furthermore, recent evidence demonstrates that Epo in the brain (and other tissues) regulates mitochondrial energy production and protects against oxidative stress (Wang et al., 2014; Plenge et al., 2012; Carraway et al., 2010; Elliot-Portal et al., 2018; Xiong et al., 2007). Accordingly, a recent report from our laboratory showed that Epo protects in adult mice by probably mitigating against oxidative imbalance and cardiorespiratory dysfunction induced by IH (Elliot-Portal et al., 2018). Despite this cluster of information, the impact of Epo as a therapeutic tool for AoP has not yet been investigated.

On the other hand, respiratory disorders that occur around birth are clearly and remarkably sex dimorphic. The clinical importance of this sex asymmetry is revealed by the fact that the morbidity and mortality related to respiratory disorders in newborn and during infancy are higher in males than in females (Mage and Donner, 2006). While most of these conditions are characterized by a reduced oxygen availability (hypoxia)(Mage and Donner, 2006), our understanding of such sex-based differences is restricted, limiting the ability of clinicians to provide optimal cares. Concerning AoP, our team recently reported that in preterm infants diagnosed with AoP and treated with caffeine, the maturation of the respiratory system may occur more rapidly in females than males, and that the resulting IH may have sex-specific consequences on the maturation of respiratory control (Bairam et al., 2018). In line, studies in animal models showed that oxidative stress has sex-specific consequences in neonatal brain injuries, with higher impact in male than female animals (Demarest et al., 2016; Khalifa et al., 2017; Robertson and Saraswati, 2015). As such, while AoP and IH are frequent in preterm boys and girls, there is no data addressing whether IH leads to sex-specific respiratory consequences, neither if drugs targeting AoP (and the associated IH-induced oxidative stress) are more effective in males or females.

Based on this background we tested the hypothesis that neonatal IH leads to sex-specific consequences. Furthermore, we compared the sex-specific impact of Epo, caffeine, and the combination of both drugs in the prevention of IH-mediated respiratory and oxidative imbalances. To test this hypothesis, we exposed male and female rats to IH in an animal model of AoP (Julien et al., 2008, 2010a,b, 2011). Our results show that IH increases the number of apneic events in male but not in female animals, but that oxidative stress occurs similarly in the brainstem of male and female pups. Furthermore, Epo and caffeine showed a comparable effect in preventing apneas in males, possibly linked to the prevention of oxidative stress in brainstem tissue.

2. Materials and methods

2.1. Animal groups

All rat pups were born and raised in our animal care facilities. Dams and males used for mating were obtained from Charles-River, St Constant, Quebec, Canada. Breeding animals were supplied with food and water *ad libitum* and maintained in standard laboratory and animal care conditions (21 °C, 12:12 dark:light cycle; lights on at 07:00 and of at 19:00 h). We used a total of 80 rat pups (about 7–9 per group). At postnatal day 3 (P3), rats pups were randomly distributed into the following groups: 1) sham, gavage with saline, exposed to room air (RA), and raised in the same conditions as animals exposed to IH, in the same room, exposed to the (slight) noise of the oxygen-regulating unit, and placed in animal chambers of similar size and aspect; 2) IH, gavage with saline and exposed to IH; 3) IH-Epo, gavage with Epo and exposed to IH 4) IH-caffeine (IH-Caf), gavage with caffeine and exposed to IH; and 5) IH-Epo-caffeine (IH-Epo-Caf), gavage with Epo and caffeine and exposed to IH. All recordings and tissue sampling were performed at P10. Laval University Animal Care Committee approved all the experimental procedures described in this manuscript; the protocols were in accordance with the guidelines detailed by the Canadian Council on Animal Care (authorization number 2015113–1).

2.2. Intermittent hypoxia

Rat pups (together with their corresponding mothers) were exposed to IH during 7 days, from P3 to P10. To do so, the whole cage containing the mother and pups were placed in a Plexiglas chamber to repeat cycles of hypoxia/normoxia 10×/h for 8 h/day (8:00–16:00). Oxygen level fluctuations were accomplished by the use of an Oxycycler (Biospherix, Lacona, NY, USA). Setup of the IH chamber was done as described in previous studies (Julien et al., 2008, 2010a, 2011). In a day, 80 cycles of normoxia–hypoxia fluctuations took place, with each cycle lasting 5 min. At the beginning of each cycle, the animals were exposed to 1 min of normoxia (21% O_2) followed by a drop from 21% to 10% O_2 over 1.5 min. Then, hypoxia was sustained for 1 min after which the oxygen level was returned from 10% back to 21% O_2 over 1.5 min. Control animals were exposed to normoxic room air (RA) under otherwise identical housing conditions.

2.3. Drug administration

From P3 to P10 pup rats received by gavage a single daily dose of saline, Epo, caffeine, or Epo + caffeine before being exposed to IH. Gavage of saline and testing drugs were administered in 0.05 mL/10 g body weight using polyethylene tubing (PE10), as described in previous studies from our team (Julien et al., 2011; Montandon et al., 2006; Bairam et al., 2015; Uppari et al., 2016). IH-Epo-treated animals received 5000 U/Kg of Epo daily. IH-Caf-treated animals received a daily dose of 7.5 mg/kg of caffeine base. As shown in our previous studies, this dose results in a plasma level similar to that observed after a loading dose or for the maintenance of therapy in human newborns (Julien et al., 2011; Montandon et al., 2006; Montandon et al., 2008). IH-Epo-Caf animals received a combined treatment of 5000 U/Kg of Epo and 7.5 mg/kg of caffeine base daily.

2.4. Respiratory recordings

Respiratory recordings were performed at postanatal day 10 (P10),

after 7 days in IH. Respiratory parameters were monitored by using the whole-body plethysmography technique in freely behaving, unrestrained rats (Emka Technologies, Paris, France) (Ballot et al., 1985). Airflow through the chamber was set at ~250 mL/min. A differential pressure transducer (Emka technologies) was connected between the recording chamber and the built-in reference chamber. Calibration was performed by a single injection of 500 μL of air inside the chamber. The signal was acquired and recorded on a computer using Spike 2 software (Cambridge Electronic Design, Cambridge, UK), and the same program was used offline to calculate respiratory frequency (Rf), tidal volume (Vt), and minute ventilation (VE = Rf x Vt). The temperature inside the plethysmograph was kept thermoneutral (at 30 °C), with a temperature control system (TCAT-2, Physytemp, Clifton, NJ). After a 30-min adaptation period, the recording was started under normoxic condition. Baseline normoxic respiratory measurements were obtained over a 90-min period. Subsequently, animals were exposed to hypoxia (10% O_2) for 20 min, which was help used to assess the hypoxic ventilatory response (HVR). Next, animals were returned to normoxia (for 15–20 min), before being exposed to hypercapnia (5% CO_2, 21% O_2, in N_2) for additional 10 min. Body weight was measured routinely after experiments to express the tidal volume in milliliters per 100 g. Body temperature was measured orally at the beginning and at the end of the experiment (Physitemp).

2.5. Apneas

Apneas were quantified from ventilatory recordings obtained under normoxic basal ventilation. Restful breathing was recorded for 90 min under normoxic conditions, which allows the evaluation of apneic events during stable and regular breathing from the respiratory recordings (Fournier et al., 2011; Perramon et al., 1983). The number of apneic episodes was evaluated during periods of regular breathing pattern (Fournier et al., 2011; Perramon et al., 1983). Any cessation of breathing lasting more than two regular breaths was considered as an apneic event. Of note, although the regularity of the pattern of breathing at rest is remarkable, we used the last 10 to 20 breaths before the apneic events as a reference to calculate the cessation of breathing. The quantification of apneas was done by visual examination of the plethysmography recordings under normoxia. Extensive details of these approaches and representative respirograms in newborn mice or rats are presented in some of our recent papers (Ballot et al., 1985; Iturri et al., 2016; Potvin et al., 2014).

