NORMOBARIC HYPEROXIA: HAEMODYNAMIC RESPONSES TO ACUTE AND LONG-TERM EXPOSURE

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Doctoral Dissertation
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Doctoral Dissertation

NORMOBARIČNA HIPEROKSIJA: HEMODINAMIČNI ODZIVI NA AKUTNO IN DOLGOTRANJO IZPOSTAVITEV

Doktorska disertacija

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"I wanted movement and not a calm course of existence. I wanted excitement and danger and the chance to sacrifice myself for my love. I felt in myself a superabundance of energy which found no outlet in our quiet life".

L. N. Tolstoy,
***Family Happiness*, 1859**

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Abstract

The aim of the present thesis was to examine the effect of acute and long-term normobaric hyperoxic exposure on selected haemodynamic and haematological responses during resting and exercise conditions in healthy aerobically well-trained males. This purpose was evaluated in four separate studies, and each of them had a specific aim listed below:

Study I evaluated the effect of a 2-hour normobaric O_2 exposure on the concentration of plasma erythropoietin (EPO). Ten healthy males were studied twice in a single blinded counterbalanced crossover study protocol. On one occasion they breathed air (NOR) and on the other 100% normobaric O_2 (HYPER). Blood samples were collected Pre, Mid and Post exposure; and thereafter, 3, 5, 8, 24, 32, 48, 72 and 96 hours, and 1 and 2 weeks after the exposure to determine [EPO]. [EPO] increased markedly 8 and 32 hours after the NOR exposure (~58% and ~52%, respectively, $P \leq 0.05$) as a consequence of its natural diurnal variation. Conversely, the O_2 breathing was followed by a ~36% decrement of EPO 3 hours after the exposure ($P \leq 0.05$). Moreover, [EPO] was significantly lower in HYPER than in the NOR condition 3, 5 and 8 hours after the breathing intervention ($P \leq 0.05$). Accordingly, the present results indicate that a short period of normobaric O_2 breathing does not increase EPO in aerobically fit healthy males. Increased O_2 tension suppresses EPO 3 and 5 hours after the exposure; thereafter EPO seems to change in a manner consistent with natural diurnal variation.

Study II investigated the effect of ten daily short-term exposures to normobaric O_2 over a 2-week period on the plasma EPO in healthy individuals. Twenty males were assigned to either an experimental (HYPER) or to a control (NOR) group. The HYPER group breathed 100% normobaric O_2 for 2 hours every weekday over a 2-week period. The NOR group breathed air within the same time protocol. Blood samples were collected the Pre, Mid and Post intervention period to determine EPO. EPO of the HYPER group was significantly lower than in the NOR group during the Mid and Post periods ($P < 0.001$). EPO of the HYPER group showed a slight, albeit statistically non-significant, decrease during the Mid (~11%) and Post (~16%) periods. Accordingly, daily short-term exposures to normobaric hyperoxia do not increase EPO. The increased O_2 tension suppresses EPO. Hence, administration of pure O_2 to enhance erythropoiesis is not warranted.

Study III mapped the cerebral, intercostal and leg muscle oxygenation of young healthy males during an acute short-term normobaric O_2 administration at rest. Ten healthy males were studied twice in a single blinded counterbalanced crossover study protocol. On one occasion they breathed air and on the other 100% normobaric O_2 for a 2-hour time period. Oxygenated ($\Delta[O_2Hb]$), deoxygenated ($\Delta[HHb]$ and total ($\Delta[tHb]$) haemoglobin in the cerebral, intercostal and vastus lateralis tissues were simultaneously monitored with near-infrared spectroscopy. The hyperoxic stimulus promptly increased the $\Delta[O_2Hb]$ (~2 μM) and decreased the $\Delta[HHb]$ (~3.6 μM) in the frontal cortex. These cerebral responses were directly and fully countered by the return to a normoxic environment. On the contrary, in both intercostal and vastus lateralis muscles only $\Delta[HHb]$ was significantly decreased by pure O_2. The aforementioned muscle changes transpired slower compared to those in the cerebral area; and they were partially

recovered during the 15-min normoxic-recovery period. Accordingly, the acute supplementation of normobaric O_2 at rest influences the cerebral, leg and respiratory muscle oxygenation of healthy individuals, but not in the same manner. The cerebral tissue appears to be more sensitive to the O_2-induced changes compared to the muscle region; a fact which is characterized by the heterogeneous vasoconstrictive response to pure O_2 and the dissimilar return to baseline levels upon the transition to normoxic environment.

Study IV investigated the effect of carbon monoxide (CO) in the inspired air as anticipated during peak hours of traffic in higly polluted urban areas on cerebral, respiratory and leg muscle oxygenation during a constant-power cyce ergometer exercise. In addition, since O_2 breathing is used to hasten elimination of CO from the blood, we examined the effect of breathing O_2 following exposure to CO on cerebral and muscle oxygenation during a subsequent exercise test under CO conditions. Nine healthy males participated in three trials: (a) 3-hr air exposure followed by a control constant-power test (CPT), (b) 1-hour air and 2-hour CO (18.9 ppm) exposure succeeded by a CPT under CO conditions (CPT_{CO_A}), and (c) 2-hour CO and 1-hour 100% normobaric O_2 exposure followed by a CPT under CO conditions (CPT_{CO_B}). All exercise tests were performed at 85% of peak power output to exhaustion. Oxygenated ($\Delta[O_2Hb]$), deoxygenated ($\Delta[HHb]$) and total ($\Delta[tHb]$) haemoglobin in cerebral, intercostal and vastus lateralis muscles were monitored with near-infrared spectroscopy (NIRS) throughout the constant-power tests. Performance time did not vary between exercise trials. However, the vastus lateralis and intercostal $\Delta[O_2Hb]$ and $\Delta[tHb]$ were lower in CPT_{CO_A} than in the CPT ($P \leq 0.05$). During the CPT_{CO_B}, the intercostal $\Delta[O_2Hb]$ and $\Delta[tHb]$ were higher than in the CPT_{CO_A} ($P \leq 0.05$). There were no differences in cerebral oxygenation between the trials. Accordingly, inspiration of 18.9 ppm CO decreases the oxygenation in the vastus lateralis and serratus anterior muscles, but does not affect endurance during cycle ergometry. Breathing normobaric O_2 moderates the CO-induced reductions in muscle oxygenation, mainly in the intercostals, but does not affect exercise endurance.

Povzetek

Cilj pričujoče raziskave je proučiti učinek akutne in dolgotrajne normobarične hiperoksije na ciljne hemodinamične in hematološke odzive zdravih in aerobno treniranih moških, med mirovanjem in med vadbo. Cilj raziskave je bil dosežen na podlagi štirih spodaj opisanih samostojnih študij.

Namen *Študije I* je bil ovrednotiti učinek 2-urnega dihanja čistega kisika (normobarične hiperoksije) na koncentracijo eritropoietina ([EPO]) v plazmi. Deset zdravih preiskovancev je sodelovalo v protokolu, ki je zajemal dva enojno-slepa križnovezana protiutežna poskusa. V enem poskusu so preiskovanci dihali zrak (NOR), v drugem pa 100% normobarični kisik. Krvne vzorce za določitev [EPO] smo jim odvzeli pred 2-urnim dihanjem dihalnega plina, med njim in po njem; nato tudi 3, 5, 8, 24, 32, 48, 72, in 96 ur, ter 1 in 2 tedna po poskusu. [EPO] se je značilno ($P < 0.05$) povečala 8 (58%) in 32 (52%) ur po dihanju zraka v NOR skupini, kot posledica cirkadialnega ritma. V nasprotju pa je dihanje čisega kisika povzročilo značilno ($P < 0.05$) 36% znižanje [EPO] tri ure po prenehanju dihanja čistega kisika. Rezultati dokazujejo, da kratkotrajno dihanje čistega kisika ne poveča [EPO] pri zdravih in aerobno treniranih preiskovancih. Povečan delni tlak kisika zavre [EPO] 3 do 5 ur po normobarični hiperoksiji. Po tem se odziv [EPO] vrne v svoj cirkadialni ritem.

Namen *Študije II* je bil proučiti učinek 10 dnevnih kratkotrajnih izpostavitev normobaričnemu kisiku v 2-tedenskem terminu na [EPO] v plazmi pri zdravih preiskovancih. Dvajset preiskovancev smo naključno razporedili v eksperimantalno (HYPER) ali kontrolno (NOR) skupino. HYPER skupina je dihala 100% normobarični kisik 2 uri vsak delovnik 2 tedna. NOR skupina je dihala zrak po enakem časovnem protokolu. Krvne vzorce smo za analizo plazma [EPO] odvzeli pred 2-tedenskim protokolom, med njim in po njem. [EPO] izmerjen v krvi preiskovancev HYPER skupine je bil značilno ($P < 0.001$) nižji kot [EPO] preiskovancev NOR skupine, tako med 2-tedenskim poskusom kot tudi po njem. Dnevna izpostavitev normobarčni hiperokisji ne poveča [EPO] ampak nasprotno, povečan delni tlak kisika zavre [EPO]. Na podlagi teh rezultatov lahko zaključimo, da dihanje čistega kisika za stimualcijo eritropoeze ni primerno.

Namen *Študije III* je bil določiti oksigenacijo med akutno kratkotrajno izpostavitvijo normobaričnemu kisiku v mirovanju istočasno v možganih, v medrebrnih mišicah in v mišici vastus lateralis Deset zadravih preiskovancev je sodelovalo v raziskavi, ki je bila zasnovana kot enojno-slepa križnovezana protiutežna raziskava. V enem poskusu so preiskovanci dihali zrak in v drugem poskusu 100% kisik. Oksi- ($\Delta[O_2Hb]$), deoksi- ($\Delta[HHb]$ in totalni ($\Delta[tHb]$) hemoglobin v cerebralnem delu možaganov, v medrebrnih mišicah in v mišici vastus lateralis smo določili z metodo bližnje infrardeče spektroskopije. Hiperoksični stimulus je povečal $\Delta[O_2Hb]$ (~2 μM) in znižal $\Delta[HHb]$ (~3.6 μM) v skorji frontalnega dela možgan; vrednosti so se vrnile na normalne vrednosti z dihanjem zraka. V medrebrnih mišicah in v mišici vastus lateralis pa smo izmerili le značilnio znižan $\Delta[HHb]$ med dihanjem čistega kisik. Odzivi v mišicah so bili znatno počasnejši od odzivov v cerebralnem delu. Vrednosti so se deloma vrnile na izhodščne vrednosti med 15 minutnim okrevanjem v normoksiji. Akutno dihanje čistega kisika v

mirovanju vpliva na oksigenacijo cerebralnega dela ter na medrebrne mišice in mišico vastus lateralis pri zdravih preiskovancih, vendar ne v enakem vzorcu. Cerebralno tkivo je bolj občutljivo na spremembe, ki so posledica normobarične hiperoksije, kot je mišično tkivo. To se vidi v heterogenem vazokonstriktornem odzivu na čisti kisik in v različih vzorcih povratka na izhodiščne vrednosti po prehodu na normobarično hiperoksijko.

Namen *Študije IV* je bil določiti učinek ogljikovega monoksida (CO) v dihalni mešanici v količinah, ki so prisotne med prometnimi konicami v gosto naseljenih urbanih predelih, na oksigenacijo. Ta učinek smo merili v sprednjem kortikalnem delu možgan, v medrebrnih mišicah in v mišici vastus lateralis med vadbo na cikloergometru. Ker se normobarični kisik uporablja za pospeševanje odstranjevanja CO iz krvi, smo proučili tudi učinek dihanja čistega kisika na cerebralno oksigenacijo, na oksigenacijo medrebrnih mišic in mišice vastus lateralis, po dihanju mešanice s 18,9 ppm CO med vadbo,. Devet zdravih preiskovancev je sodelovalo v treh poskusih: (a) 3-urna izpostavitev zračni mešanici in nato aerobni test moči (CPT), (b) eno-urna izpostvitev zračni mešanici, nato 2-urna izpostavitev normoksični mešanici, ki je vsobovala 18,9 ppm CO, in nato aerobni test moči (CPT_{CO_A}), in (c) 2-urno dihanje normoksične mešanice, ki je vsobovala 18,9 ppm CO, nato 1-urno dihanje 100% normobaričnega kisika, in nato aerobni test moči (CPT_{CO_B}). Vse aerbne teste moči so preiskovanci izvajali na cikoergometru nastavljenem na 85% največje dosežene obremenitve. Oksi- ($\Delta[O_2Hb]$), deoksi- ($\Delta[HHb]$) and totalni ($\Delta[tHb]$) hemoglobin v cerebralnem delu, v medrebrnih mišicah in v mišici vastus lateralis smo med aerobnimi testi moč merili z bližnjo infrardečo spektroskopijo. Dosežen čas vadbe je bil enak za vse skupine. $\Delta[O_2Hb]$ in $\Delta[tHb]$ v mišici vastus lateralis in v medrebrnih mišicah je bil nižji ($P \leq 0.05$) med CPT_{CO_A} kot pri CPT. Med CPT_{CO_B} sta bila $\Delta[O_2Hb]$ in $\Delta[tHb]$ večja ($P \leq 0.05$) kot pri CPT_{CO_A}. Med vsemi tremi poskusi ni bilo razlike v oksigenaciji cerebralnega dela. Dihanje mormoksične mešanice, ki vsebuje 18,9 ppm CO, zmanjša oksigenacijo v mišicah vastus lateralis in serratus anterior, vendar ne vpliva na aerobni test moči, oziroma na dosežen čas zmogljivosti na cikloergometru. Predhodno dihanje čistega kisika zmanjša učinek CO v mišicah, predvsem v medrebrnih mišacah. Ne učinkuje na vzdržljivost.

Abbreviations

CaO_2	=	arterial oxygen content
CNS	=	central nervous system
CO	=	carbon monoxide
CO_2	=	carbon dioxide
COHb	=	carboxyhaemoglobin
COMb	=	carboxymyoglobin
DAP	=	diastolic arterial pressure
DPF	=	differential path-length factors
D-RPE	=	dyspnoea-respiratory discomfort
EPO	=	erythropoietin
[EPO]	=	erythropoietin concentration
EPO-Rs	=	receptors of erythropoietin
FiO_2	=	inspired oxygen fraction
FEV_1	=	forced expiratory volume in 1 second
FS	=	feeling scale
FVC	=	forced vital capacity
GSH	=	glutathione
Hb	=	haemoglobin
Hct	=	haematocrit
HIF	=	hypoxia inducible factor
HR	=	heart rate
HYPER	=	hyperoxic group
[La]	=	lactate concentration
L-RPE	=	leg effort
MAP	=	mean arterial pressure
Mb	=	myoglobin
MVV	=	maximum voluntary ventilation
NIRS	=	near-infrared spectroscopy
NO	=	nitric oxide
NOR	=	normoxic group
O_2	=	oxygen
OFR	=	oxygen-free radicals
PaO_2	=	partial pressure of arterial oxygen
$PaCO_2$	=	partial pressure of arterial carbon dioxide
PEF	=	peak expiratory flow
$P_{ET}CO_2$	=	partial pressure of end-tidal carbon dioxide
$P_{ET}O_2$	=	partial pressure of end-tidal oxygen
PFT	=	pulmonary function test
PO_2	=	partial pressure of oxygen
PPO	=	peak power output
Q	=	cardiac output
RBC	=	red blood cell
RPE	=	rate of perceived exertion

ROS	=	reactive oxygen species
SD	=	standard deviation
SE	=	standard error
SAP	=	systolic arterial pressure
SpO_2	=	arterial oxygen saturation
SV	=	stroke volume
SVC	=	slow vital capacity
$\dot{V}E$	=	minute ventilation
VHL	=	Von Hipple Lindau protein
$\dot{V}O_2$	=	oxygen consumption
$\dot{V}O_{2max}$	=	maximal oxygen uptake
$\Delta T_{calf\text{-}toe}$	=	calf-toe skin temperature gradient
Δ[tHb]	=	total haemoglobin
$\Delta[O_2Hb]$	=	oxyhaemoglobin
Δ[HHb]	=	deoxyhaemoglobin

1 Introduction

In 1774, the English natural scientist Joseph Priestley discovered the element of *"l'air vital"* for aerobic organisms that was termed *"air dephlogistique"* (Priestley, 1775). This was oxygen (O_2), the essential element for the existence and sustainment of life. Since then, O_2 has become one of the most effective, widely available and cheap pharmacon for treatment or prevention of hypoxaemia and tissue hypoxia through increased O_2 saturation levels of arterial haemoglobin (SpO_2) and enhanced partial pressure of O_2 dissolved in arterial plasma (PaO_2) (cf. Treacher and Leach, 1998). Namely, hyperoxia is defined as any level of partial O_2 pressure (PO_2), inspired (in vivo) or superfused (in vitro), greater than the maximal level of inspired PO_2 (>160 Torr) in our natural environment (Dean et al., 2004). The level of supplemental O_2 that humans can be exposed to in medical settings varies: it is administered either at normal atmospheric pressure (1 ATA; normobaric hyperoxia) or at increased pressure (hyperbaric hyperoxia), up to 2280 Torr (~3 ATA pure O_2). The present thesis focuses on normobaric hyperoxia, which is extensively used in several clinical and non-clinical conditions.

Notwithstanding the vital role of O_2, hyperoxia is a powerful drug that could be harmful, in view of the fact that continuous exposure to increased O_2 concentrations for hours or days induces an excessive production and accumulation of reactive O_2 species (ROS) that have a subtle, but toxic action on neurological function in the immature central nervous system (CNS) (Grave et al., 1972) and a modulating influence on neuronal excitability in the mature CNS (Bickford et al., 1999). In particular, normobaric hyperoxia seems to cause detrimental effects on the human lungs (Beckett and Wong, 1988; Caldwell et al., 1966; Dean et al., 2004; Deneke and Fanburg, 1980), which eventually might lead to decease, as has already been reported in studies with animals (Crapo et al., 1980). The precise concentration of O_2 that may be toxic to the lungs is still unknown; it depends on several characteristics of the exposed individual, such as age, nutrition, endocrine status and previous exposure to O_2 or other oxidants (cf. Deneke and Fanburg, 1980).

Normobaric O_2 is still administered mostly empirically, and the haemodynamic and haematological responses to it are still poorly understood. In fact, there are evidences to believe that hyperoxia may be self-defeating in an attempt to increase tissue oxygenation (Reinhart et al., 1991). For instance, the notion that supplemental O_2 is not hazardous has been challenged by the findings that normobaric hyperoxia induces a significant reduction in cardiac output (Q) and increased systemic vascular resistance in patients with congestive cardiac failure (Haque et al., 1996; Saadijan et al., 1999). In this regard, there is a debate whether the administration of pure O_2 might be dangerous due to such a hyperoxia-induced vasoconstrictive effect, which partly offsets the increased O_2 delivery (cf. Forkner et al., 2007a; 2008; Her, 2008). Despite a paucity of data supporting the notion that hyperoxia stimulates erythropoiesis, normobaric hyperoxia is currently being advocated as an efficient treatment of primary anaemia and an adjuvant therapy for cancer patients suffering from chemotherapy-induced anaemia (Balestra et al., 2010b; Burk, 2007; Calzia et al., 2010; De Bels et al., 2011). Furthermore, based on the controversial clinical evidence that normobaric hyperoxia alleviates carbon monoxide (CO) poisoning (cf. Weaver, 2009), as it hastens the elimination of CO from the blood (Weaver et al.,

2000), it has been proposed that pre-exposure to pure O_2 might enhance exercise performance when it is carried out in an environment with high CO concentration (Shephard, 1984).

Accordingly, any benefits of administering pure O_2 to healthy and unhealthy individuals must outweigh its toxic effects, and must be based on clear and convincing evidence concerning its effectiveness. The present thesis deals with the examination of selected haemodynamic and haematological responses to acute and long-term exposure to normobaric hyperoxia in young, healthy individuals.

1.1 Cardiovascular responses to normobaric hyperoxia

Administration of pure O_2 induces a number of haemodynamic and circulatory responses both in animals and humans, the most consistent being bradycardia, and cerebral and microvascular vasoconstriction (Bulte et al., 2007; Daly and Bondurant, 1962; Rousseau et al., 2005; 2007). These responses seem to constitute a defense of the exposed tissue against the high arterial O_2 tensions (Bird and Telfer, 1966). Still, it is difficult to have an overall view of the O_2-induced effects, given the contrasting findings of different studies. There may be several explanations for the discrepancy between results obtained in different investigations including different experimental protocols, heterogeneous study populations and the wide range of inter-individual responses to O_2 exposure. Current findings with regard to the main cardiovascular responses to acute normobaric hyperoxic exposure at rest will be briefly described in the following paragraphs.

Systemic haemodynamics responses. The most consistent response to normobaric hyperoxia is a reduction of heart rate (HR) (Crawford et al., 1997; Daly and Bondurant, 1962; Gole et al., 2011; Larsson et al., 2010; Litscher et al., 1990; Parkinson, 1912; Rousseau et al., 2005; Waring et al., 2003), caused by reduced sympathetic tone (Seals et al., 1991) and enhanced vagal efferent discharge (Gole et al., 2011; Lund et al., 1999; Milone et al., 1999). However, the exact afferent pathway that triggers the O_2-induced bradycardia is still unclear and hypothetical, and may include either central and/or peripheral mechanisms. In particular, it has been suggested that the high levels of the arterial O_2 tension during hyperoxic exposures reduce arterial chemoreceptors activation, which in turn decreases HR (Daly and Bondurant, 1962; Gole et al., 2011). Yet, Lodato and Jubran (1993) argued that the arterial chemoreflex acting through cardiac vagal efferents requires only a few seconds to be fully activated, and thus cannot entirely explain the bradycardic response to hyperoxia that needs approximately 5 minutes to evolve.

A second proposed mechanism to the hyperoxia-induced drop of HR is increased arterial baroreflex activity ensued by enhanced vagal activation (Rousseau et al., 2005). However, the role of the baroreceptors has been questioned because of the inconsistent findings concerning the mean arterial pressure (MAP) response to O_2 breathing. Namely, some of the studies have shown a slight rise in both systolic (SAP) and diastolic (DAP) pressure during the hyperoxic exposure (Alvery and Brody, 1948; Daly and Bondurant, 1962; Waring et al., 2003; Whitehorn et al., 1946), while others have not observed any changes (Behnke et al., 1935; Dufour et al., 2010; Gole et al., 2011; Larsson et al., 2010; Rousseau et al., 2005; Yamazaki et al., 2007).

Aside from the aforementioned central parasympathetic activity, the O_2-induced reductions in blood perfusion might have a negative chronotropic and inotropic effect on the heart (Rousseau et al., 2005). Namely, it has been hypothesized that the vasoconstriction at the microvascular level tends to raise the MAP, an effect which is dampened by activation of the baroreceptors, which not only reduces precapillary vasomotor tone, but also lowers HR (Rousseau et al., 2005). The hyperoxia-induced

peripheral vasoconstriction probably results from the endothelium by either an enhanced release of vasoconstrictors (O_2 radicals or serotonin) (Gustafsson and Sjoberg, 1996; Rubanyi and Vanhoutte, 1986) or a diminished effect of vasodilators (prostaglandin E2, endothelium-derived relaxing factor) (Messina et al., 1994). It has been suggested that even the red blood cells (RBC) might have a direct role in the regulation of the vascular tone; given the fact that they are not only the major carriers of O_2, but also serve as O_2 sensors and affectors of changes in O_2 delivery via its release of ATP (Dietrich et al., 2000; Ellsworth et al., 1995; 2000; 2009; Sprague et al., 2010).

The majority of the studies support that administration of pure O_2 slightly decreases Q (Asher et al., 1988; Gole et al., 2011; Lodato, 1989; Waring et al., 2003), although the physiological significance of the small change (10-15%) is uncertain (Mathieu et al., 2006). Conversely, there are several studies that have not observed any change in Q during the hyperoxic exposure (Joachimsson et al., 1996; Kety and Schmidt, 1948; Matalon et al., 1982; Smith et al., 1963). Likewise, the findings concerning the effect of hyperoxia on stroke volume (SV) are unclear, since there are only few studies that have reported reduced SV in healthy individuals (Gole et al., 2011; Rousseau et al., 2005). Hyperoxia has more consistently reduced SV in studies on patients with congestive cardiac failure (Mak et al., 2001), suggesting that these patients may be more sensitive or respond differently to pure O_2 (Thomson et al., 2006).

Cerebral and muscle blood flow. The response of cerebral blood flow to supplemental O_2 was first described in humans by Kety and Schmidt (1948), who observed a mean decrease of ~13%. Subsequent studies confirmed these findings, and suggested that hyperoxia causes a reflex cerebral vasoconstriction that leads to a marked reduction of cerebral blood flow (Bulte et al., 2007; Floyd et al., 2003; McLeod et al., 2003; Tisdall et al., 2009; Watson et al., 2000). However, it is still not clear whether the O_2-induced changes in cerebral perfusion are dominated by the reduction in arterial CO_2 pressure ($PaCO_2$) or by the increase of arterial PO_2 (PaO_2) *per se*. Namely, a typical, although paradoxical, response to O_2 exposure is a slight hyperventilation (cf. Dean et al., 2004), which reduces end-tidal PCO_2 ($PETCO_2$) resulting in hypocapnia (Becker et al., 1996), which in turn is accompanied by the vasoconstriction in arterioles reducing the cerebral blood flow (Ogoh et al., 2010). It has also been suggested that the increased PaO_2 might also have a direct vasoconstrictive effect (Floyd et al., 2003; Kolbitsch et al., 2002) to the cerebral parenchyma (Phillis, 1989) and/or the cerebrovascular endothelium (Demchenko et al., 2002) and/or specific brain O_2 sensitive neurons (Golanov and Reis, 1996) and/or the red blod cells (Dietrich et al., 2000; Sprague et al., 2010).

On the other hand, the findings as regards the effect of hyperoxia on muscle blood flow are inconsistent. Namely, the majority of the studies have shown a significant decrement during normobaric hyperoxic exposure (Bredle et al., 1988; Crawford et al., 1997; Rousseau et al., 2005), while a few studies have not detected any change in muscle blood flow during hyperoxia (Dufour et al., 2010; Plewes and Farhi, 1983). It has thus been suggested that precapillary vessels may respond to pure O_2 with stronger vasoconstriction in the cerebral than in the skeletal muscle tissue (Larsson et al., 2010); although this hypothesis has not been tested by any specific experiment.

1.2 Normobaric hyperoxia and erythropoiesis

The glycoprotein hormone erythropoietin (EPO) is essential for the production of RBC, inasmuch as it activates the proliferation, differentation and maturation of the bone marrow erythroid progenitor cells (Jelkman et al., 2011). Therefore, recombinant human EPO is mainly used in patients with anaemia (e.g. HIV/AIDS patients, chemotherapy and radiotherapy induced anaemia in cancer patients) and in particular, in patients suffering

from anaemia due to the EPO deficiency resulting from chronic renal failure (Jelkman et al., 1992; Mastromarino et al., 2011). It has also been shown that EPO treatment enhances maximal O_2 uptake ($\dot{V}O_{2max}$) and endurance exercise performance by increasing the O_2 transport capacity (Audran et al., 1999; Lundby et al., 2008; Robach et al., 2008; Thomsen et al., 2007). Additionally, a number of newly discovered EPO receptors (EPO-Rs) in non-haematopoietic tissues have illuminated the non-erythropoietic functions of EPO (Buemi et al., 2002; Jelkman et al., 2007; Lundby and Olsen, 2011). Namely, it has been suggested that EPO has beneficial effects on patients with ischaemic stroke (Ehrenreich et al., 2002; 2009), hypoxic retinal disease (Grimm et al., 2002), gastrointestinal ischaemia (Squadrito et al., 1999), ischaemic spinal cord injury (Celik et al., 2002), renal ischaemic injury (Bahlmann et al., 2004) and ischaemic heart disease (Mastromarino et al., 2011). Moreover, high doses of EPO seem to modulate the neural processing and thus might improve the cognitive function of healthy (Miskowiak et al., 2008a; 2008b; Ninot et al., 2006) and non-healthy (i.e. schizophrenia, depression) (Ehrenreich et al., 2007; Miskowiak et al., 2010) individuals.

The earliest documented observation inferring to EPO was by Jourdanet (1863), who observed that inhabitants at high altitude had more viscous blood. However, the relationship between the O_2 content of the blood and erythropoiesis was first described about 100 years ago by the French histologist Francois-Gilbert Viault (1890), who noted that the number of erythrocytes in his blood increased significantly during an expedition to the highlands of Morococha in Peru (~4500 m). The observation was confirmed by several others (Miescher et al., 1893a; 1893b; Muntz, 1891). Indeed, it has been well-documented that the cells controlling the synthesis of EPO are regulated by the O_2 availability (cf. Jelkman, 1992; Jelkman, 2011); and it is widely accepted that acute (Friedmann et al., 2005; Mackenzie et al., 2008) and chronic (Berglund et al., 2002; Chapman et al., 1998; Ge et al., 2002; Gunga et al., 1994) hypoxia induce an enhancement of EPO (cf. Jelkman, 1992; Weidemann and Johnson, 2009) in the kidneys (Bauer and Kurtz, 1989), and, in minor amounts, in the liver (Clemons et al., 1986) and the brain (Pagel et al., 1989; von Wussow et al., 2005). A dose-response relationship exists between the degree of hypoxia and the release of EPO, the degree of hypoxia being determined both by inspired O_2 fraction (FiO_2) and the duration of the hypoxic exposure (MacKenzie et al., 2008). Namely, it has been shown that either a short period (~70-120 min) of continuous hypoxic exposure (Eckardt et al., 1989; Knaupp et al., 1992; MacKenzie et al., 2008; Rodriguez et al., 2000) or intermittent hypoxia induced by repeated voluntary maximal duration apnoeas (de Bruijn et al. 2008; Richardson et al., 2009) markedly increase the levels of EPO.

In contrast to a hypoxic stimulus to EPO synthesis and erythropoiesis, hyperoxia seems to induce an eryhtropoietic suppression in both animals and humans (Table 1.1). Although the mechanisms underlying the hypoxia-inducible upregulation of the EPO gene are understood in considerable detail, the mechanisms for the O_2-induced suppression of EPO remain unresolved. Considering the shape of the oxyhaemoglobin binding curve, the O_2 content of arterial blood does not increase substantially during the exposure to pure O_2, and hence it is unlikely that the hyperoxia-induced drop of EPO is attributable to increased O_2 availability in the renal tissue (Jelkman, 1992). In addition, renal blood flow does not appear to be influenced by normobaric hyperoxia judging from studies performed on conscious rats (Flemming et al., 2000; Torbati et al., 1979) and dogs (Berry et al., 1998). Thus, it is questionable whether the renal cortex is the principal origin of diminished EPO secretion. Recent studies regarding the presence of humoral factor(s) stemming from the hypothalamic-hypophyseal system – i.e. adrenocorticotropic hormone, growth hormone, thyroid hormones and sex steroid hormones (Jelkman, 1992)

– that, at least in part, have a regulating effect on renal EPO secretion (Pagel et al., 1989; von Wussow et al., 2005), and given the existence of sensors in the brain capable of detecting changes in arterial PO_2 (Lahiri et al., 2006), the role of the CNS on the suppression of [EPO] should be taken into account. Thus, the mechanisms underlying the O_2-induced suppression of EPO remain speculative and need to be further investigated.