2.6. Statistical analysis of ventilatory parameters

Ventilatory recordings during normoxia, hypoxia, and hypercapnia were evaluated through Two-way ANOVA, followed by a Fisher's LSD test. Hypoxic (HVR) and hypercapnic (HcVR) ventilatory responses were calculated as percentage from baseline. Then, a one-way ANOVA followed by a Fisher's LSD test was calculated. All analysis and graphs were done with the GraphPad prism 6.0 software (La Jolla, CA, USA). The reported values are means ± SEM. Differences were considered significant at $p < 0.05$.

2.7. Protein extraction and assays

At postnatal day 10, after the end of the plethysmographic recordings, the animals were deeply anesthetized (Ketamine 160 mg/kg IP; Xylazine 10 mg/kg IP), and the brainstem was rapidly dissected, immediately frozen on dry ice, and kept at −80 °C until further analysis. After homogenization in phosphate buffer, different centrifugations (all at 4 °C) were used to isolate the cytosolic fraction. Briefly, the samples were centrifuged for 4 min at 1500*g*, followed by a second centrifugation for 10 min at 12,000*g*, after which the supernatant (cytosolic fraction) was collected. This fraction was stored at −80 °C until analyses. The protein concentration was determined by a standard colorimetric BCA assay kit (Thermo Scientific, catalogue #23225) and all subsequent measurements were normalized to the total protein concentration of the sample.

2.8. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined by the degree of inhibition of the reaction between O_2^- (produced by a hypoxanthine–xanthine oxidase system) and NTB. SOD activity was assessed using a cocktail containing nitroblue tetrazolium (NTB—2.2 mM in water), Tris-HCL pH 8 (2.8 mM), diethylene-triamine-penta-acetic acid (1.3 mM in Tris-HCl). A fresh solution of xanthine oxidase (XO - 1.02 units/mL) was prepared, and then we added 20 μL of sample, 250 μL of cocktail, and 20 μL of XO to each well, and mixed the plate for 4–5 s at room temperature. The absorbance was quickly read at 450 nm, every 50 s for 5 min. For this assay, 4 wells were used as blanks, with 20 μL of PBS 1 × rather than samples. SOD activity corresponded to the difference between the slopes of the formation of formazan blue by time of the blank versus each sample.

2.9. Glutathione peroxidase (GPX)

Activity of glutathione peroxidase (GPX) was determined using the same cocktail described for SOD, plus a fresh solution of glutathione reductase, NADPH (1.7 mM), and reduced glutathione (1.6 mM in water) using H_2O_2 (0.036% in water) as substrate (Laouafa et al., 2017). We added 20 μL of sample, 200 μL of PBS 1 ×, 30 μL of the cocktail solution, and 30 μL of H_2O_2 solution to each well and mixed the plate 4–5 s at room temperature. The absorbance was quickly read at 340 nm every 50 s for 5 min. GPX activity was measured as the slope of the NADPH extinction by time.

2.10. NADPH oxidase

NADPH oxidase (NOX) activity was assessed using the same cocktail described for SOD, plus a fresh solution of NADPH (1 mM). Twenty μL of sample, 250 μL of cocktail, and 30 μL (100 μM/well) of NADPH were added to each well and the plate was shaken for 2 min at room temperature. The absorbance was read at 560 nm, every 50 s for 10 min. NOX activity corresponded to the slope of the formation of formazan blue by time.

2.11. Statistical analysis of the enzymatic activity

We used GraphPad prism 6.0 software for all analyses. All data were analyzed using a one-way ANOVA followed by a post hoc test (Fisher's LSD). All data are shown as boxplots displaying minimum, first quartile, median, third quartile, and maximum. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Epo and caffeine prevent the increase of apneic episodes induced by IH in male but not in female animals

The total number of apneic events and the time duration of apneas were evaluated during 1 h of continuous normoxic conditions. IH significantly increased both the total number of apneic episodes and the time duration of apneas in male (Fig. 1A, B), but not in female (Fig. 1C, D) animals. Furthermore, the treatment with both Epo and caffeine significantly decreased the number of apneic events and the time duration of apneas in male pups (Fig. 1A, B). However, when Epo and caffeine were administrated together to male animals they did not provide cumulative beneficial effects to reduce apnea frequency or duration (Fig. 1A, B). On the other hand, Epo, caffeine, and Epo + caffeine did not have any effect on female animals (Fig. 1C, D).

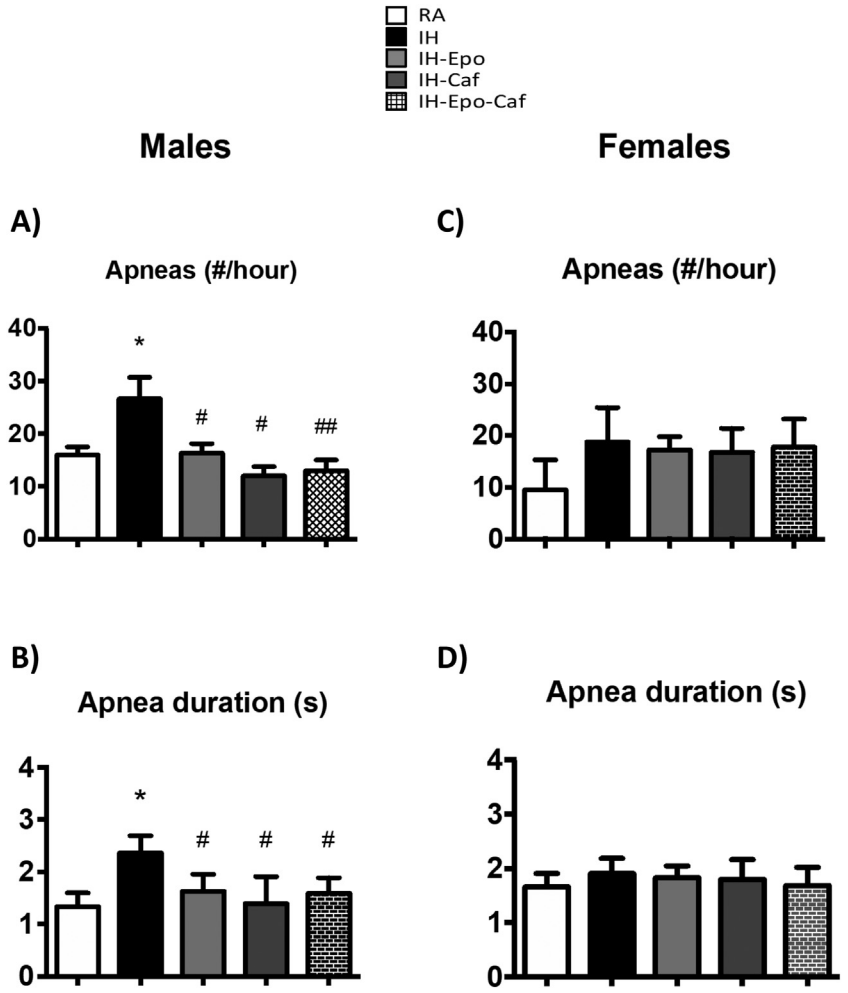

Fig. 1. Apneas in P10 male and female animals were calculated over a period of 90 min of sleep. A, C frequency of apneic events. B, D Apnea duration (n = 7–9 males; n = 6–8 females). * $p < 0.05$ vs RA. # $p < 0.05$ vs IH. ## $p < 0.01$ vs IH.