Table 1.1: *Summary of studies investigating the erythropoietin concentration ([EPO]) response to normobaric hyperoxia in animals and humans.* ↓: decrease; ↑: increase; ∅: no change.

Study	Subjects	Protocol	Findings
Fletcher et al., (1973)	1. 60 healthy rats 2. 280 healthy rats	100% O_2 for intervals of: a. 24, 48, 72 & 120 hours b. 0-20 days	↓ [EPO]
Morshchakova et a., (1980)	healthy rats	100% O_2, 4 hours	↓ [EPO]
Kokot et al., (1994a)	19 patients with chronic renal failure	100% O_2, 2 hours	↓ [EPO]
Kokot et al., (1994b)	15 hypertensive patients and 15 healthy	100% O_2, 2 hours	↓ [EPO] in both groups
Balestra et al., (2006)	16 healthy ♂ and ♀	100% O_2, 2 hours	↑ [EPO]
McGuire et al., (2006)	6 healthy ♀	100% O_2, 2 hours	∅ [EPO]
Momeni et al., (2011)	31 healthy ♂ and ♀	100% O_2, 90 min	∅ [EPO]
Embury et al., (1984)	3 patients with sickle-cell anaemia	100% O_2, 5 days	↓ [EPO]
Tsangaris et al., (1999)	16 patients with acute or chronic respiratory failure	100% O_2, 6 days	↓ [EPO], Hb, Hct

In contrast to the studies suggesting that hyperoxia suppresses EPO production, two recent investigations have reported that the initial decrease of serum EPO is followed by a significant increase 24 and 36 hours after the cessation of a 2-hour normobaric O_2 breathing (Balestra et al., 2004; 2006). The suggested mechanism for such a hyperoxia-induced EPO production, which is coined the "normobaric O_2 paradox", is the sudden and sustained decrease in tissue O_2 level ("relative hypoxia") upon the transition from hyperoxic to normoxic breathing conditions (Balestra et al., 2006). The authors have proposed a potential molecular mechanism in order to interpret these findings (Figure 1.1), which however, remains speculative and needs to be further investigated.

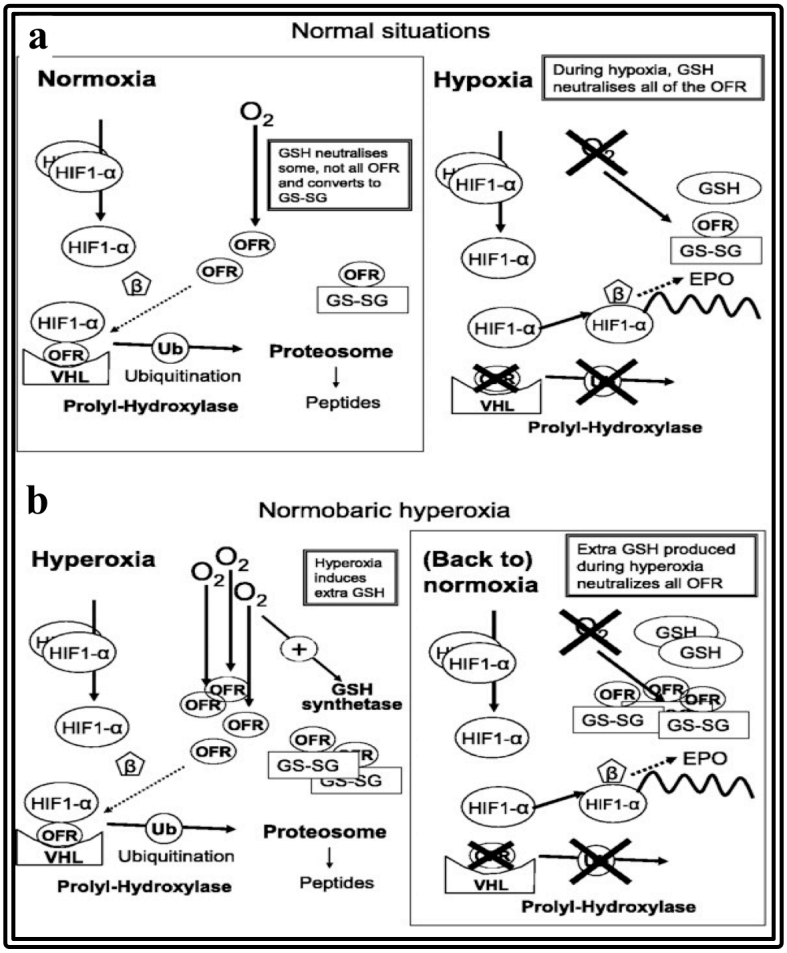

Figure 1.1: Schematic representation of the suggested mechanism for the "normobaric O_2 paradox" theory. (a): exposure of mammalian cells to hypoxia activates a transcriptional response pathway mediated by hypoxia inducible factor (HIF). These responses include the upregulation of genes involved in erythropoiesis, angiogenesis and glycolysis. HIF is a heterodimeric transcription factor composed of a hypoxia-inducible α subunit and a constitutively expressed β subunit, also known as the aryl hydrocarbon receptor nuclear translocator. HIF1-α is continuously produced, but also continuously ubiquitinated through reactions involving Von Hipple Lindau (VHL) protein and prolyl-hydroxylase and then degraded by proteosomes; this reaction needs O_2-free radicals (OFR), some of which are neutralized by glutathione (GSH): thus intracellular HIF1-α concentrations remain low. During hypoxia, GSH can neutralize all OFRs, and HIF1-α and –β dimerization can occur and proceed to the EPO gene expression (Haddad et al., 2000). (b): during normobaric hyperoxia, OFRs stimulate GSH production (GSH synthetase) and thus, on returning to normoxic conditions, all OFRs are neutralized by the increased intracellular GSH. This induces EPO gene expression similarly to hypoxia, and this situation has been termed the "normobaric O_2 paradox". (modified from Balestra et al., 2006).

The paradoxical increase of endogenous EPO after a brief period of O_2 breathing could not been confirmed by two similar studies. Namely, McGuire et al., (2006) failed to detect any significant difference in plasma EPO in healthy females after a single O_2 administration, when compared to their pre-exposure values. Likewise, Momeni et al., (2011) did not find any increment of EPO following 90 min of pure O_2 breathing in healthy non-smokers. The discrepancy between the results in these studies and in those by Balestra and co-workers might be explained by the wide inter-individual variability of EPO response to external stimuli (Friedmann et al., 2005; MacKenzie et al., 2008) that might be linked to specific genetically inherited traits (Ou et al., 1998; Jedlickova et al., 2003). In addition, the diurnal variation of EPO may have contributed to the serendipitous

observation of the "normobaric O_2 paradox", though results from studies examining the diurnal variation of EPO are equivocal (Gunga et al., 2007): some of the studies have detected pronounced changes during the course of the day, described by nadir values of EPO in the morning hours and zenith levels during the evening and night hours (Cahan et al., 1992; Cotes and Brozovic, 1982; Klausen et al., 1993; Kokot et al., 1994b; Wide et al., 1989), while others have not observed any daily variation (Gunga et al., 1996; Miller et al., 1981; Roberts and Smith, 1996).

Despite the conflicting evidence regarding the erythropoietic potential of pure O_2, the "normobaric O_2 paradox" is recommended as an efficient method to enhance erythropoiesis in healthy and non-healthy individuals (Balestra et al., 2004; 2006; 2011). Normobaric O_2 is advocated as a simple, inexpensive and advantageous treatment both for primary anaemia and as an adjuvant therapy for cancer patients suffering from chemotherapy-induced anaemia (Balestra et al., 2010b; Burk, 2007; Calzia et al., 2010; Ciccarella et al., 2011; De Bels et al., 2011). It is also suggested as an ergogenic method to increase endurance performance in athletes (Balestra and Germonpre, 2010). Yet, it appears that the recommendations for this therapy are based on the findings of a single study investigating the effect of an acute exposure to normobaric hyperoxia (Balestra et al., 2006), disregarding the inter-individual variability and the diurnal variation of the EPO, as well as the paucity of data on the long term-effects of O_2 breathing on erythropoiesis. Last but not least, as it was mentioned above, any benefits of administering O_2 at such high doses must exceed its toxic action on the lungs (Deneke and Fanburg, 1980). *Ergo*, it appears warranted to evaluate the "normobaric O_2 paradox" theory in a homogenous group of healthy individuals through the explication of an acute and long-term hyperoxic stimulus, the latter potentially having clinical relevance.

1.3 Carbon monoxide and exercise performance: the potential action of normobaric hyperoxia

CO is a colorless, odorless and tasteless diatomic gas that is considered as a toxic byproduct of environmental and industrial processes (Durante et al., 2006). Its toxic effect is well-known, and mainly resides in its strong affinity for haemoglobin (Hb) that is nearly 245 times that of O_2. The binding of CO with Hb results in the formation of carboxyhaemoglobin (COHb), which in turn leads to a marked reduction in O_2 carrying capacity (Asmussen and Chiodi, 1941). In addition, the partial occupation of CO at the heme binding sites inhibits the release of O_2 from the remaining heme groups, shifts the Hb-O_2 dissociation curve to the left, decreases the O_2 delivery to the tissues and leads to tissue hypoxia (Roughton and Darling, 1944). Moreover, higher concentrations of CO can bind with the myoglobin (Mb), forming carboxymyoglobin (COMb) (King et al., 1987), and with the cytochrome *c* oxidase (Piantadosi et al., 1987), which could further amplify the detrimental actions of CO (Piantadosi, 2002).

Due to the aforementioned physiological mechanisms, CO has also been considered a major air pollutant capable of affecting exercise performance in healthy individuals. Several studies have observed that moderate levels of CO inhalation during exercise markedly reduce aerobic capacity and endurance performance (Aronow and Cassidy, 1975; Ekblom and Huot, 1972; Horvath et al., 1975; Nielsen, 1971; Pirnay et al., 1971; Vogel and Gleser, 1972). It has been suggested that an increase in COHb above 2.7% leads to a significant drop of aerobic performance (Raven et al., 1974). A number of recent studies, which have analyzing athletes' performance times in city races (i.e. running, cycling) and correlating it to the monitored levels of air pollutants of the area that the competitions were held (i.e. Athens, Beijing), have shown that the current levels of inspired CO do not seem to be the most detrimental factor limiting exercise

performance (Flouris, 2006; Lippi et al., 2008; Marr and Ely, 2010; Nassis and Geladas, 2002). However, it is still unknown whether, or to what extent, the current levels of CO recorded in the modern megalopolises (Figure 1.2) could have an intoxicating action to exercise performance of healthy individuals.

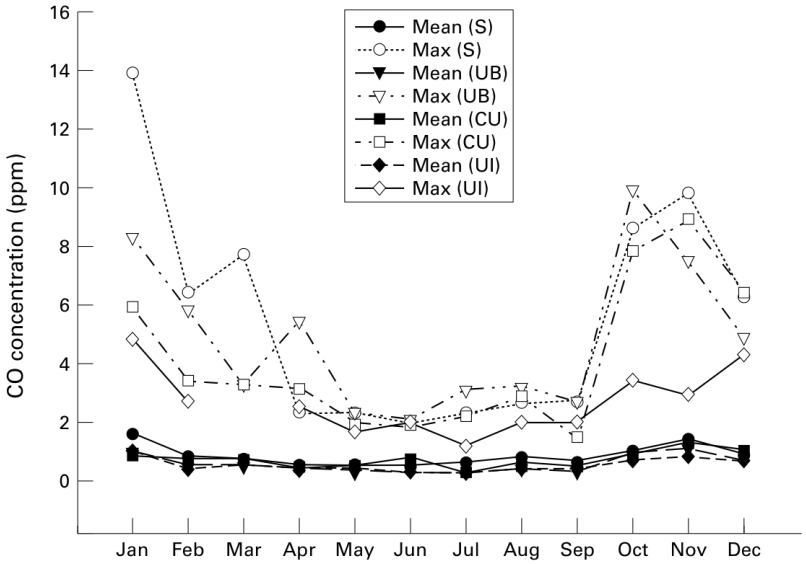

Figure 1.2: *Arithmetic mean and maximum hourly average monthly statistics of 1997 carbon monoxide (CO) levels at four British sites from the UK National Monitoring Network.* S: suburban; UB: urban background; CU: central urban; UI: urban industrial. (Carlisle and Sharp, 2001).

The regular therapy for CO poisoning has traditionally been the administration of normobaric O_2 (Piantadosi, 2004; Weaver, 2009), since it hastens the elimination of CO from blood (Weaver et al., 2000). In line with this, it has been suggested that pre-exposure to normobaric hyperoxia might improve exercise performance in environmental conditions with increased CO concentration (Shephard, 1984), although no study has been performed specifically investigating this hypothesis.

2 Aims and Hypotheses

The *overall aim* of the present thesis was to examine the effect of acute and long-term normobaric hyperoxic exposure on select haemodynamic and haematological responses during rest and exercise in healthy aerobically well-trained males. This general purpose was evaluated in four separate studies, and each of them had a specific aim and hypothesis listed below:

- **Study I.** This study investigated the effect of a 2-hour normobaric hyperoxic exposure on the plasma EPO in healthy individuals.

We hypothesized that an initial phase of suppressed EPO production will transpire a few hours after the normobaric hyperoxic intervention, and that such hyperoxia-induced drop in EPO will not be followed by any further increment compared to in the control condition.

- **Study II.** This study investigated the effect of ten daily short-term exposures to normobaric hyperoxia over a 2-week period on the plasma EPO in healthy individuals.

We hypothesized that although the evidence of erythropoiesis following a single hyperoxic exposure is equivocal, repeated stimuli of increased O_2 tension might give rise to a cumulative effect, which would potentially have clinical relevance.

- **Study III**. This study mapped the cerebral, intercostal and leg muscle oxygenation of young healthy males during an acute short-term normobaric O_2 administration at rest.

We hypothesized that the high levels of arterial O_2 tension will influence primarily the cerebral frontal cortex, and to a lesser degree the limb and respiratory muscles.

- **Study IV**. This study investigated the effect of low levels of CO in the inspired air on cerebral, respiratory and leg muscle oxygenation during constant-power cycle ergometry. A secondary purpose was to examine the effect of pre-exposure to pure O_2 on cerebral and muscle oxygenation during such constant-power exercise under CO conditions.

We hypothesized that the CO administration:

[a] will reduce exercise performance,

[b] will decrease leg and respiratory muscle oxygenation via formation of COHb and a leftward shift of the O_2 dissociation curve, but

[c] will not influence the cerebral oxygenation due to the unaltered levels of PO_2.

In addition, we hypothesized that short-term pre-exposure to normobaric hyperoxia will not increase exercise performance, but will improve muscle oxygenation as a result of regional elimination of CO.

3 Thesis structure

The hypotheses outlined above have been addressed in four separate research studies, which are reported in four separate chapters (Chapter 4-7).

In particular, Chapter 4 presents the effect of acute exposure to normobaric hyperoxia on plasma [EPO] in healthy males.

Chapter 5 presents the effect of long-term exposure to normobaric hyperoxia on plasma [EPO] in healthy males.

Chapter 6 presents the effect of acute exposure to normobaric hyperoxia on cerebral, respiratory and leg muscle oxygenation.

Chapter 7 presents the effect of CO exposure on cerebral and muscle oxygenation during exercise performance, and the role of pre-exposure to normobaric hyperoxia.

The findings of the present thesis are summarized in Chapter 8.