3.2. Epo and caffeine have a minor impact in the regulation of basal ventilation of animals exposed to IH

Normoxic basal ventilation (VE), respiratory frequency (Rf), and tidal volume (VT) were evaluated in male and female pups. In male animals, IH significantly increased basal ventilation (RA = 72.4 ± 3.7 vs. IH = 144.6 ± 19.9) due to an increase of VT rather than Rf (Fig. 2 A,B,C). Furthermore, neither the treatment with Epo (IH-Epo = 146.5 ± 12.1), nor caffeine (IH-Caf = 138.8 ± 22.5) was able to decrease the IH-mediated increase in basal ventilation (Fig. 2 A). However, when Epo and caffeine were administrated together (IH-Epo-Caf = 129.6 ± 12.9) no significant differences between the basal ventilation of control (RA) and treated animals were found (Fig. 2 A). In female animals, IH was unable to alter basal ventilation (RA = 81.7 ± 9.9 vs. IH = 117.4 ± 7.0) (Fig. 2 D), despite VT was significantly increased by IH (RA = 0.5 ± 0.10 vs. IH = 0.7 ± 0.05) (Fig. 2 F). While the treatment with Epo (IH-Epo = 136.5 ± 6.4) and caffeine (IH-Caf = 129.2 ± 18.5) did not alter basal ventilation of females exposed to IH, Epo + caffeine (IH-Epo-Caf = 139.3 ± 22.2) significantly increased the basal ventilation compared to the control RA group (Fig. 2 D). However, this increase in basal ventilation was not accompanied by significant augmentation of Rf or VT (Fig. 2 E, F).

3.3. Epo and caffeine were unable to prevent the decrease of HVR induced by IH

The ventilation under hypoxic (10% O_2) condition, was evaluated over a time period of 20 min. As expected, control (RA) male (Figs. 3A and 4A) and female (Figs. 3D and 4D) pups showed a strong response to the hypoxic stimulus, which occurred due to a significant augmentation of Rf (Fig. 3B, E) and VT (Fig. 3C, F). The HVR (calculated as % of baseline) was significantly blunted in both male and female animals exposed to IH (Fig. 4A, D). The reduction of HVR in male and female pups was due to a significant reduction in VT (Fig. 4C, F), rather than Rf (Fig. 4B, E). Furthermore, the treatment of male and female pups with Epo, caffeine, and Epo + caffeine were unable to prevent the IH-mediated reduction of HVR induced by IH (Fig. 4A, D). Such effect was linked to an incapacity of treatments to restore VT (Fig. 4C, F), rather than Rf (Fig. 4B, E).

3.4. Epo and caffeine had no effect in the regulation of the hypercapnic ventilatory response

The hypercapnic ventilatory response (HcVR) to 5% CO_2, was evaluated over a time period of 10 min. Compared to the corresponding basal (normocapnic) ventilation, the absolute values of ventilation under hypercapnic conditions were higher in all tested groups in males (Fig. 5A), and in IH and IH-Epo groups in females (Fig. 5A). Such increase was due to an increased VT (Fig. 4C, F), rather than Rf (Fig. 4B,

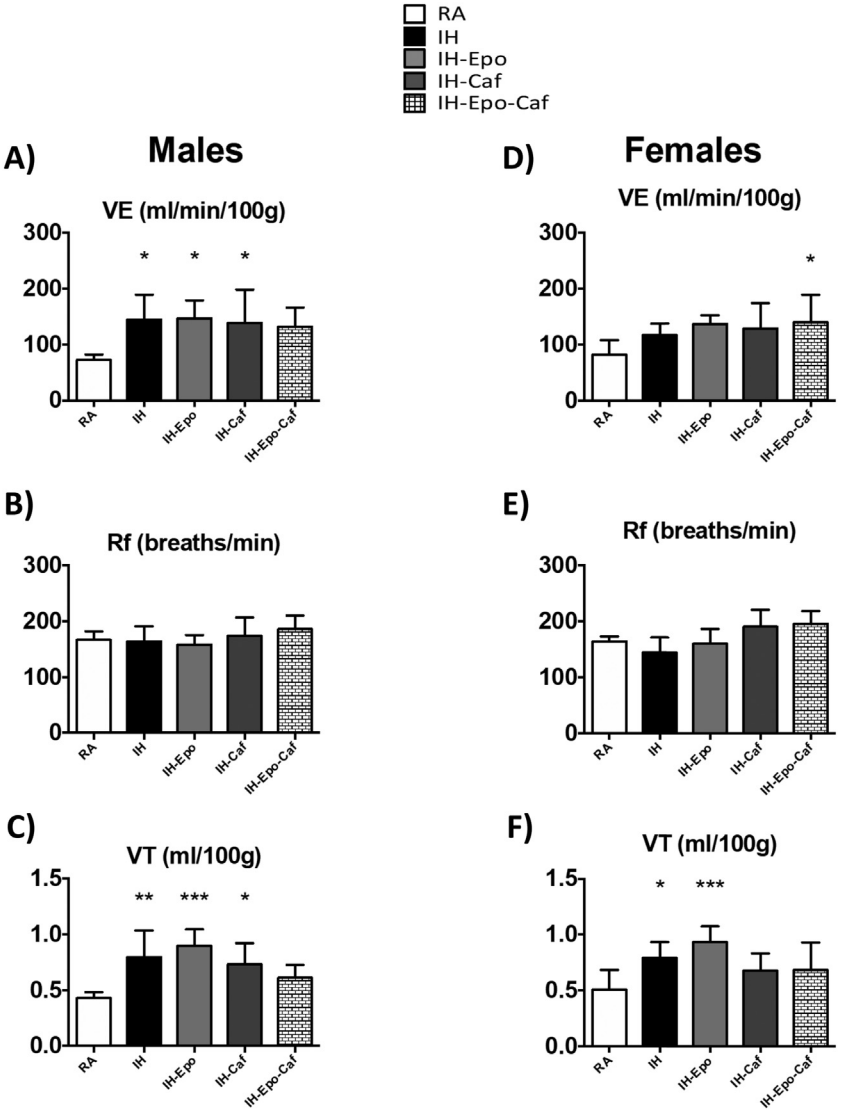

Fig. 2. Ventilatory parameters evaluated under normoxic (basal) conditions. Minute ventilation (VE), respiratory frequency (Rf), and tidal volume (VT) (n = 7–9 males; n = 6–8 females). * $p < 0.05$ vs RA. ** $p < 0.01$ vs RA. *** $p < 0.001$ vs RA.

E). Moreover, the calculation of the HcVR (calculated as % of baseline) in male and female animals was unaffected by the exposure to IH and treatment (Fig. 6).

3.5. Epo and caffeine have protective antioxidant effects in the brainstem of male and female pups exposed to IH

Exposure to IH induced a significant reduction of SOD in the brainstem of male (Fig. 7A) and female (Fig. 7C) rat pups. Moreover, the treatment of animals with Epo and Epo + caffeine (but not caffeine alone), were able to prevent the decrease of SOD induced by IH both in male and female animals (Fig. 7A, C). In addition, compared to the control (RA) group, the combined treatment of Epo + caffeine in male and female pups promoted a significant increase in SOD activity (Fig. 7A, C).

Apart from SOD, the activity of GPX was also evaluated in brainstem tissue of pups exposed to IH. Exposure to IH had no effect of GPX activity in male (Fig. 7B) and female (Fig. 7D) rat pups. Moreover, as showed in Fig. 7B, and C, no effect of Epo and Epo + caffeine over the activity of this enzyme was observed. However, the treatment of IH-exposed animals with caffeine promoted a significant reduction of GPX activity in the brainstem of male and female rats (Fig. 7B, D).

3.6. Caffeine, but not Epo, reduces the NOX activity in the brainstem of male and female pups

Our enzymatic activity measurements showed that the exposure to IH does not alter the NOX activity in the brainstem of male and female animals (Fig. 8A, C). In addition, neither the treatment of Epo, nor of Epo + caffeine promoted any alteration in the activity of this enzyme. However, the treatment of IH-exposed male and female animals with caffeine significantly decreased the activity of NOX in the brainstem tissue (Fig. 8A, C).

3.7. Epo, caffeine, and Epo + caffeine have a protective impact over the oxidative imbalance induced by IH in the brainstem of male and female animals

In an attempt of calculating the balance between the pro- and anti-oxidant activity in the brain, the ratio NOX/SOD was determined. Exposure to IH significantly increased the ratio NOX/SOD in both male and female animals (Fig. 8B, D). Furthermore, all the tested treatments (Epo, caffeine, and Epo + caffeine) promoted a significant prevention of the oxidative balance in the brainstem. However, compared to Epo, caffeine and Epo + caffeine induced stronger reduction of NOX/SOD in

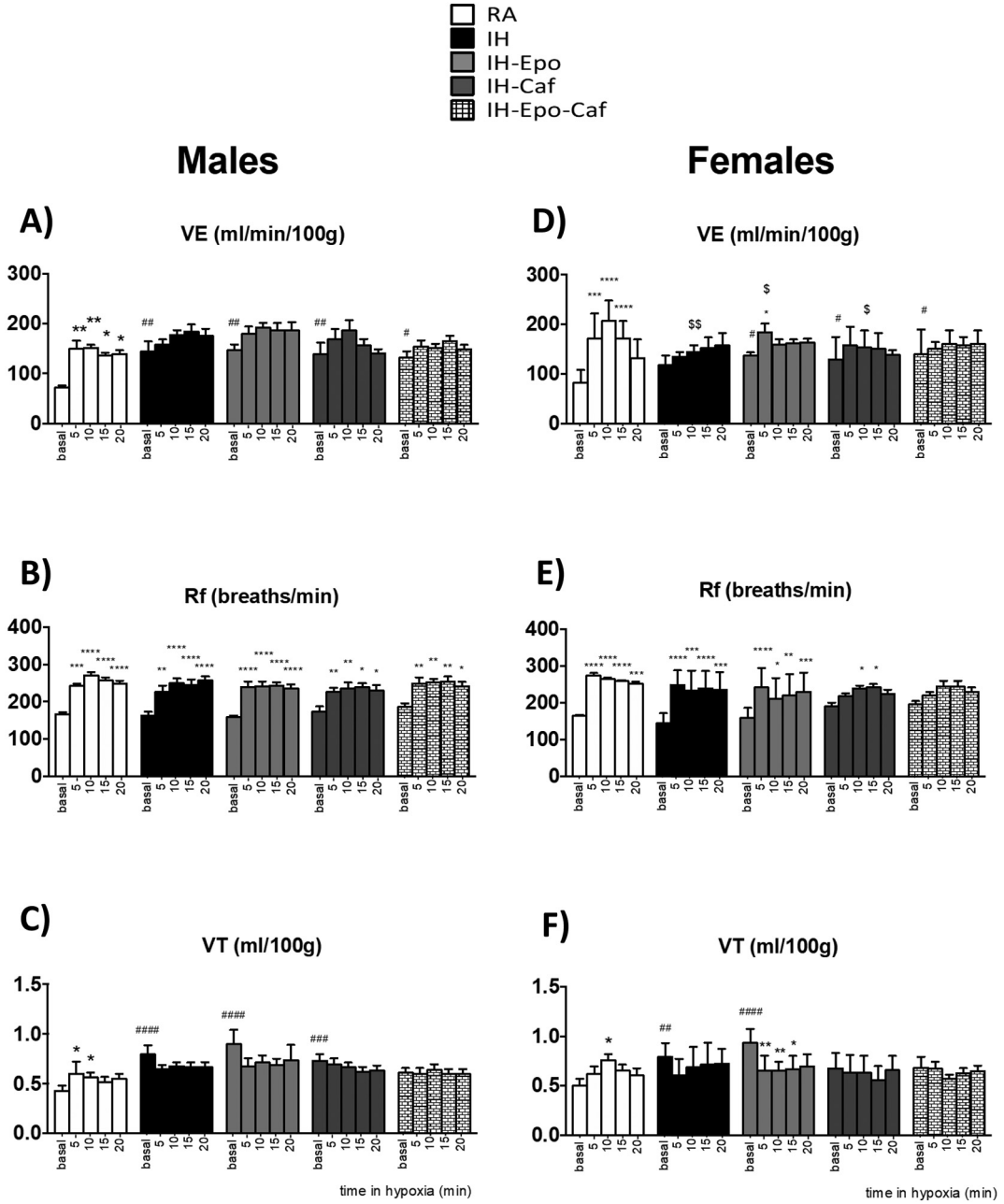

Fig. 3. Ventilatory parameters evaluated under 20 min of hypoxic (10% O_2) condition. Minute ventilation (VE), respiratory frequency (Rf), and tidal volume (VT). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs baseline, same group (n = 7–9 males; n = 6–8 females). # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$ vs RA, at same exposure time.

male and females (Fig. 8B, D). Furthermore, in females, caffeine and Epo + caffeine were able to significantly reduce the ratio NOX/SOD to values lower than the showed in the control group (Fig. 8D).

4. Discussion

In this study we used male and female rat pups, to test the hypothesis that the exposure to neonatal IH (a reliable laboratory model to study AoP) leads to sex-specific augmentation of apneic events, alteration of central and peripheral chemosensitivity, and oxidative stress in the brainstem. Keeping in mind that caffeine is used as the treatment of choice for AoP, and that Epo at neonatal ages is a potent respiratory stimulant and powerful neural regulator of oxidative stress, we also compared the (individual and combined) impact of these drugs. Our results clearly show that IH increases the number of apneas and ventilation in males, but not in females. Remarkably however, IH induced oxidative stress in the brainstem of male and female animals. The treatment of male animals exposed to IH showed that Epo exerts a similar protective effect as caffeine in decreasing apneas, and that Epo and caffeine prevent the redox imbalance in the brainstem of male and female pups. Accordingly, our results support previous findings suggesting that the maturation of the respiratory system in females occurs more rapidly than in males, and thus, they are less susceptible to respiratory-associated consequences induced by oxidative stress.

4.1. IH and sex dimorphism

Newborn males are more at risk of developing respiratory disorders than females (Mage and Donner, 2006). This sexual dimorphism is also apparent in infant respiratory mortality rates worldwide, with females