4 Acute normobaric hyperoxia and plasma [EPO]

Erythropoietin (EPO) is a glycoprotein hormone that is produced primarily by the adult kidney. It stimulates the proliferation, differentiation and maturation of the bone marrow erythroid progenitor cells, and accordingly regulates the production rate of red blood cells (Gunga et al., 2007; Jelkmann, 1992; 2010). The secretion of EPO is regulated by the relative amount of O_2 availability to the tissues, and it is broadly accepted that acute (Friedmann et al., 2005; Mackenzie et al., 2008) and chronic (Berglund et al., 2002; Chapman et al., 1998; Ge et al., 2002; Gunga et al., 1994) hypoxia lead to an enhancement of EPO formation. In particular, it has been shown that either a short period (~70 to 120 min) of continuous hypoxic exposure (Eckardt et al., 1989; Knaupp et al., 1992; Mackenzie et al., 2008; Rodriguez et al., 2000) or intermittent hypoxia induced by repeated voluntary maximal duration apneas (de Bruijn et al., 2008) markedly increase the levels of EPO.

In contrast, an erythropoietic suppression following hyperoxic exposure in animals (Fletcher et al., 1973; Morshchakova et al., 1980) and humans (Kokot et al., 1994a; 1994b) has been reported, although the underlying physiological mechanisms are still unclear. Recent studies have reported that the initial decrease of serum EPO is followed by a significant increase 24 and 36 hours after the cessation of a 2-hour normobaric O_2 breathing (Balestra et al., 2004; 2006). The authors suggested that such hyperoxia-induced EPO production, which they term the "normobaric O_2 paradox", is due to the sudden and sustained decrease in tissue O_2 level ("relative hypoxia") upon the transition from a hyperoxic to a normoxic breathing mixture. These findings were not confirmed by a similar study conducted by McGuire et al., (2006), who did not detect any significant differences compared to pre-exposure values after the O_2 breathing intervention.

One factor that might contribute to the aforementioned discrepancy is the wide inter-individual variability of the EPO response (Friedmann et al., 2005; Mackenzie et al., 2008). Moreover, the diurnal variation of EPO may have contributed to the observation of the "normobaric O_2 paradox"; though the results from studies investigating the diurnal variation of [EPO] are inconsistent (Klausen et al., 1993; Roberts and Smith, 1996; Wide et al., 1989).

Accordingly, the purpose of the present study was to investigate the effect of a 2-hour normobaric hyperoxic exposure on the [EPO]. To minimize the inter-individual variability of the EPO response, a homogenous group of healthy aerobically well-trained males participated in this single-blinded counterbalanced crossover study. To account for the contribution of diurnal rhythm, we monitored EPO at regular intervals for 2 weeks following a 2-hour period of breathing either air or 100% normobaric O_2. We hypothesized that an initial phase of suppressed EPO production will transpire few hours after the normobaric hyperoxic intervention, and that such hyperoxia-induced drop in EPO will not be followed by any further increment compared to in the control condition.

4.1 Materials and Methods

4.1.1 Subjects

Ten healthy males participated in the present study (Table 4.1). All were physically active on a recreational basis; however, none of them was engaged in a formal sport-training program. They were non-smokers, had no history of any renal, haematological, heart or lung disease, and had not used any drugs acting as prostaglandin inhibitors during the month preceding the experiments. The subjects were informed in detail about the experimental procedures and risks involved, and gave their written consent. They were instructed to abstain from consuming alcohol or any caffeinated product prior to, and during the study. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia and conformed to the Declaration of Helsinki.

The sample size for the present study was determined using the reported mean (SD) responses of [EPO] reported by Balestra et al., (2006), and setting the level of statistical significance at 0.05. The analysis assumed that a 15% difference in the mean (SD) values between the two experimental conditions would be statistically significant, and that the power of the test would be 0.85. *Post hoc* analysis revealed that the power of the statistical test performed was 0.95 (Cohen, 1988).

Table 4.1: *Descriptive characteristics of the subjects that participated in studies I and III.* Values are mean ± SD. FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; PEF: peak expiratory flow; SVC: slow vital capacity; $\dot{V}O_{2max}$: maximal O_2 uptake; PPO: peak power output.

Variables	
Age (years)	25.5 ± 3.0
Body mass (kg)	74.3 ± 6.5
Stature (cm)	180.4 ± 6.8
Body fat (%)	9.3 ± 4.2
FVC (L)	5.5 ± 0.6
FEV_1 (L)	4.6 ± 0.5
PEF ($L \cdot s^{-1}$)	10.5 ± 1.5
SVC (L)	5.1 ± 0.6
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	55.4 ± 5.1
PPO (W)	348 ± 30

4.1.2 Experimental protocol

On the first visit to the laboratory, subjects were thoroughly familiarized with the equipment and the experimental procedures. The experimental protocol consisted of a preliminary session during which an incremental exercise test to exhaustion was performed, followed after one week by two experimental sessions (Figure 4.1): a 2-hour 100% normobaric O_2 exposure (HYPER) and a 2-hour air exposure (NOR). The order of the two experimental sessions was randomized.

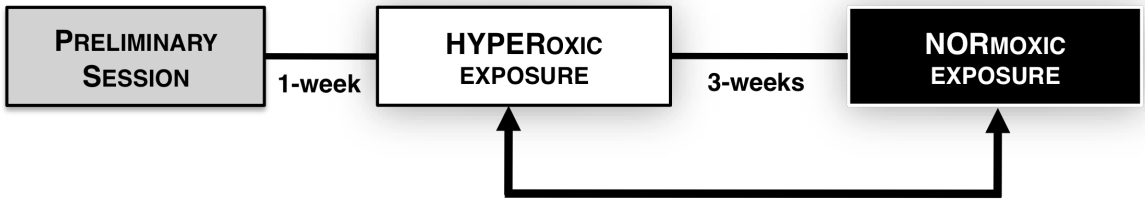

Figure 4.1: *Schematic representation of the overall protocol of studies I and III.*

4.1.2.1 Preliminary session

To ensure that subjects had similar levels of aerobic fitness, they performed an incremental exercise test to exhaustion on an electrically braked cycle-ergometer (Daun Electronic, Furth, Germany) to determine their $\dot{V}O_{2max}$ and PPO. $\dot{V}O_2$ and ventilation ($\dot{V}E$) were measured on-line with a metabolic cart (Quark CPET, Cosmed, Rome, Italy). HR was measured continuously using a HR monitor (S810i, Polar, Kempele, Finland). The blood lactate concentration ([La]) was measured from the tip of the left index finger at the 3rd min of recovery (Accutrend Lactate, Roche, Basel, Switzerland).

4.1.2.2 Air and hyperoxic exposure

One week after the preliminary session, the participants conducted the main experimental trials in a single-blind manner. Five subjects breathed 100% normobaric O_2 for 2-hours, and the rest of the participants breathed air (O_2: 20.93%) for the identical time. After a 22-day washout period, the experiments were repeated with the subjects crossed in conditions.

During both exposures, the participants were in a supine position for 2 hours and breathed through a low resistance two-way respiratory valve (Model 2, 700 T-Shape, Hans Rudolph, Inc. Shawnee, USA). The inspiratory side of the respiratory valve was connected via respiratory corrugated tubing to a 200 L Douglas bag filled with the pre-mixed humidified breathing mixture. Throughout the 2-hour period, HR was recorded continuously using a HR monitor (S810i, Polar, Kempele, Finland); and ratings of perceived exertion (RPE; scale 0-10; Wilson and Jones, 1991) for dyspnoea-respiratory discimfort (D-RPE) were requested every 15 min.

All tests were conducted at the same time of the day (beginning of the exposure at 8:00 to 8:30) to ensure that the effect of diurnal variations was similar in both trials. The environmental conditions were kept constant and thermoneutral during both exposures: the mean ambient temperature, relative humidity and barometric pressure were 21.5 ± 1.0°C, 41.0 ± 1.9% and 978 ± 9 mb, respectively. The participants were instructed not to engage in any strenuous activity a day before and throughout the first week after the exposure. Apart from that, they followed their normal daily routines (no more than 3-5 hours of exercise per week) during the next two weeks. During the entire experimental period, they were asked to record their physical activity in individual diaries.

4.1.3 Blood analyses

4.1.3.1 Erythropoietin

The participants reported to the laboratory at 7:30 in the morning, after an overnight fast. Venous blood samples were collected in EDTA tubes from an antecubital vein immediately before (Pre), in the middle (Mid) and at the end (Post) of the breathing interventions (Figure 4.2). Thereafter, blood samples were collected 3, 5, 8, 24, 32, 48, 72 and 96 hours; and 1 and 2 weeks after the cessation of O_2- or air-breathing intervention. The blood was immediately centrifuged and the plasma was frozen to -80°C for the subsequent analysis.

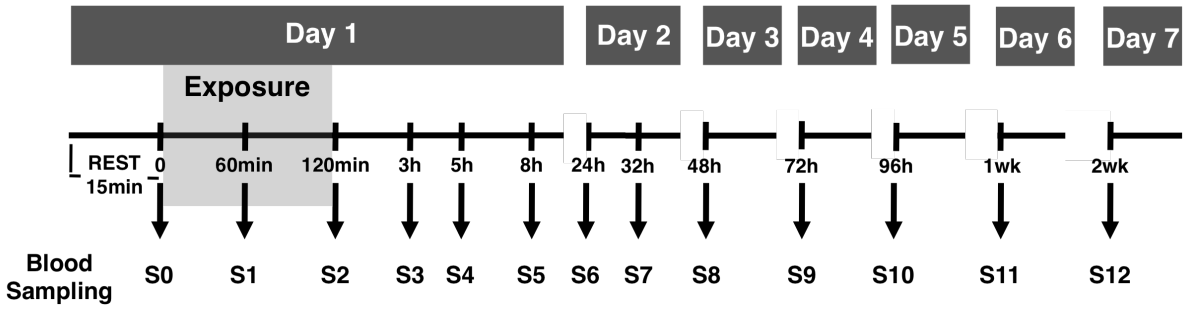

Figure 4.2: *Schematic representation of the blood sampling in the hyperoxic (HYPER) and control (NOR) phases during study I.*

The [EPO] was determined by sandwich enzyme-linked immunoassay (Quantikine IVD EPO ELISA, R&D Systems, Minneapolis, USA) using 100 µL of plasma. Optical density was quantified on a microplate reader Quant (Bio-Tek instruments, Winooski, USA) set at 450 nm and corrected at 600 nm. The current method has been validated before (Sakata et al., 1995). All techniques and materials were in accordance with the protocol provided by the company. All samples were assayed in triplicate; and one microplate was used for each subject. The estimated coefficient of variation of the analysis was 3.1%, and the sensitivity of the measurement was 0.6 $mU \cdot mL^{-1}$.

4.1.3.2 Complete blood count and reticulocyte count

Venous blood samples (500 µL) were collected for a complete blood count and reticulocyte count Pre, Mid, Post, and 1 and 2 weeks after the breathing interventions (Figure 4.2). The complete blood count, including analysis of total RBCs, Hb and haematocrit (Hct), and the reticulocyte count were obtained with an automated laser-based haematology analyzer (Advia 120, Siemens, Munich, Germany) within 6 hours after the blood sampling. The apparatus was calibrated before each measurement. All samples were assayed in duplicate. The estimated coefficient of variation for the RBC, Hb, Hct and reticulocyte count was 1.1, 0.8, 1,3 and 8.1%, respectively.

4.1.4 Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft, Inc., Tulsa, USA). All data are reported as mean (SD), unless otherwise indicated. A two-way analysis of variance (ANOVA) for repeated measures was used for the haematological variables (condition × time). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVA revealed significant F-ratio for main effects. Moreover, Pearson's correlation analysis was used for selected variables. The alpha level of significance was set *a priori* at 0.05.

4.2 Results

4.2.1 $\dot{V}O_{2max}$ testing

The average values of $\dot{V}O_{2max}$ and PPO are presented in Table 4.1. Moreover, the maximal HR, $\dot{V}E$ and [La] were 185 ± 11 beats · min^{-1}, 152.9 ± 16.6 L · min^{-1} and 14.6 ± 3.1 mmol · l^{-1}, respectively.

4.2.2 Air and hyperoxic exposure

The mean HR was significantly lower during the HYPER than the NOR condition (NOR: 59 ± 6 beats · min^{-1}; HYPER: 55 ± 6 beats · min^{-1}; $P \leq 0.05$). There was no difference in D-RPE between the exposures [NOR: 0 (0-3); HYPER 0.5 (0-5); $P > 0.05$].

4.2.3 Erythropoietin

The mean absolute values of [EPO] throughout the experimental period are presented in Figure 4.3, which also reveals the wide individual variability of plasma [EPO]. Figure 4.4 shows the relative changes of [EPO] in both conditions up to the blood sample 48 hours after the breathing interventions. During NOR, EPO showed an initial increase of approximately 35% 5 hours after the cessation of the breathing intervention; the increase was more pronounced 8 and 32 hours after the exposure (~58% and ~52%, respectively; $P \leq 0.05$). Conversely, the 100% normobaric O_2 breathing was followed by a ~36% decrement of [EPO] 3 hours after the intervention ($P \leq 0.05$); EPO returned to pre-exposure values 8 hours after the exposure. Furthermore, EPO levels were significantly lower in the HYPER than in the NOR condition 3, 5 and 8 hours after the intervention ($P \leq 0.05$); no more differences were observed between the two conditions at any other time-point.

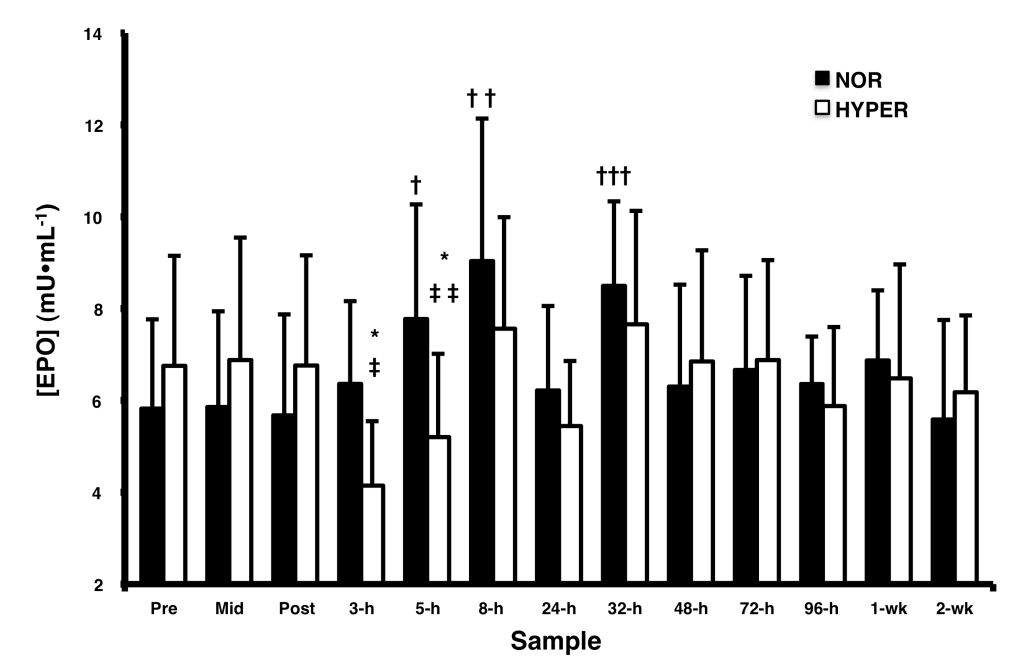

Figure 4.3: *Absolute values of erythropoietin concentration ([EPO]) Pre, Mid, Post, 3, 5, 8, 24, 32, 48, 72, 96 hours, 1 and 2 weeks after air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are mean ± SD. Significantly different: † from Pre, Mid, Post and 1 week; †† from Pre, Mid, Post, 3, 24, 48, 72, 96 hours and 1 week; ††† from Pre, Mid, Post, 3, 24, 48, 96 hours and 2 weeks; ‡ from Pre, Mid, Post, 8, 32, 48, 72 hours, 1 and 2 weeks; ‡‡ from 8 and 32 hours. * Significant difference between normoxia and hyperoxia ($P \leq 0.05$).