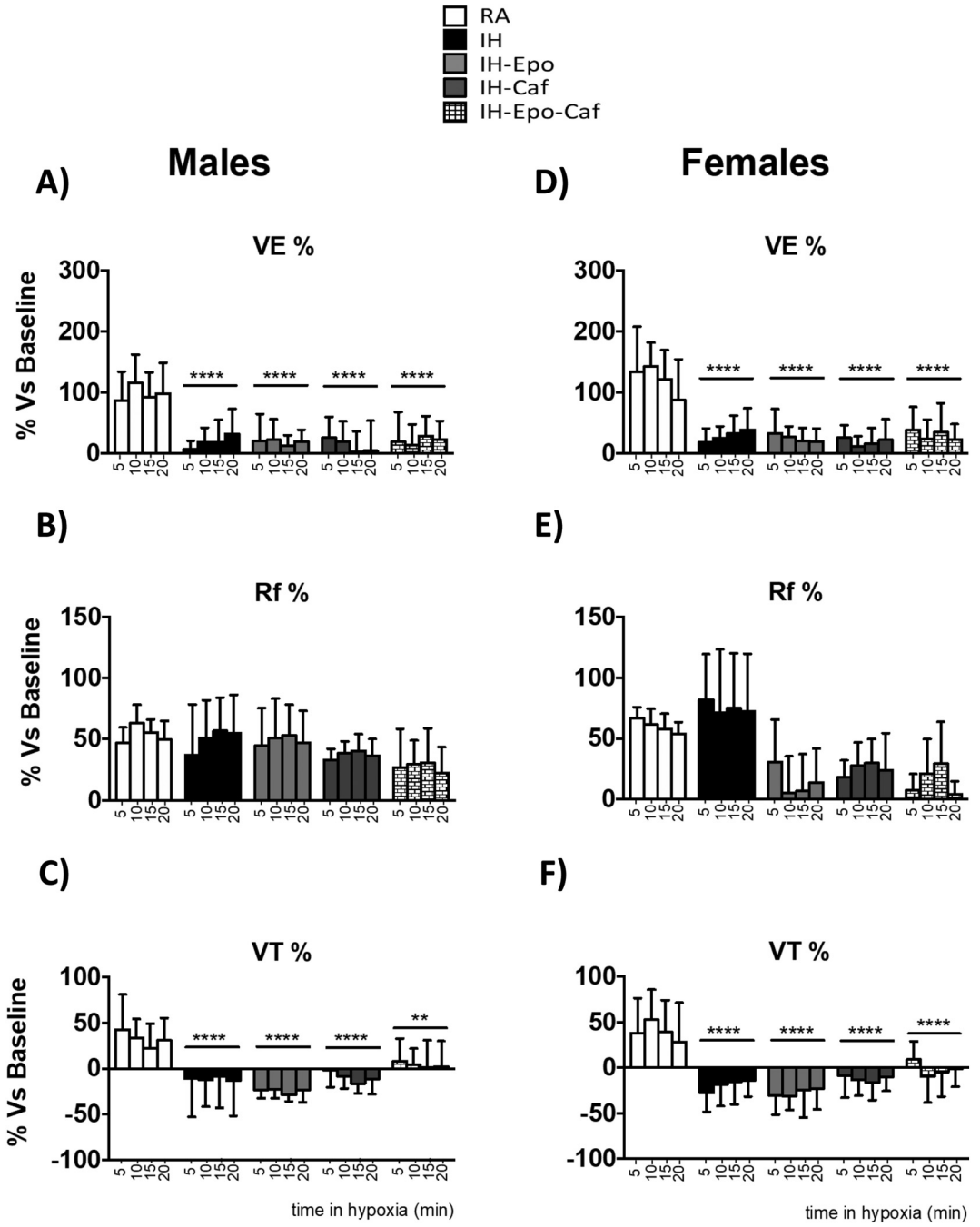

Fig. 4. Hypoxic ventilatory response (HVR) calculated as percentage of baseline. Minute ventilation percentage (VE %), respiratory frequency percentage (Rf %), and tidal volume percentage (VT %) (n = 7–9 males; n = 6–8 females). ** $p < 0.01$; **** $p < 0.0001$ vs RA.

showing a one-third lower rate than males, as revealed by the pediatric mortality data from the Centers for Disease Control and Prevention and the World Health Organization (Mage and Donner, 2006). Our understanding of such heterogeneity remains limited (Fournier et al., 2011). This relatively higher protection for the female is especially apparent under conditions of O_2 deprivation, and early experiments performed in rodents showed that, under progressive exposure to low O_2 concentrations, males died faster and in a larger proportion than females (Perramon et al., 1983; Britton and Kline, 1945). In newborn humans, this sexually dimorphic resistance to hypoxia is apparent in the cases of deaths that are linked to respiratory distress syndrome (RDS), sudden infant death syndrome (SIDS) and suffocations in childhood (Mage and Donner, 2006). The critical developmental period during which most of these diseases occur coincides with the immaturity of the brain (Bissonnette, 2000; Cohen et al., 1997). In fact, due to such brain immaturity AoP occurs in the majority of infants born preterm. However, while AoP is frequent in preterm boys and girls, there is not yet data addressing whether AoP (and the associated IH) leads to sex-specific consequences. In an attempt to shed light on this issue, we recently reported that in a cohort of 24.387 AoP preterm babies (born between gestational ages 24–34 weeks) there were slightly more females diagnosed with AoP than males, but that females required fewer days of caffeine therapy than males. These observations suggested that the maturation of the respiratory system may occur more rapidly in females than males, and/or that the resulting IH has sex-specific consequences on the maturation of the respiratory control system (Bairam et al., 2018). In line with these results, data from the current investigation demonstrate that when neonatal male and female rats are exposed to

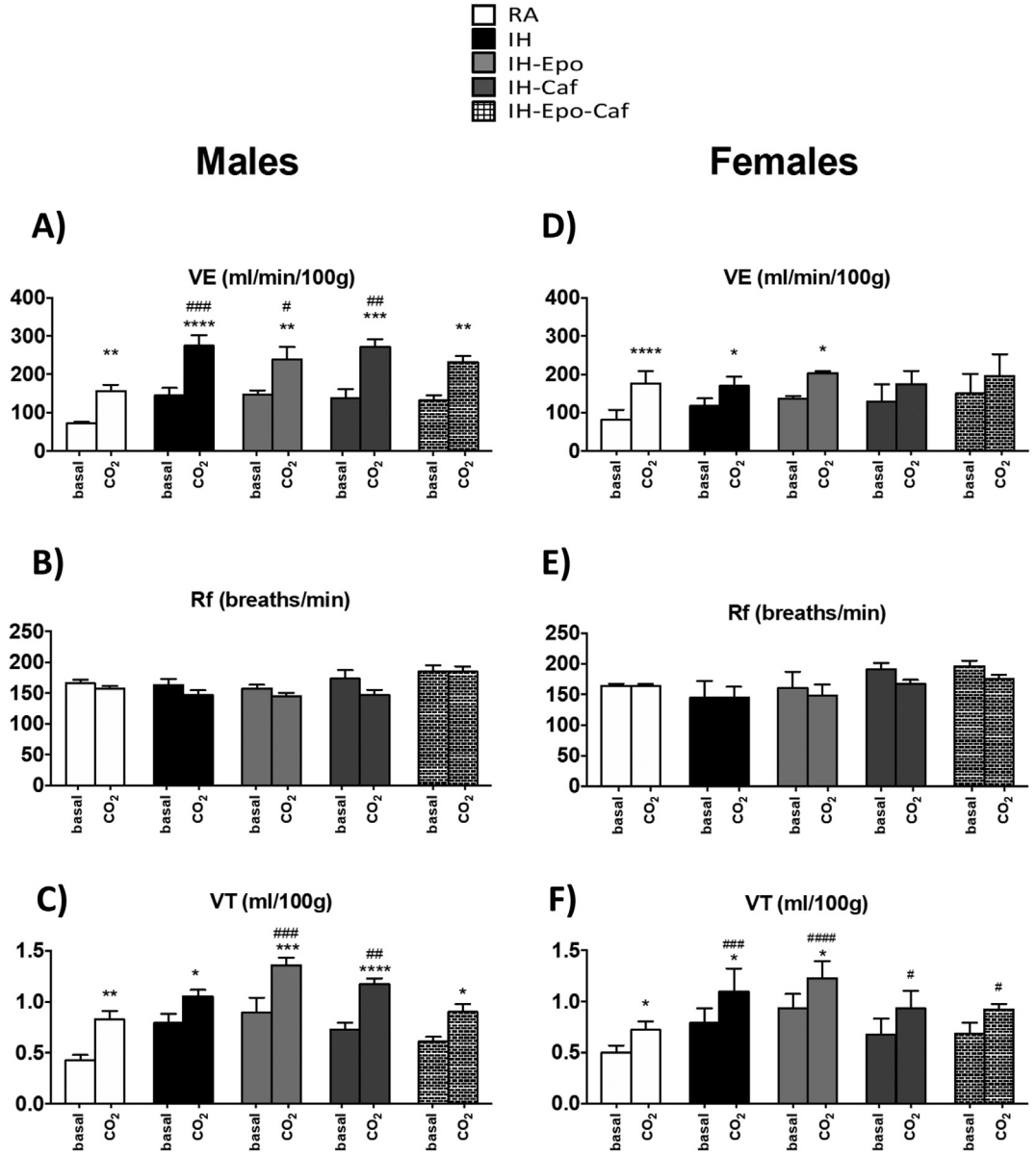

Fig. 5. Ventilatory parameters evaluated under 10 min of hypercapnic (5% CO_2) condition. Minute ventilation (VE), respiratory frequency (Rf), and tidal volume (VT) (n = 7–9 males; n = 6–8 females). ** $p < 0.01$; *** $p < 0.001$; vs RA. (n = 7–9).

IH, only males presented an increased number of apneic events. Mechanism on any link of sexual dimorphism and maturation of the neural circuitry in the respiratory system remains to be elucidated. However, it is known that during late gestation and around birth, surges of testosterone promote the development of brain areas involved in masculine behavior (a process called masculinization), and repress the development of feminine behavior (defeminization) (Hsu et al., 2001; MacLusky and Naftolin, 1981; Toran-Allerand, 1984). In line with these data and the results obtained in our work, it is tempting to suggest that processes of masculinization and defeminization occurring in the brain in male animals significantly enlarge the window in which the brain is highly susceptible to hypoxic insults.

4.2. Impact of Epo and caffeine in IH-exposed animals

To avoid IH exposure, AoP in neonates are systematically treated, and caffeine is the most-widely used treatment (Di Fiore et al., 2016b). Caffeine is an adenosine receptor antagonist, which increases central respiratory drive, thus reducing the frequency of apneas (Schmidt et al., 2007). Furthermore, the therapeutic value of caffeine for AoP has been further reinforced by the CAP (Caffeine for Apnea of Prematurity) trial performed in infants of < 1250 g of body weight at birth (Schmidt et al., 2007; Schmidt, 2005). This trial confirmed the short- and long-term safety of caffeine, as it shortens the time of mechanical ventilation support, and improves the rate of survival without neurodevelopmental disabilities at two, five, and eleven years (Schmidt et al., 2007; Schmidt, 2005). However, whether the impact of caffeine has a sex-specific effect is yet unknown. Results from our study clearly show that caffeine administration to IH-exposed rat pups prevents increase of apneic events in male animals, while no effect of caffeine was observed in female rats. These results strongly support the suggestion that the quicker recovery observed in AoP female babies (Bairam et al., 2018), is associated with a more rapid maturation of the neural respiratory network, rather than to the administration of caffeine.

Apart from caffeine, studies performed in our laboratory suggest that Epo appear to be a candidate of choice against AoP. Epo and its receptor (EpoR) are extensively expressed in the fetal human (Juul et al., 1998) and rodent (Kumral et al., 2011) brain. Epo is essential for

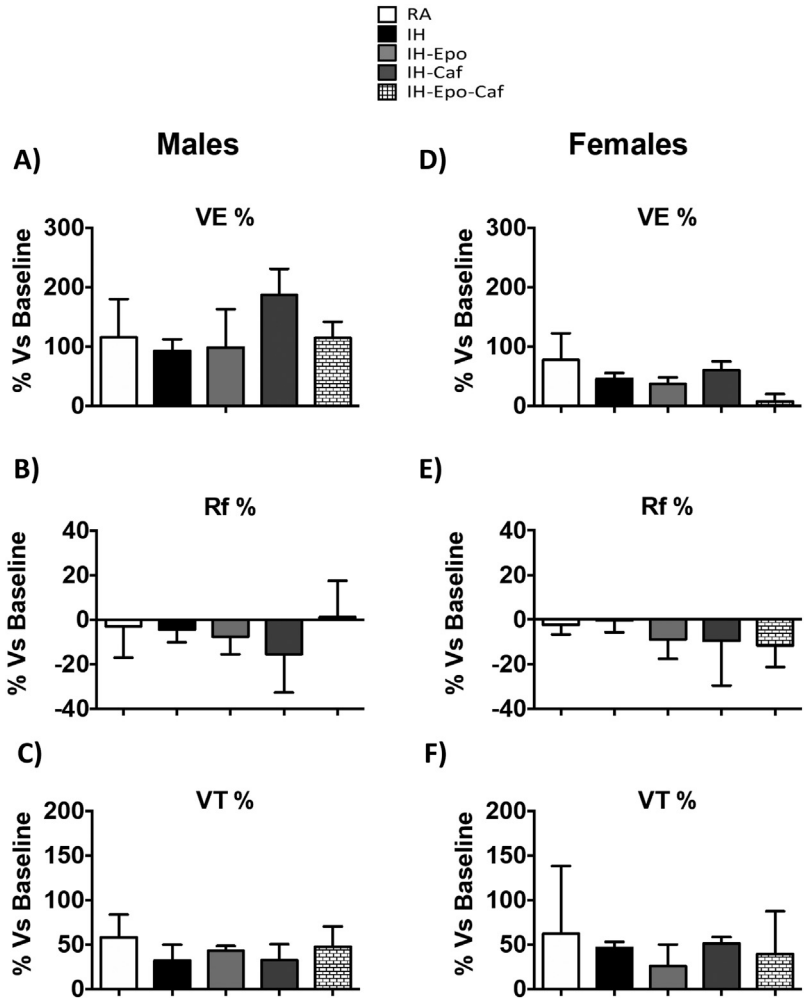

Fig. 6. Hypercapnic ventilatory response (HcVR) calculated as percentage of baseline. Minute ventilation percentage (VE %), respiratory frequency percentage (Rf %), and tidal volume percentage (VT %)(n = 7–9 males; n = 6–8 females). ** $p < 0.01$; **** $p < 0.0001$ vs RA.

adequate brain development and fetal survival: mice lacking Epo or EpoR have increased neuronal apoptosis, decreased number and defective migration of neural progenitor cells and oligodendrocytes in subventricular zone, and incomplete neural tube closure (Alnaeeli et al., 2012). Moreover, studies performed using the in vitro "en-bloc" brainstem-spinal cord preparation showed that Epo has a direct effect on the central respiratory rhythm generator, and reverses the respiratory depression induced by hypoxia in this model (Khemiri et al., 2011). In addition, by using respiratory recordings in non-anesthetized newborn mice, we showed that a competitive antagonist of EpoR injected directly in the brainstem decreases normoxic ventilation (−38% in males, −59% in females), and leads to respiratory depression, asphyxia (gasping), and high mortality when mice were exposed to severe hypoxia (6% O_2)(Ballot et al., 1985). We also reported that under severe hypoxia, at postnatal days 7, 15 and 21, Epo reduces the frequency of apnea in newborn male, but not in female mice in a dose dependent manner (Iturri et al., 2016). In line, a clinical study aiming to improve the transfusion needs in very preterm neonates (gestational age < 30 wks) showed, as collateral effect, that Epo reduced the need for assisted ventilation and O_2 supplementation (Tempera et al., 2011). Finally, in adult transgenic mice exposed to IH we recently showed that overexpression of Epo in the brain only (Tg21 transgenic mice), significantly reduced the frequency of post-sigh apnea (Elliot-Portal et al., 2018). Concerning mechanism, it was recently reported in neonatal (0- to 5-old) Wistar rats that Epo is produced in response to hypoxia in rostroventrolateral medulla (RVLM) neurons, promoting the increase of blood pressure (Oshima et al., 2018). Furthermore, histological examination showed that EpoRs are present in bulbospinal RVLM neurons and some co-expressed with TH immunoreactivity, suggesting that catecholaminergic RVLM neurons at neonatal ages express EpoRs (Oshima et al., 2018). This finding proposes also that, at least in part, Epo might regulate ventilation and promote a decrease of apneic events by modulating the catecholaminergic synthesis in the brainstem. However, an earlier study performed in infants suggested that AoP may not be related to the immaturity of catecholamine pathways (Bhat et al., 1982). As such, it is tempting to suggest that Epo may reduce apneas by direct interaction with mitochondrial generation and/or detoxification of reactive oxygen species (see below). Altogether, these findings suggest that Epo may be used as an alternative therapeutic tool against AoP. Based on pharmacokinetic studies (Juul et al., 2015), in this work IH-exposed rat pups were treated with 5000 U/kg of Epo daily for 7 days. Of note, it was previously proved that this dose crosses the blood-brain barrier in rodents (Banks et al., 2004; Brines et al., 2004; Statler et al., 2007). Our results show that Epo has (as caffeine) a sex-specific effect in the prevention of apneic events, with impact restricted to male animals. Moreover, the protective impact of Epo in males was just as effective as caffeine. Surprisingly however, the combined administration of Epo + caffeine was unable to induce a cumulative beneficial effect. The molecular explanation of this results seems to be related with the incapability of these drugs to reduce oxidative stress below physiological values (see below). On the other hand, keeping on mind that the effectivity of caffeine is limited to about 50% of cases