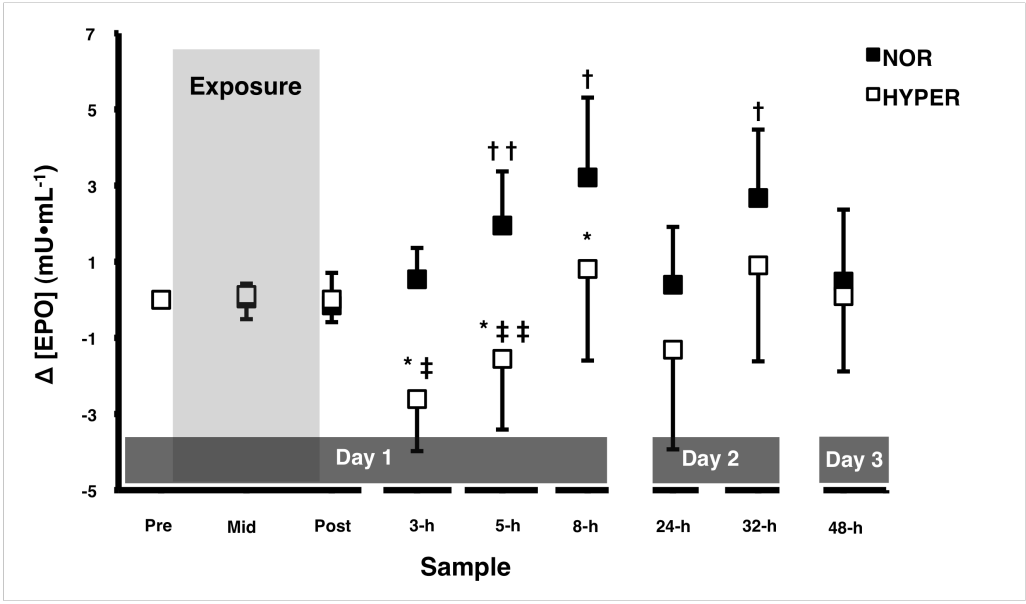

Figure 4.4: *Changes from Pre values of erythropoietin concentration (Δ[EPO]) Mid, Post, 3, 5, 8, 24, 32, 48 hours after air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are mean ± SD. Significantly different from: † Pre, Mid, Post, 3, 24 and 48 hours; †† from Pre, Mid and Post; ‡ from Pre, Mid, Post, 8, 24, 32 and 48 hours; ‡‡ from 8 and 32 hours. * Significant difference between normoxia and hyperoxia ($P \leq 0.05$).

4.2.4 Complete blood count and reticulocytes

The mean values of Hb, Hct, reticulocyte count and RBC are summarized in Table 4.2. In

both conditions, Hct and reticulocyte count did not alter throughout the experimental period ($P > 0.05$). Hb and RBC increased slightly 1 and 2 weeks after the breathing intervention ($P \leq 0.05$). However, there were no differences in any of the measured variables between the two conditions.

Table 4.2: *Haematological variables Pre, Mid, Post, 1 and 2 weeks after the air (NOR) and 100% normobaric O_2 (HYPER) breathing intervention*. Values are means ± SD; Hb: haemoglobin, Hct: haematocrit, RBC: red blood cell count. † Statistically significant different from Pre, Mid and Post; ‡ Statistically significant different from Mid; †† Statistically significant different from Pre and Mid; ($P \leq 0.05$).

	NOR					HYPER				
	Pre	Mid	Post	1-week	2-weeks	Pre	Mid	Post	1-week	2-weeks
Hb ($g \cdot dL^{-1}$)	15.0± 0.7	14.9± 0.7‡	15.0± 0.8	15.4± 0.5‡	15.5± 0.6†	14.8± 0.6	14.7± 0.4	14.8± 0.5	15.3± 0.6††	15.3± 0.7†
Hct (%)	0.44± 0.03	0.43± 0.02	0.44± 0.03	0.44± 0.02	0.45± 0.02	0.43± 0.02	0.43± 0.01	0.43± 0.02	0.44± 0.02	0.44± 0.02
Reticulocyte count (%)	0.8± 0.3	0.8± 0.2	0.8± 0.3	0.8± 0.3	1.0± 0.3	1.0± 0.3	0.9± 0.2	0.9± 0.3	0.9± 0.3	0.9± 0.2
RBC ($10^{12} \cdot L^{-1}$)	5.0± 0.2	5.0± 0.2	5.0± 0.2	5.2± 0.2†	5.2± 0.2†	4.9± 0.2	4.9± 0.2	5.0± 0.2	5.1± 0.2†	5.1± 0.2†

4.3 Discussion

The principal finding of the present study is that a short period of 100% normobaric O_2 breathing caused an initial erythropoietic suppression that was not followed by a marked increase of EPO. Moreover, besides the decrement 3 and 5 hours after the normobaric O_2 exposure, EPO seemed to change in a manner consistent with natural diurnal variation. The present results are contrary to those previously reported by Balestra et al., (2006), who detected a marked increase in [EPO] 32 hours after the exposure, but in agreement with the results of McGuire et al., (2006), who did not observe any significant changes in [EPO] after a period of breathing normobaric O_2.

It is widely accepted that tissue hypoxia is the primary stimulus of EPO production; and it is assumed that the O_2-sensitive sensor triggering the synthesis of EPO is located in the renal cortex (Bauer and Kurtz, 1989). In particular, the cells controlling the synthesis of EPO appear to respond to changes in the O_2 capacity, the O_2 tension and the O_2 affinity of the blood, and to the renal blood flow (Jelkmann, 1992). In the present study, a general decline of [EPO] was observed 3 and 5 hours after the end of O_2 breathing intervention, which confirms findings by others (Fletcher et al., 1973; Kokot et al., 1994a; 1994b; Morshchakova et al., 1980). This decrement in [EPO] is of interest in view of the shape of the haemoglobin O_2 binding curve, because the O_2 content of fully oxygenated arterial blood increases very little when the O_2 tension is enhanced above normal. Furthermore, renal blood flow does not appear to be affected by normobaric hyperoxia as suggested by studies in conscious rats (Flemming et al., 2000; Torbati et al., 1979) and dogs (Berry et al., 1998). Thus, it is questionable whether the renal cortex is the principal origin of diminished EPO secretion or increased EPO elimination. Recent studies have suggested the presence of humoral factor(s) stemming from the hypothalamic-hypophyseal system that, at least in part, has a regulating effect on renal EPO secretion (Pagel et al., 1989; von Wussow et al., 2005). The hypophysial influence on EPO production is likely mediated

by the concerted action of several hormones, including adrenocorticitropic hormone, growth hormone, thyroid hormones, and sex steroids hormones (Jelkmann, 1992), but the exact mechanisms are still undefined. Since it has been demonstrated that the brain comprises sensors for the detection of changes in arterial PO_2 (Lahiri et al., 2006), the prospect that high PO_2 suppresses EPO secretion via the central nervous system should also be considered. Thus, the mechanisms underlying O_2-induced suppression of EPO remain speculative and need to be further investigated.

4.3.1 "Normobaric O_2 paradox"

The suppression of [EPO] was diminished 5 hours after the cessation of the O_2 breathing intervention, and it was reversed to the basal values 8 hours later. Despite this, [EPO] remained lower than in NOR; and the difference disappeared one day later, in contrast to Balestra et al., (2006), who observed a marked [EPO] increase at a specific point in time following the hyperoxic exposure. The authors suggested that such hyperoxia-induced EPO production is due to the sudden and sustained decrease in renal O_2 tension ("relative hypoxia") upon the transition from a hyperoxic to a normoxic breathing condition. However, such mechanism was not confirmed by the present results.

Indeed, the EPO secretion seems to follow a natural diurnal variation, which, in the present study, might have been disturbed by the short period of O_2 breathing. The results of studies regarding the circadian rhythm on [EPO] are equivocal (Gunga et al., 2007). Some of them have detected pronounced changes during the course of the day (Brozovic, 1982; Cahan et al., 1992; Cotes and Wide et al., 1989; Klausen et al., 1993; Kokot et al., 1994b), while others have not (Gunga et al., 1996; Miller et al., 1981; Roberts and Smith, 1996). The observed diurnal variation is described by nadir values of [EPO] in the morning hours, and zenith levels during the evening and night hours. Even though the present data do not permit us to draw firm conclusions regarding circadian rhythm of EPO secretion, it is noteworthy that they are indeed consistent with such a rhythm, characterized by the lowest values at 8:00 to 9:00 AM and peak values at 18:00 to 19:00 PM. These changes are unlikely to be caused by differences in volume distribution along the body axis (Kirsch et al., 2005) as a consequence of body movements (Gunga et al., 1996) or intense exercise tasks (Roberts et al., 2000; Schwandt et al., 1991), since the participants were instructed to remain in the laboratory throughout the testing day either in a supine or in a sitting position and to refrain from any strenuous physical activity the day before the tests. Furthermore, the total amount of blood (30 mL) that was collected up to the sample 48 hours after the exposure could not, in itself, be responsible for any changes in the [EPO] (Roberts and Smith, 1996).

4.3.2 Inter-individual variability of EPO response

In the present study, only three out of ten males exhibited higher values of [EPO] in the HYPER compared to NOR condition 32 hours after the breathing intervention (Figure 4.5). These responses were not related to subjects' aerobic fitness ($r = -0.10$) or basal values of Hb ($r = 0.23$). Likewise, based on unpublished observations, Balestra et al., (2004; 2006) reported that only two out of five divers markedly increased their [EPO] levels after a series of breath-hold dives. In this regard, several investigations have confirmed the wide inter-individual variability of EPO response to acute (Friedmann et al., 2005; Mackenzie et al., 2008) or chronic hypoxic stimulus (Chapman et al., 1998; Ge et al., 2002; Gunga et al., 1994) that may be linked to specific genetically inherited traits (Jedlickova et al., 2003; Ou et al., 1998). Hence, we cannot exclude genetic determinants of individual variability of the EPO response to normobaric hyperoxia that may enlighten

the inconsistencies between the present findings and those of Balestra et al., (2004; 2006).

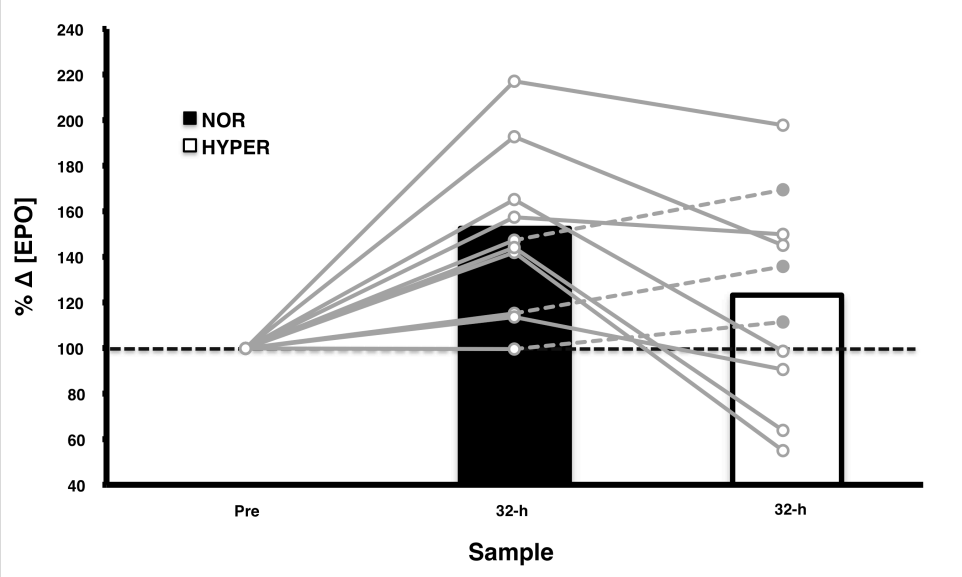

Figure 4.5: *Mean and individual percentage changes from Pre values of erythropoietin concentration (Δ[EPO]) 32 hours after air (NOR) and hyperoxic (HYPER) breathing intervention.*

4.3.3 Clinical perspectives

Following the observation that [EPO] increased in one patient suffering from chemotherapy-induced anaemia after repeated exposures to O_2 (Burk, 2007), and based on the theory of a "normobaric O_2 paradox" (Balestra et al., 2006), O_2 treatment has been advocated as a means of increasing EPO in anaemic patients (Balestra et al., 2010b; De Bels et al., 2011). It appears that longitudinal studies regarding the effect of repeated exposures of O_2 on haematological variables are required before considering O_2 as an adjuvant therapy in anaemic patients. Notably, the present single O_2 exposure did not significantly increase reticulocyte count or RBC from baseline values.

4.3.4 Methodological considerations

In the present study, the [EPO] was determined in plasma, in contrast with Balestra et al., (2004; 2006), who measured it in the serum. However, it is unlikely that the different results could be explained by the different specimen, given that no concentration differences have been detected between serum and plasma samples in previous studies (Eckardt et al., 1988; Jedlickova et al., 2003; Lindstedt and Lundberg, 1998). Moreover, the high sensitivity and the low coefficient of variation of the current analysis enforce the reliability and validity of the present findings.

5 Long-term normobaric hyperoxia and plasma [EPO]

Recent publications advocate the use of pure O_2 breathing as a treatment for anaemia (Balestra et al., 2010b) and as an adjuvant therapy for cancer patients (De Bels et al., 2011). The justification for these recommendations is based on a finding that the transition from an acute hyperoxic exposure (breathing 100% O_2 for 2 hours) to normoxia (breathing normal room air) induced erythropoiesis. The term "normobaric O_2 paradox" was coined to define the serendipitous observation of a marked paradoxical increase in the endogenous [EPO] 24 and 36 hours after the cessation of an acute normobaric O_2 breathing (Balestra et al., 2004; 2006). The suggested mechanism for such a hyperoxia-induced EPO production is the sudden and sustained decrease in tissue O_2 level ("relative hypoxia") upon the transition from hyperoxic to normoxic breathing conditions (Balestra et al., 2006).

This theory conflicts with the reported O_2-induced erythropoietic suppression in animals (Fletcher et al., 1973; Morshchakova et al., 1980) and humans (Debevec et al., 2011; Keramidas et al., 2011b; Kokot et al., 1994a; 1994b; McGuire et al., 2006). Keramidas et al., (2011b) observed that a 2-hour exposure to normobaric hyperoxia did not increase the production of EPO in healthy males during a single-blinded counter-balanced crossover investigation. On the contrary, the increased O_2 tension suppressed plasma [EPO] 3 to 5 hours after the breathing intervention, confirming similar previous findings (Fletcher et al., 1973; Morshchakova et al., 1980, Kokot et al., 1994a; 1994b). Thereafter, the plasma [EPO] levels appeared to resume their natural circadian rhythm (Cahan et al., 1992; Wide et al., 1989). Likewise, McGuire et al., (2006) and Momeni et al., (2011) failed to detect any significant differences in plasma [EPO] in healthy individuals after an acute O_2 breathing intervention.