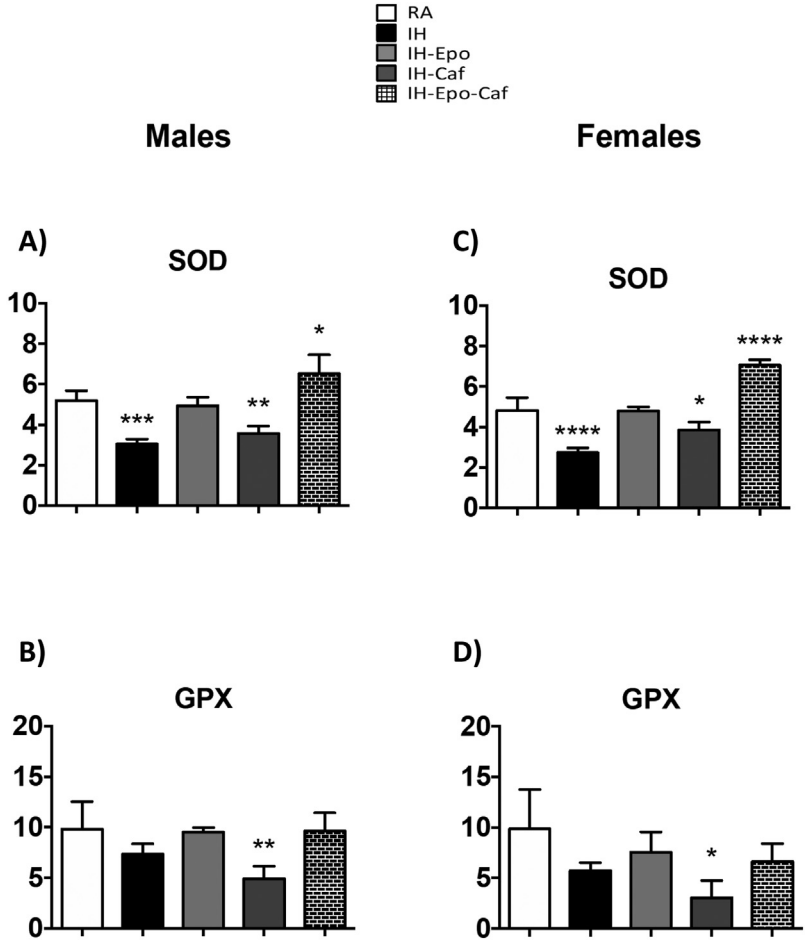

Fig. 7. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity in the brainstem of male (A, B), and female (C, D) animals (n = 6–8 males; n = 6–7 females). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs RA.

(only 1/3 of treated infants have a total reduction of apneic events, and in about half of treated infants apnea frequency remains elevated) (Erenberg et al., 2000; Tabacaru et al., 2017), our results suggest that Epo could be used as an alternative treatment. In fact, Epo is widely used in clinic at neonatal ages and there is extensive preclinical data showing that in rodents Epo reduces oxidative stress and inflammation following hypoxic-ischemia (Juul and Pet, 2015; Juul et al., 2015). Thus, results from this work gather proof-of-concept evidence necessary to perform a clinical trial.

4.3. IH and altered chemosensitivity

Apart from an increased number of apneas, preterm neonates with AoP show an excessive ventilatory response to hypoxia apparently due to an excessive peripheral chemoreceptor stimulation. In fact, IH alters the function of the carotid bodies, which are the main peripheral chemoreceptor responding to low arterial levels of O_2 to induce protective cardio-respiratory responses (Kumar and Prabhakar, 2012). In response to IH exposure, however, the activity and sensitivity of the carotid bodies are increased, and exaggerated responses could contribute to the destabilization of the breathing pattern, and promote the development of apnea (Prabhakar et al., 2015). In preterm neonates with apneas, the contribution of peripheral chemoreceptors to baseline breathing is increased (Rigatto et al., 1975), and it is clear from a large array of studies that excessive inputs from peripheral chemoreceptors contribute to respiratory instabilities and AoP (Di Fiore et al., 2016b). Animal studies have shown that in newborn rats exposed to short term of IH (16 h), the sensory response of peripheral chemoreceptors to hypoxia was increased (in vitro), as well as the HVR (in vivo) (Peng et al., 1985; Prabhakar et al., 2007). In this study, we found that the HVR was reduced in males and females. However, these results are not necessarily contrary to those previously reported because ventilatory parameters in this work were evaluated after a longer term of IH (7 days). Furthermore, keeping in mind that IH induces long term facilitation in the phrenic motor output of postnatal rats (Tadjalli et al., 2008), it is possible to suggest that the reduced HVR in our animals results from an increased basal ventilation (after 7 days of IH), rather than a decreased chemosensory stimulation. In support of this suggestion, our results show that Epo, caffeine, and Epo + caffeine are unable to prevent the IH-mediated increase of basal ventilation. Indeed, this effect makes sense considering that both drugs are potent respiratory stimulants. Finally, concerning hypercapnia, it was reported that preterm neonates have a reduced HcVR, which is more marked in preterm neonates with AoP (Paolillo and Picone, 2013). No impact of HcVR in male and female animals was observed in our data. These results suggest that the IH paradigm used in this work had a minor effect in the central chemosensitivity.

4.4. IH and oxidative stress in brainstem tissue

IH exposure increases the production of ROS and tissue injury (Laouafa et al., 2017; Prabhakar et al., 2007; Lavie, 2015), and represents a major component of several cardiovascular and cerebral pathologies (Prabhakar et al., 2015; Lavie, 2015; Betteridge, 2000; Douglas et al., 2010). Moreover, the brain is especially vulnerable to oxidative stress because of its lower antioxidant enzyme activities

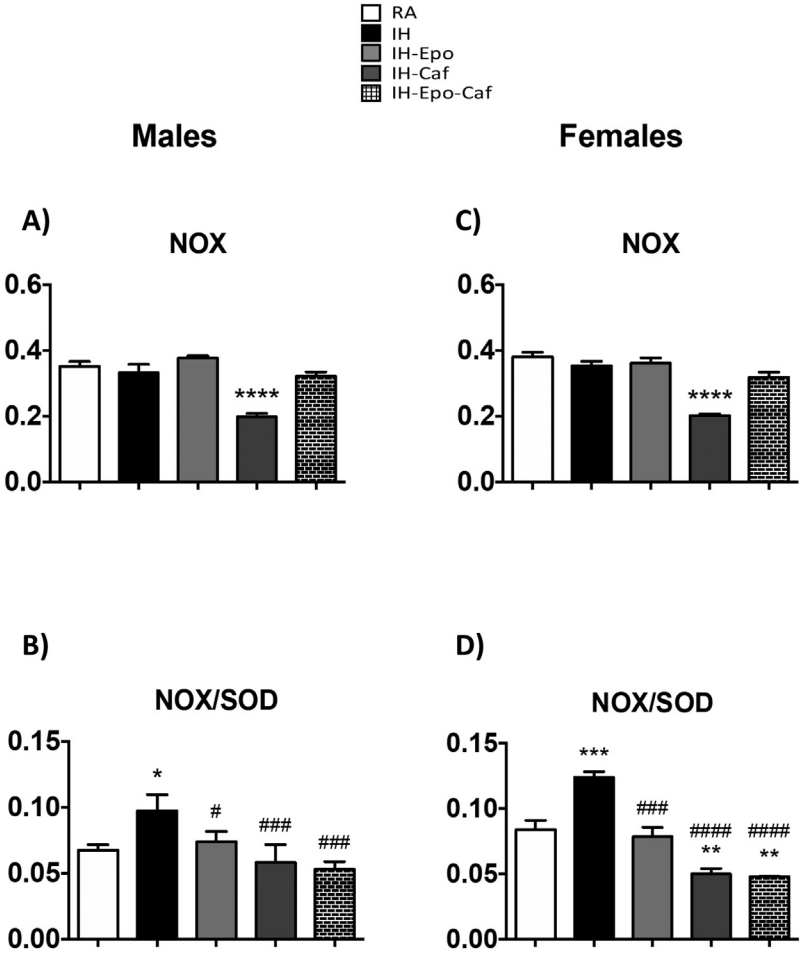

Fig. 8. NADPH oxidase (NOX) activity and ratio of NOX to SOD activity in the brainstem of male (A, B), and female (C, D) animals (n = 6–8 males; n = 6–7 females). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs RA. # $p < 0.05$; ### $p < 0.001$; #### $p < 0.0001$ vs IH.