Despite conflicting evidence regarding the erythropoietic potential of pure O_2, the "normobaric O_2 paradox" continues to be promoted as an efficient treatment for the enhancement of Hb for several clinical conditions (i.e. anaemia, cancer, sepsis) (Balestra et al., 2010b; Burk et al., 2007; Calzia et al., 2010; De Bels et al., 2011). However, advocates of this therapy base their recommendation on the findings of a study investigating the effect of a single acute exposure to hyperoxia (Balestra et al., 2006), disregarding the paucity of data on the long-term effects of O_2 breathing on erythropoiesis. It may be argued, that the response of plasma EPO to a single 2-hour period of breathing pure O_2 is of limited value. For O_2 therapy to be of any practical clinical value in this context, it would need to provide a long-term stimulus for erythropoiesis, which may require repeated hyperoxic exposures.

Accordingly, the present study evaluated whether the cumulative effect of daily hyperoxic exposures induces erythropoiesis. Specifically, the "normobaric O_2 paradox" was challenged by investigating the effect of ten daily short-term exposures to normobaric O_2 over a 2-week period on plasma [EPO] in healthy aerobically well-trained males. We hypothesized that although the evidence of erythropoiesis following a single hyperoxic exposure is equivocal, repeated stimuli of increased O_2 tension might give rise to a cumulative effect, which would potentially have clinical relevance.

5.1 Materials and Methods

5.1.1 Subjects

Twenty healthy males participated in the study. All of them were near-sea level residents, and were not exposed to altitude >500 m during the month preceding the experiments. They were non-smokers (Tanabe et al., 1997) and had no history of any renal, haematological, heart or lung disease (Miller et al., 1981; Payne et al., 1960; Westenbrink et al., 2010). They had not used any drugs acting as prostaglandin inhibitors (Fisher, 1980) during the month preceding the experiments. All subjects were physically active on a recreational basis; however, none of them were engaged in a formal sport-training program nor were any of them scuba divers. The subjects were informed in detail regarding the experimental procedures and risks involved, and gave their written consent. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia and conformed to the Declaration of Helsinki.

5.1.2 Experimental Protocol

The experimental protocol consisted of ten 2-hour sessions, during which either 100% normobaric O_2 or room air was inspired. Each condition comprised three testing phases (Figure 5.1): (a) Pre tests: one day before the 1st exposure session, (b) Mid tests: one day after the 5th exposure session, and (c) Post tests: one day after the 10th exposure session. During the three testing phases, blood samples were collected. Moreover, in the Pre testing phase, the subjects performed an incremental exercise test to exhaustion ($\dot{V}O_{2max}$) to ensure that they had similar levels of aerobic fitness.

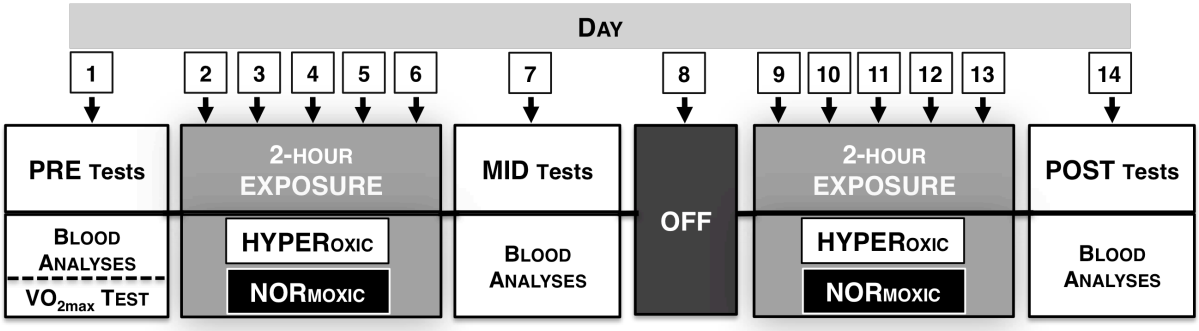

Figure 5.1: *Schematic representation of the overall protocol of study II.*

Throughout the 2-week experimental period, the subjects followed their normal daily routine of physical activity (no more than 3-5 hours of exercise per week), and they were asked to record their activities in individual diaries. However, they were instructed not to engage in any strenuous activity, and to abstain from consuming alcohol or any caffeinated product a day before the tests.

5.1.2.1 Air and Hyperoxic Exposure

After completing all baseline tests, the subjects were assigned to either the control group that breathed air (O_2: 20.93%) for 2 hours (NOR) or the experimental group that breathed 100% normobaric O_2 for the identical time (HYPER); groups being balanced with respect

to age and aerobic capacity (Table 5.1).

Table 5.1: *Descriptive characteristics of the control (NOR) and hyperoxic (HYPER) group of study II*. Values are mean ± SD. $\dot{V}O_{2max}$: maximal O_2 uptake; PPO: peak power output.

	NOR group (n = 10)	HYPER group (n = 10)
Age (years)	24.0 ± 3.2	25.1 ± 3.1
Body mass (kg)	78.9 ± 8.3	75.6 ± 7.3
Stature (cm)	181.1 ± 5.4	181.1 ± 7.5
Body fat (%)	11.8 ± 4.1	12.0 ± 8.2
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	50.6 ± 6.7	49.4 ± 6.3
PPO (W)	348 ± 30	331 ± 38

All subjects were exposed to normobaric O_2 or air 5 days per week for 2 weeks, each exposure being supervised by the same investigator. The exposures were conducted at the same time of the day for each participant (between 8:00 to 17:30); and the environmental conditions were kept constant: the mean ambient temperature, relative humidity and barometric pressure were 24.0 ± 0.8°C, 35.9 ± 3.9% and 979 ± 4 mb, respectively.

The subjects were naive regarding the breathing gas. During each session they were seated while breathing the gas mixture through a low resistance two-way respiratory valve (Model 2, 700 T-Shape, Hans Rudolph, Inc. Shawnee, USA). The inspiratory side of the respiratory valve was connected via respiratory corrugated tubing to a 200 L Douglas bag, which was continuously filled with the pre-mixed humidified breathing mixture. Throughout each exposure, HR was recorded continuously using a HR monitor (RS800CX, Polar, Kempele, Finland); and the subject provided ratings of perceived exertion (RPE; scale 0-10; Wilson and Jones, 1991) for D-RPE at 15-min intervals. Moreover, during each breathing breathing intervention, capillary oxyhaemoglobin saturation (SpO_2) was monitored continuously using a finger pulse oxymeter (BCI 3301, Waukesha, WI, USA) throughout the exposure; subjects were not provided any feedback from the physiological responses.

5.1.3 Blood analyses

During the tests (Pre, Mid, Post), each subject reported to the laboratory at 8:00 in the morning, after an overnight fast. He rested for 5 min in a semi-reclining chair, and venous blood samples were collected in two different EDTA tubes from the antecubital vein to determine the [EPO], the complete blood count and the reticulocyte count. The blood sample for the EPO determination was immediately centrifuged and the plasma frozen to -80°C for subsequent analyses. During the subsequent blood analyses, the investigator was blinded to each specimen.

5.1.3.1 Erythropoietin

The [EPO] was determined by sandwich enzyme-linked immunoassay (Quantikine IVD EPO ELISA, R&D Systems, Minneapolis, USA) using 100 µL of plasma. Optical density was quantified on a microplate reader Quant (Bio-Tek instruments, Winooski, USA) set at 450 nm and corrected at 600 nm. The current method has been validated previously (Sakata et al., 1995). All techniques and materials were in accordance with the protocol provided by the company. All samples were assayed in duplicate. The estimated coefficient of variation of the analysis was 3.1%, and the sensitivity of the measurement was 0.6 $mU \cdot mL^{-1}$.

5.1.3.2 Complete blood count and reticulocyte count

Venous blood samples (2 mL) were collected for complete blood count and reticulocyte count. The complete blood count, including analysis of total RBC, Hb and Hct, and the reticulocyte count were obtained with an automated laser-based haematology analyzer (Advia 120, Siemens, Munich, Germany) within 6 hours after the blood sampling. The apparatus was calibrated before each measurement. All samples were assayed in duplicate. The estimated coefficient of variation for the RBC, Hb, Hct and reticulocyte count was 1.4, 0.6, 2.1 and 3.1%, respectively.

5.1.4 Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft, Inc., Tulsa, USA). All data are reported as mean (SD), unless otherwise indicated. A two-way analysis of variance (ANOVA) for repeated measures was used for the haematological variables (group × testing period). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVA revealed significant F-ratio for main effects. Differences in median (range) D-RPE were evaluated with a Wilcoxon matched pairs non-parametric test. The alpha level of significance was set *a priori* at 0.05.

5.2 Results

5.2.1 Air and hyperoxic exposures

The mean HR during all the breathing sessions was significantly lower in the HYPER than in the NOR group (NOR: 66 ± 8 beats·min^{-1}; HYPER: 57 ± 7 beats·min^{-1}; $P = 0.03$) (Figure 5.2). There was no difference in D-RPE between the exposures [NOR: 0.5 (0.5-1); HYPER: 0 (0-0.5); $P > 0.05$].

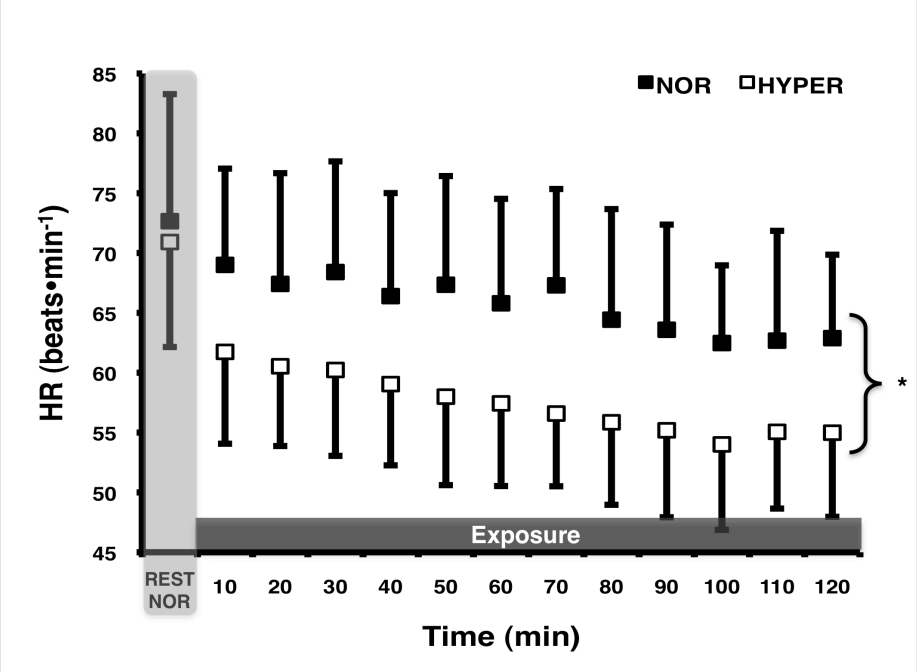

Figure 5.2: *The mean heart rate (HR) during all breathing sessions of the control (NOR) and hyperoxic (HYPER) group.* Values are mean ± SD. * Significant difference between the NOR and HYPER group ($P \leq 0.05$).

5.2.2 Erythropoietin

Figure 5.3 and 5.4 present the mean absolute values and the relative changes of [EPO] of both groups, respectively. Pre values of [EPO] were similar in the NOR and HYPER groups, whereas both Mid and Post EPO values were lower ($P < 0.001$) in the HYPER than in the NOR group. [EPO] of the NOR group was increased during the Mid period compared to Pre values (~41%; $P = 0.02$). The increase seemed to be maintained in the Post period (~31%), but the difference was not statistically significant compared to the Pre levels ($P > 0.05$). Conversely, [EPO] of the HYPER group showed a slight decrease during the Mid (~11%) and Post (~16%) tests that was not statistically different from the Pre values (Mid: $P = 0.88$; Post: $P = 0.67$).

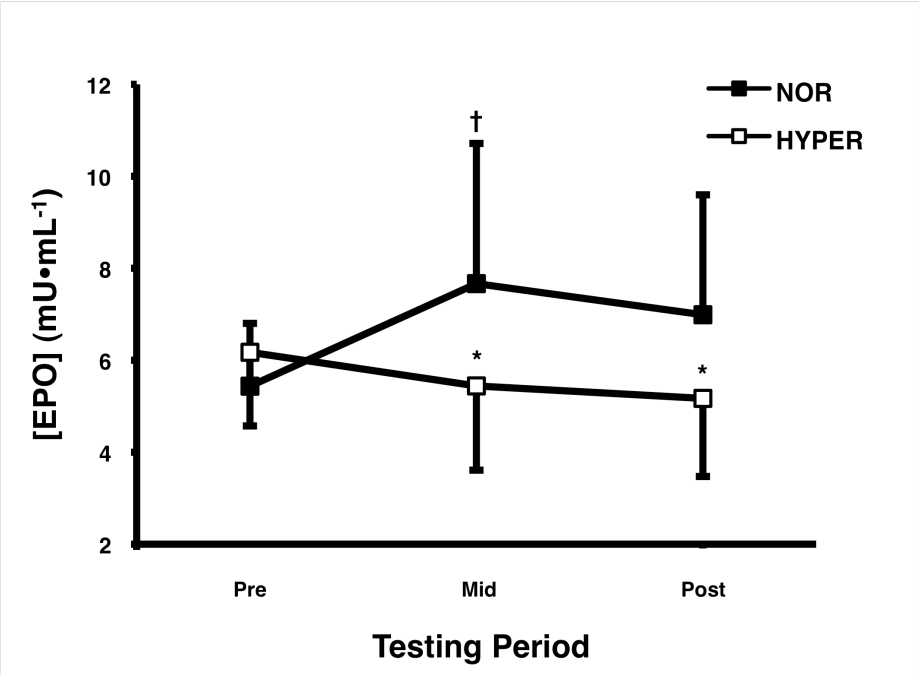

Figure 5.3: *Absolute values of the erythropoietin concentration ([EPO]) during the Pre, Mid and Post breathing intervention periods for the control (CON) and hyperoxic (HYPER) group.* Values are mean ± SD. † Significant different from the Pre-testing period. * Significant difference between the NOR and HYPER group ($P \leq 0.05$).

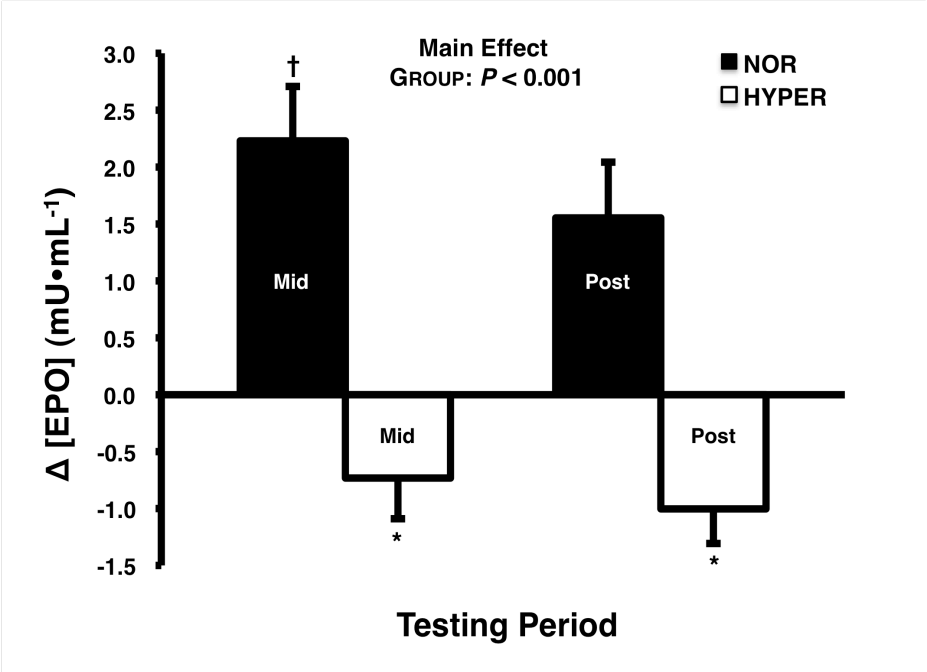

Figure 5.4: *Changes from the Pre values of erythropoietin concentration ([EPO]) during the Mid and Post breathing intervention periods for the control (NOR) and hyperoxic (HYPER) group.* Values are mean ± SD. † Significant different from the Pre-testing period. * Significant difference between the NOR and HYPER group; ($P \leq 0.01$).