(Brannan et al., 1981), and high quantity of lipids (which are targets of lipid peroxidation) (Milder and Patel, 2012; Souza et al., 2013). In addition, the brain: 1) has a very large amount of metal ions (able to react with H_2O_2 to form the hydroxyl radical through the Fenton reaction) (Connor and Benkovic, 1992); 2) consumes large amount of oxygen that promotes the production of ROS (Lagranha et al., 2017; Halliwell, 2001); and 3) uses large amounts of glutamate as an excitatory neurotransmitter leading to an elevated concentration of intracellular Ca^{2+} that activates pro-oxidant systems (such as phospholipase A2 and neuronal nitric oxide synthase - nNOS) (Lagranha et al., 2017). The brain vulnerability to oxidative stress is further exacerbated in preterm neonates (Perrone et al., 2010). ROS are mainly produced by mitochondrial respiration and by cytosolic enzymes such as NADPH oxidase (NOX) and xanthine oxidase (Wang et al., 2010), while super oxide dismutase (SOD), glutathione peroxidase (GPX) and catalase are the main antioxidant enzymes (Lavie, 2015). Furthermore, both Epo and caffeine are potent antioxidant factors. Epo in the brain (and other tissues) increases mitochondrial oxygen consumption thereby, reducing ROS production (Wang et al., 2014; Plenge et al., 2012; Carraway et al., 2010; Elliot-Portal et al., 2018; Xiong et al., 2007). Caffeine is a radical scavenger, particularly for ·OH (Shi et al., 1991), and increases the activity of antioxidant enzymes (glutathione reductase and SOD) (Abreu et al., 2011), and prevents against oxidative damage in brain tissue (Prasanthi et al., 2010). In line, the results of our study show that IH lead to oxidative stress in the brainstem of male and female animals, and that both Epo and caffeine are able to prevent such alteration. Interestingly however, the molecular mechanism activated by each drug was different. While Epo (but not caffeine) prevented the decrease of SOD, caffeine (but not Epo) promoted the decrease of NOX. In line, Epo is able to compensate in part the antioxidant properties of mitochondrial SOD deficiency (Liu et al., 2005). In fact, when calculating the ratio NOX/SOD as a relative index of redox imbalance, Epo, caffeine, and both drugs combined were able to prevent the oxidative stress induced by IH in brainstem tissue. Importantly, because ROS are also increasingly being recognized for their involvement in the activation of crucial cellular functions and molecular pathways, there is a subtle balance between their beneficial and deleterious effects (Schieber and Chandel, 2014). As such, antioxidant treatments have to be finely regulated (Schieber and Chandel, 2014). In line, it is important to notice that Epo and caffeine do not reduce the NOX/SOD ratio below control values. This fact suggests that Epo and caffeine were administrated at optimal doses. Finally, our results propose that, at least in part, the IH-mediated oxidative imbalance in neural tissue is responsible of the increase of apneas in male rat pups. This effect can be observed clearer when plotting apneas versus oxidative stress (Fig. 9). In fact, IH in males decreases the activity of antioxidant enzymes (Fig. 9A), rather than promoting an increase of the activity of prooxidant enzymes Fig. 9B). Nevertheless, the most effective strategy to avoid an increase of apneas seems to be decreasing the activity of NOX (as caffeine does – Fig. 9B, C), rather than preventing the reduced activity of SOD (as Epo does – Fig. 9A, C). In female animals, the absence of correlation between apneas and oxidative stress (Fig. 9D, E, F) strongly support the hypothesis that the brain of female animals matures more rapidly than in males, as was suggested previously (Bairam et al., 2018).

In conclusion, neonatal IH induces sex-specific consequences by

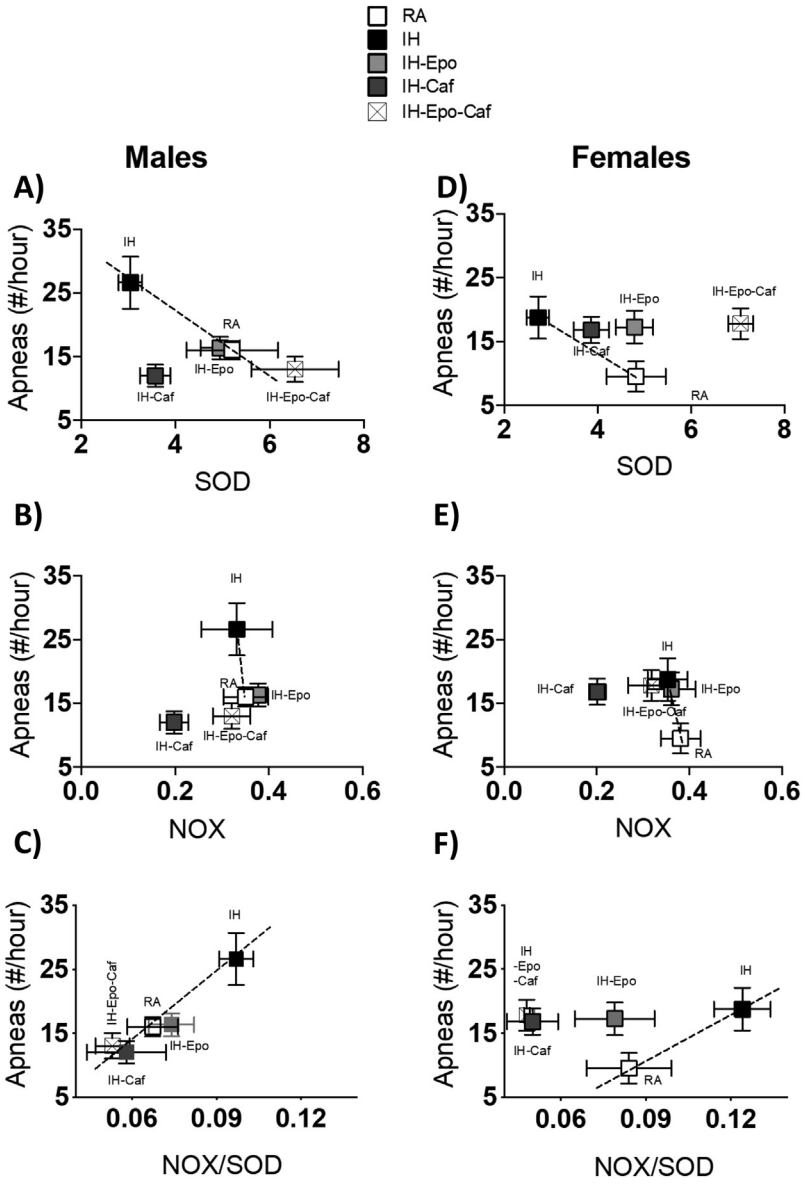

Fig. 9. Correlation between apneic events and oxidative stress induced by intermittent hypoxia (IH). Superoxide dismutase (SOD), and NADPH oxidase (NOX).

increasing apneic events in male animals only. Caffeine (the most widely used treatment for AoP), and Epo have similar prevention impact of apneas in males, and no effects in females. Furthermore, our results suggest that IH-associated oxidative stress in the brainstem of male animals is responsible, at least in part, for the observed increase of apneas. Our results also support the hypothesis that the brain of female animals mature more rapidly, and thus is better protected against oxidative stress insults. All together our data suggest that Epo could be used as an alternative target drug to protect against deleterious consequences of AoP.

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