5.2.3 Complete blood count and reticulocytes

Mean values of Hb, Hct, reticulocyte count and RBC are summarized in Table 5.2. In both groups, Hb, Hct and RBC decreased in the Mid test ($P \leq 0.05$); there were no differences in any of these variables between the groups. The reticulocyte count did not alter throughout the experimental period in either of the groups ($P > 0.05$).

Table 5.2: *Haematological variables Pre, Mid and Post breathing intervention period of the control (NOR) and hyperoxic (HYPER) group.* Values are mean ± SD; Hb: haemoglobin, Hct: haematocrit; RBC: red blood cell count. † Statistically significant different from the Pre testing period; ($P \leq 0.05$).

	NOR group			HYPER group		
	Pre	Mid	Post	Pre	Mid	Post
Hb ($g \cdot dL^{-1}$)	15.5 ± 0.7	14.9 ± 0.6†	14.9 ± 0.7	15.6 ± 1.1	14.9 ± 0.9†	15.4 ± 0.9
Hct (%)	0.48 ± 0.02	0.46 ± 0.02†	0.47 ± 0.02	0.48 ± 0.04	0.47 ± 0.03†	0.48 ± 0.03
Reticulocyte count (%)	1.5 ± 0.5	1.6 ± 0.4	1.5 ± 0.4	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
RBC ($10^{12} \cdot L^{-1}$)	5.3 ± 0.3	5.2 ± 0.3†	5.2 ± 0.3	5.3 ± 0.5	5.1 ± 0.4†	5.3 ± 0.4

5.3 Discussion

The present study clearly demonstrates that a daily 2-hour exposure to normobaric O_2 over a 2-week period does not increase plasma [EPO] in aerobically fit males, confirming the established O_2-induced eryhtropoietic suppression, and disapproving the notion of a

"normobaric O_2 paradox" in healthy individuals. Advocating the use of pure O_2 breathing as a method to enhance erythropoiesis is thus not warranted.

The term "normobaric O_2 paradox" was coined to define the supposedly paradoxical increase in endogenous [EPO] 24 and 36 hours after the cessation of a short-term normobaric O_2 breathing in response to a sharp decrease in tissue O_2 level ("relative hypoxia") upon the transition from a hyperoxic to a normoxic condition (Balestra et al., 2006). Notwithstanding, the present findings disprove the aforementioned notion; and, in fact, confirm the already known suppressive role of pure O_2 to erythropoiesis (Keramidas et al., 2011b; Kokot et al., 1994a; 1994b). Namely, in a previous study (Keramidas et al., 2011b), we observed that a short-term exposure to normobaric O_2 causes a marked decrease in [EPO] 3 and 5 hours after the cessation of breathing. Eventually, that drop was followed by a slow return of EPO to its natural circadian rhythm and the reversal to its basal values 8 hours later. However, a small decline of [EPO] (~11%) was still evident 24 hours after the breathing intervention, despite the lack of any statistical difference from the control values. Likewise, in the current study, we detected an equivalent drop, albeit statistically non-significant decrease in [EPO] of the HYPER group during the Mid (~11%) and Post (~16%) testing period.

Contrary to the HYPER group, [EPO] increased significantly in the NOR group during the Mid and Post periods. This increment was unexpected, since the CON group breathed normoxic air during all the sessions; and none of the participants performed any strenuous activity a day before the blood tests nor did they change their normal daily routines throughout the 2-week period (Roberts et al., 2000), as evident from their individual activity diaries which they maintained during this period. Present results do not allow us to discern which mechanisms might underlie this enhancement of [EPO]. Conceivably, it is attributable to the slightly diminished levels of Hb and/or Hct (Gunga et al., 1996; 2007; Jelkmann, 1992; Misago et al., 1986); which, in turn, might reflect a seasonal variation of Hct (Thirup, 2003) (the study was performed during June and July) and/or the level of hydration of the subjects. That the drop in Hb correlated to the increase in [EPO] in the NOR group ($r = -0.63$; $P = 0.03$) and that no such correlation was observed in the HYPER group ($r = -0.39$; $P > 0.05$), supports the notion that the [EPO] increased in the NOR group was secondary to the Hb drop and that the O_2 intervention prevented such [EPO] increase in the HYPER group. Regardless of the underlying mechanisms, both groups presented similar decrements of Hb and Hct during the course of the breathing regimen. Therefore, and because [EPO] was considerably lower in the HYPER than the NOR group during and immediately after the breathing regimen present results support the notion that normobaric hyperoxia suppresses erythropoiesis.

By contrast, Balestra et al., (2006) did not detect any significant decline of [EPO] at least during the initial period following the O_2 breathing. This disparity might be due to the wide inter-individual variability of the EPO response to the O_2-stimulus (Chapman et al., 1998; Friedmann et al., 2005; Ge et al., 2002) and as regards the natural circadian rhythm of EPO (Cahan et al., 1992; Wide et al., 1989).

The mechanism for the O_2-induced suppression of EPO remains unresolved. Considering the shape of the oxyhaemoglobin binding curve (Jelkmann, 1992), the O_2 content of arterial blood will increase very little (~5%) during O_2 breathing, and hence it is unlikely that the hyperoxia-induced suppression of EPO release is attributable to increased O_2 availability in the renal tissue. Recent studies regarding the presence of humoral factor(s) stemming from the hypothalamic-hypophyseal system that, at least in part, have a regulating effect on renal EPO secretion (Pagel et al., 1989; von Wussow et al., 2005), and given the existence of sensors in the brain capable of detecting changes in arterial PO_2 (Lahiri et al., 2006), the role of the central nervous system on the suppression of EPO should be taken into account.

5.3.1 Clinical perspectives

Despite the implausible premise for the existence of a "normobaric O_2 paradox" in healthy individuals and the lack of evidence its haematopoietic effects in patients, O_2 breathing is being advocated as an effective treatment both for primary anaemia and as an adjuvant therapy for cancer patients suffering from chemotherapy-induced anaemia (Balestra et al., 2010b; Burk, 2007; Calzia et al., 2010; De Bels et al., 2011). It is noteworthy that the erythropoietic suppression resulting from a long-term ($\leq$ 5 days) normobaric O_2 breathing has been confirmed in anaemic subjects (Embury et al., 1984; Tsangaris et al., 1999). Therefore, and because long-term treatment with normobaric O_2 might have side effects (cf. Haque et al., 1996; Saadjian et al., 1999), to emphasize the use of O_2 breathing as a treatment to increase EPO in anaemic patients might be unwarranted. In addition, considering that potential confounders (e.g. drugs, sleep apnoea) are common in patients, anecdotal findings regarding erythropoiesis in single anaemic individuals should be interpret circumspectly. Thus, specific randomised controlled long-term studies are required before considering O_2 therapy as an adjuvant treatment for anaemia.

6 Cerebral and muscle oxygenation during acute normobaric hyperoxic exposure at rest in healthy individuals

Normobaric O_2 is widely used in several clinical and non-clinical conditions to treat or prevent hypoxaemia and tissue hypoxia. Basically, the increased inspired fraction of O_2 (FiO_2) increases the arterial partial pressure of O_2 (PaO_2) and the arterial oxyhaemoglobin saturation (SaO_2) (for review see Treacher and Leach, 1998), which leads to a decreased binding of CO_2 to haemoglobin (Hb) (*Haldane* effect), hyperventilation and vasoconstriction (Becker et al., 1996; Bulte et al., 2007; Rousseau et al., 2005; 2007).

Several studies have confirmed a vasoconstrictive effect of normobaric hyperoxia in cerebral (Bulte et al., 2007; Floyd et al., 2003; Tisdall et al., 2009; Watson et al., 2000) and skeletal muscle (Bredle et al., 1988; Rousseau et al., 2005) tissues. Yet, it has been suggested that precapillarry vessels in these two tissues might differ in responsiveness to an acute O_2-breathing stimulus both during rest and exercise. Nielsen et al., (1999) have observed that supplementation with moderate hyperoxia (FiO_2 0.30) throughout a maximal exercise test diminished the exercise-induced impairment in cerebral oxygenation and maintained it at the resting levels, while the reduction in the muscle oxygenation was not affected by the O_2. Likewise, Amann et al., (2007) and Subudhi et al., (2008) have demonstrated that during maximal exercise, the switch to moderate hyperoxia (FiO_2 0.30 and 0.60, respectively) at the task failure point increased rapidly and substantially cerebral oxygenation, but only minutely oxygenation in the working leg muscles. However, to elucidate whether arterioles of the two regions differ in their sensitivity to acute O_2 stimulus, responses need to be elucidated during rest. To our knowledge, there is no study reported in the literature that has simultaneously monitored the changes in oxygenation in cerebral and skeletal muscle tissues during acute hyperoxic exposure at rest.

Near-infrared spectroscopy (NIRS) offers noninvasive, real-time assessment of local differences in the balance between O_2 consumption ($\dot{V}O_2$) and delivery (Van Beekvelt et al., 2001). The reliability of the technique has been verified in previous studies, in which the changes in oxygenation measured by NIRS were compared with the venous occlusion mercury strain gauge plethysmography (Edwards et al., 1993; Van Beekvelt et al., 2001) and the jugular venous oximetry (McLeod et al., 2003). NIRS is generally used to monitor the cerebral, intercostal and leg muscle oxygenation in resting (Edwards et al., 1993; Elwell et al., 1994) and exercise (Amann et al., 2007; Keramidas et al., 2011a; Nielsen et al., 1999; Subudhi et al., 2008) conditions; and has been employed in both healthy (Larsson et al., 2010; Tisdall et al., 2009) and non-healthy (McLeod et al., 2003) populations.

Accordingly, the purpose of the present study was to simultaneously map the cerebral, intercostal and leg muscle oxygenation in young healthy males during an acute short-term normobaric O_2 administration at rest. Based on previous findings, mainly derived from the studies on exercise conditions, we hypothesized that high levels of arterial O_2 tension will increase oygenation predominantly in the cerebral frontal cortex, and to a lesser degree in the limb and respiratory muscles. A complete description of the response of each region to pure O_2 administration might constitute useful information when designing

oxygen therapy protocols.

6.1 Materials and Methods

6.1.1 Subjects

Ten healthy males participated in the present study (Table 4.1). All individuals were physically active on a recreational basis; however, none of them were engaged in a formal sport-training program. They were non-smokers, had no history of any renal, haematological, neurological, cardiovascular or pulmonary disease, and had not received any medication during the month preceding the experiments. The subjects were informed in detail about the experimental procedures before giving their written consent to participate. They were instructed not to engage in any physical activity and to refrain from consuming alcohol or any caffeinated product 24 hours prior to the testing days. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia and conformed to the Declaration of Helsinki.

During their first visit to the laboratory, the subjects were thoroughly familiarized with the equipment and procedure. The experimental protocol consisted of a preliminary session during which an incremental exercise test to exhaustion was performed, followed after one week by two experimental sessions (Figure 4.1): (a) a 2-hour 100% normobaric O_2 exposure (HYPER), and (b) a 2-hour air exposure (NOR). Five subjects breathed 100% normobaric O_2 for 2 hours, and the rest of the subjects breathed air (O_2: 20.93%) for the identical time. After a 22-day washout period, the experiments were repeated with the subjects crossed in conditions. The two breathing exposures were conducted in a single-blind manner, and their order was randomized.

The breathing interventions commenced with a 15-min baseline period in normoxia, followed by a 2-hour exposure (NOR or HYPER), and finishing with a 15-min recovery period in normoxia. During both trials, subjects were in a resting semi-reclining position (25° head-up tilt) and breathed through a low resistance two-way respiratory valve (Model 2, 700 T-Shape, Hans Rudolph, Inc. Shawnee, USA). The inspiratory side of the respiratory valve was connected via respiratory corrugated tubing to a 200 L Douglas bag filled with the pre-mixed humidified breathing mixture.

All the tests were conducted at the same time of the day (start of exposure at 08:00 to 08:30). The environmental conditions were kept constant and thermoneutral during both exposures: the mean ambient temperature, relative humidity and barometric pressure were 21.5 ± 1.0°C, 41.0 ± 1.9% and 978 ± 9 mb, respectively. To prevent cooling throughout the exposure, a thin cotton blanket covered the trunk and the lower extremities of the subjects.

6.1.2 Instrumentation

6.1.2.1 Cerebral and muscle oxygenation

During both breathing exposures, the cerebral, intercostal and leg muscle oxygenation was monitored by three pairs of continuous-wave infrared spectroscopy (NIRS) probes (Artinis Medical System, Oxymon MKIII, Zetten, the Netherlands). The cerebral probe was positioned over the left prefrontal cortex between Fp1 and F3, according to the modified international EEG 10-20 system; the respiratory muscle probe was positioned over the right 7th intercostal space of the serratus anterior muscle and the leg muscle probe above the vastus lateralis, ~15 cm above the proximal line of the patella and ~5 cm

laterally to the midline of the thigh (Figure 6.1). The probes consisted of one emitter and one detector housed in a black, plastic holder that was stabilized on the shaved and cleaned skin with double-sided adhesive tape. A bandage covered and stabilized each probe holder in order to reduce the intrusion of external light and the loss of transmitted NIR light from the measuring area. The inter-optode distance was kept at 4.5 cm to minimize the influence of skin blood flow. All technical considerations (probe position and stabilization) were taken into account according to the previously published reports using the same NIRS device (Billaut et al., 2009; Keramidas et al., 2011a; Subudhi et al., 2007).

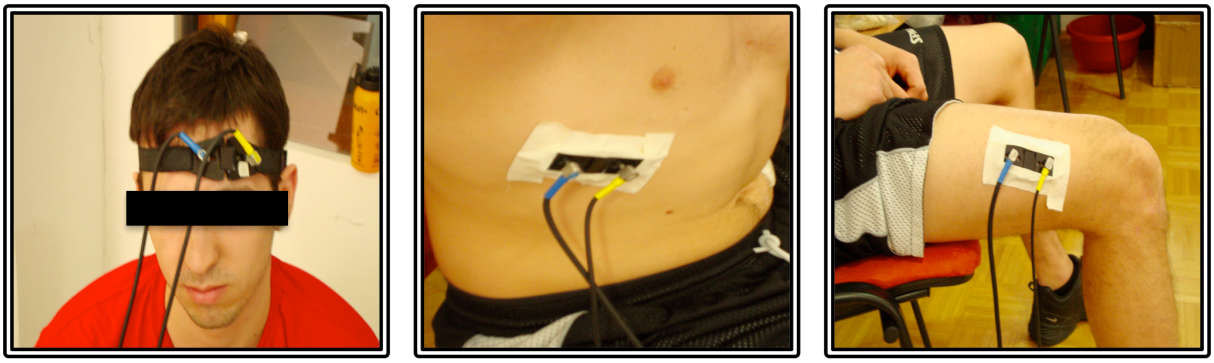

Figure 6.1: *NIRS probes placed on the left prefrontal cortex* (left figure)*, the right 7th intercostal space of the serratus anterior muscle* (middle figure) *and the right vastus lateralis muscle* (right figure).

The NIR light consisted of two wavelengths (780 and 850 nm) and the micromolar changes in tissue oxygenation - oxygenated ($\Delta[O_2Hb]$) and deoxygenated ($\Delta[HHb]$) haemoglobin - were calculated from the age-dependent differential path-length factors (DPF; range: 4.95-6.12) (Duncan et al., 1995). In addition, total haemoglobin ($\Delta[tHb]$), which is the sum of $\Delta[O_2Hb]$ and $\Delta[HHb]$, was used as an index of change in regional blood volume (Van Beekvelt et al., 2001).

The theory, limitations and reliability of the cerebral and muscle measurements obtained with the NIRS device have been detailed previously (Ferrari et al., 2004; Perrey, 2008; Subudhi et al., 2007). Briefly, the NIR light is absorbed by heme groups both within Hb and Mb. However, the relative contribution of Hb seems to be substantially greater than that of Mb, since the Hb is tetrameric and exists in appreciably greater concentrations than the monomeric Mb pigment (Wilson et al., 1989). Moreover, it is known that the thickness of adipose tissue may influence the NIRS signal (McCully and Hamaoka, 2000), potentially biasing the comparison of signal magnitudes between subjects.

NIRS data were recorded at 50 Hz and were stored in a PC for further analysis. Because the exact DPF was unknown, the cerebral and muscle measurements were normalized to reflect the magnitude of the changes from the 15-min normoxic baseline period of each trial (arbitrarily defined as 0 μM) (Keramidas et al., 2011a; Subudhi et al., 2007).

6.1.2.2 Systemic haemodynamic variables

Beat-to-beat systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressures were assessed by finger photoplethysmography (Finometer, Finapres Medical Systems BV, Amsterdam, the Netherlands) on the middle phalanx of the index of the right hand (Figure 6.2). Prior to the beginning of the experimental protocol, blood pressure recordings with a

noninvasive auscultatory method (300B, Speidel & Keller, Jungingen, Germany) on the same arm were used to confirm the accuracy of the Finometer measurements. In case of noticeable differences between the two methods, either the finger cuff was replaced or the hand was warmed with an electrical blanket until agreement between the two methods was reached. Subjects were instructed to, throughout the trials, keep their hand immobile at waist level with reference pressure transducer positioned at the level of the heart. The system was calibrated according to the manufacturer's instructions before each measurement. The Finometer has been shown to track acute changes in BP at rest providing accurate and reliable estimates of intra-arterial pressure (Parati et al., 1989).

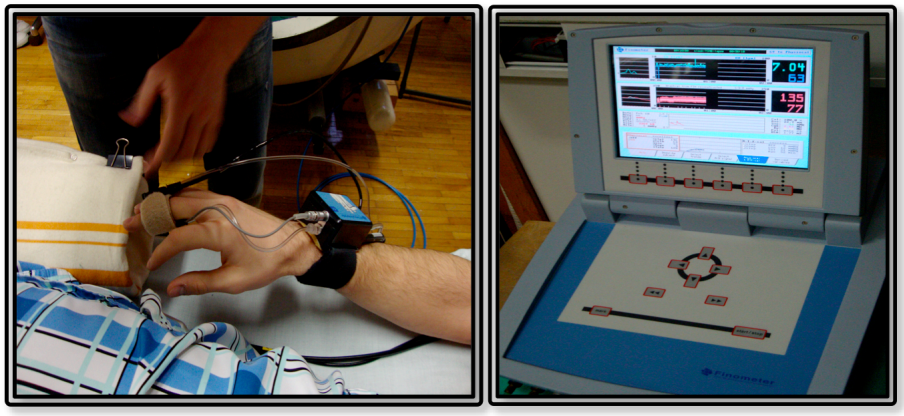

Figure 6.2: The *finger cuff of the Finometer placed on the midle phalanx of the index of the right hand.*

HR was measured and recorded continuously throughout the experimental trial using a HR monitor (S810i, Polar, Kempele, Finland).

6.1.2.3 Index of skin vasomotor tone

The calf-toe skin temperature gradient ($\Delta T_{calf\text{-}toe}$) of the left leg was calculated from two skin thermistors (MSR145, Henggart, Switzerland), which were attached with sticking plaster on the big toe pad and on the lateral aspect of the calf. The accuracy of the current method to determine skin vasoconstriction and vasodilatation thresholds has been validated in previous studies (House and Tipton, 2002; Rubinstein and Sessler, 1990).

6.1.2.4 Psychometric response scales

During both breathing interventions, subjects were requested to provide ratings of perceived exertion for dyspnoea – respiratory discomfort (D-RPE; modified Borg's scale from 0-nothing at all to 10-maximal; Wilson and Jones, 1991) and for mood/feeling (FS; a 10-point scale from -5-very bad to +5-very good; Hardy and Rejeski, 1989). Both scales were explained to the subjects by the investigator during the familiarization session and prior to the experimental trials. Scale readings were obtained at baseline period, every 15 minutes during the exposure and at recovery period.

6.1.3 Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft, Inc., Tulsa, USA). All data are reported as mean (SE), unless otherwise indicated. The normoxic baseline and recovery data were averaged over the 15-min period. Data obtained during the NOR and HYPER exposure were averaged every 5 minutes. A two-way analysis of variance

(ANOVA) for repeated measures was used for the haemodynamic variables (condition × overtime). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVA revealed significant F-ratio for main effects. Due to technical problems with the NIRS probe on the serratus anterior muscle of two subjects, the number of subjects for that specific parameter was eight. Differences in D-RPE and FS were evaluated with a Wilcoxon matched pairs non-parametric test. The alpha level of significance was set *a priori* at 0.05.

6.2 Results

6.2.1 Cerebral and muscle oxygenation

Changes in cerebral oxygenation are shown in Figure 6.3. $\Delta[O_2Hb]$, $\Delta[HHb]$ and $\Delta[tHb]$ remained unchanged throughout the NOR trial. However, during the HYPER trial, $\Delta[O_2Hb]$ was significantly increased after the 50th min of the exposure ($P = 0.01$), and $\Delta[HHb]$ was significantly decreased throughout the entire period ($P \leq 0.001$). Both variables directly returned to baseline values after the cessation of the O_2 breathing intervention. Furthermore, from 5th to 20th minute, $\Delta[tHb]$ was significantly lower in the HYPER trial than in the NOR; but there was no statistically significant difference between the trials afterwards ($P = 0.29$).

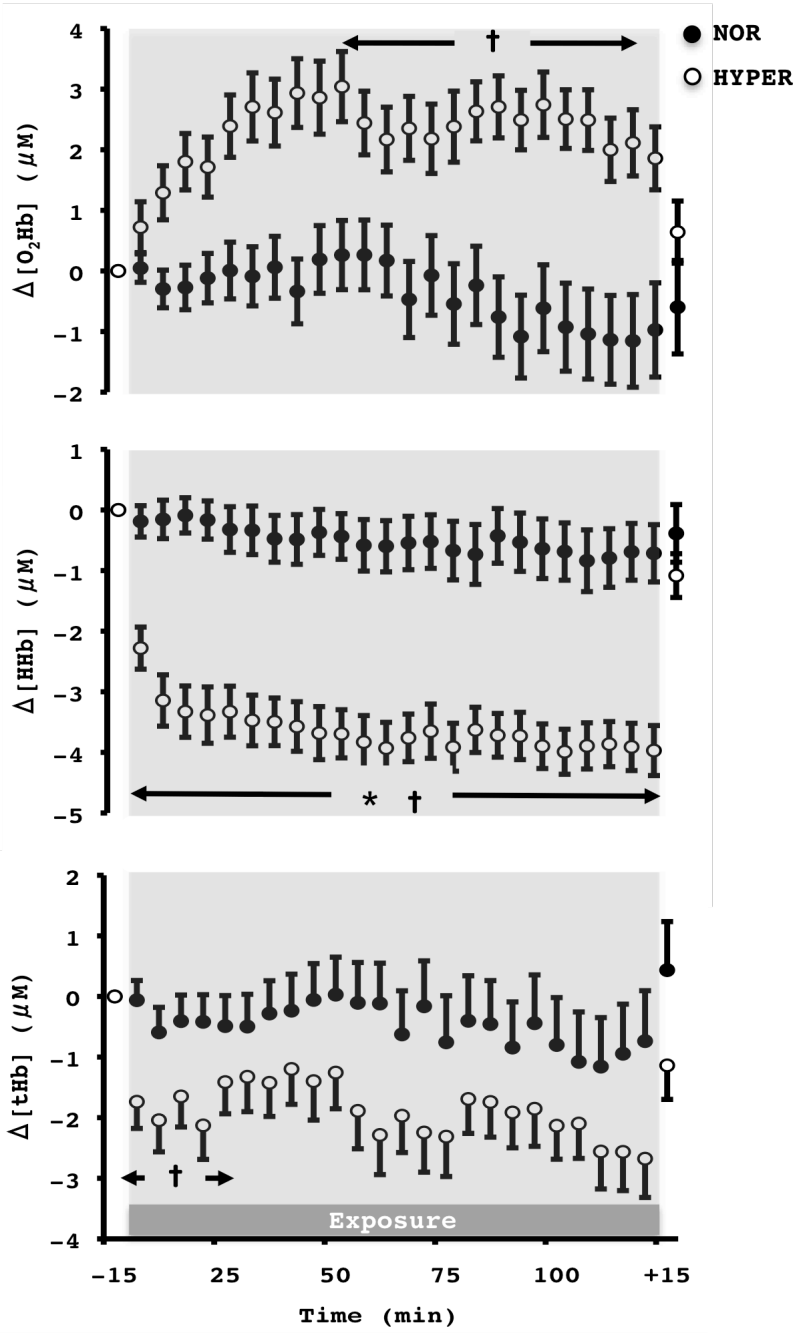

Figure 6.3: *Changes from baseline values in cerebral total haemoglobin (Δ[tHb]), oxyhaemoglobin (Δ[O_2Hb]) and deoxyhaemoglobin (Δ[HHb]) during air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are means ± SE. * Significant differences from baseline values; † Significant differences between NOR and HYPER; ($P \leq 0.05$).

In both trials, Δ[O_2Hb] and Δ[tHb] of the vastus lateralis gradually increased compared to baseline values, with no differences between trials (Figure 6.4a). Δ[HHb] presented significantly different responses in the two trials. It remained unaffected during the NOR trial, but markedly decreased (~2.7 μM) after the 15th min in the HYPER trial ($P = 0.04$) (Figure 6.4a). The difference between the trials remained during the recovery period.

Δ[O_2Hb] and Δ[tHb] of the serratus anterior increased significantly in both trials ($P = 0.05$), but the statistical analysis did not reveal any *post hoc* difference between the trials (Figure 6.4b). After the 65th min, Δ[HHb] was significantly lower (~0.7 μM) in the HYPER trial than in the NOR trial ($P = 0.05$) in which it remained constant (Figure 6.4b).

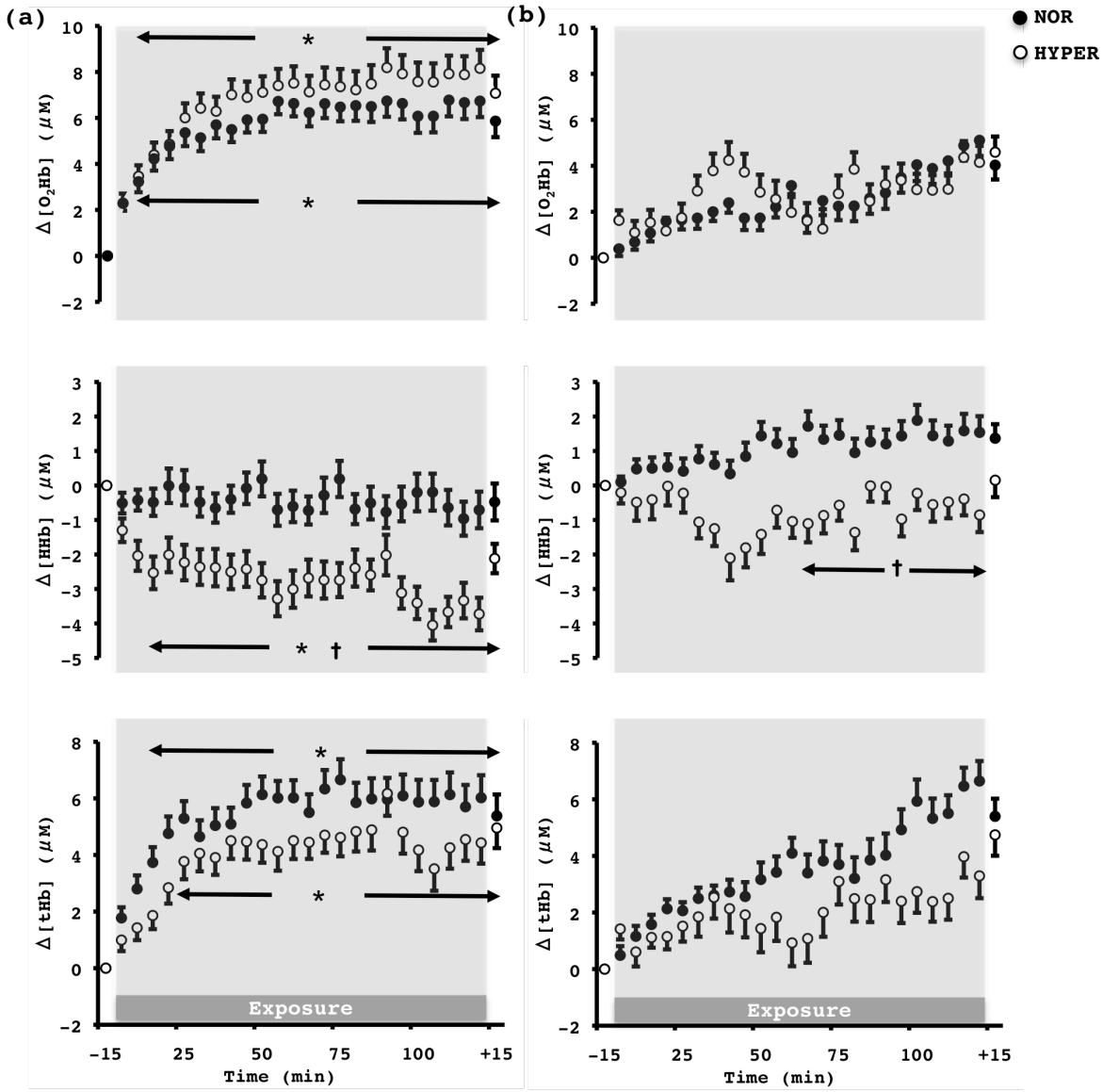

Figure 6.4: *Changes from baseline values in (a) vastus lateralis and (b) serratus anterior total haemoglobin (Δ[tHb]), oxyhaemoglobin (Δ[O_2Hb]) and deoxyhaemoglobin (Δ[HHb]) during air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are means ± SE. * Significant differences from baseline values; † Significant differences between NOR and HYPER; ($P \leq 0.05$). Data of serratus anterior Δ[O_2Hb] and Δ[HHb] were significantly different from baseline values without *post hoc* differences. n = 8 for the serratus anterior muscle.

6.2.2 Systemic haemodynamic responses

The group mean time series for HR during the NOR and HYPER trials are presented in Figure 6.5. HR decreased significantly during both breathing interventions (NOR: -7%; HYPER: -12%), but the drop was more pronounced (~4 beats·min^{-1}) in the HYPER than in the NOR trial (P = 0.05). HR fully reverted to normoxic baseline levels after the cessation of O_2 breathing. In both trials, SAP, DAP and MAP increased slightly during the course of the experiment ($P \leq 0.001$) (Figure 6.6). No differences between trials were observed for SAP, DAP and MAP.

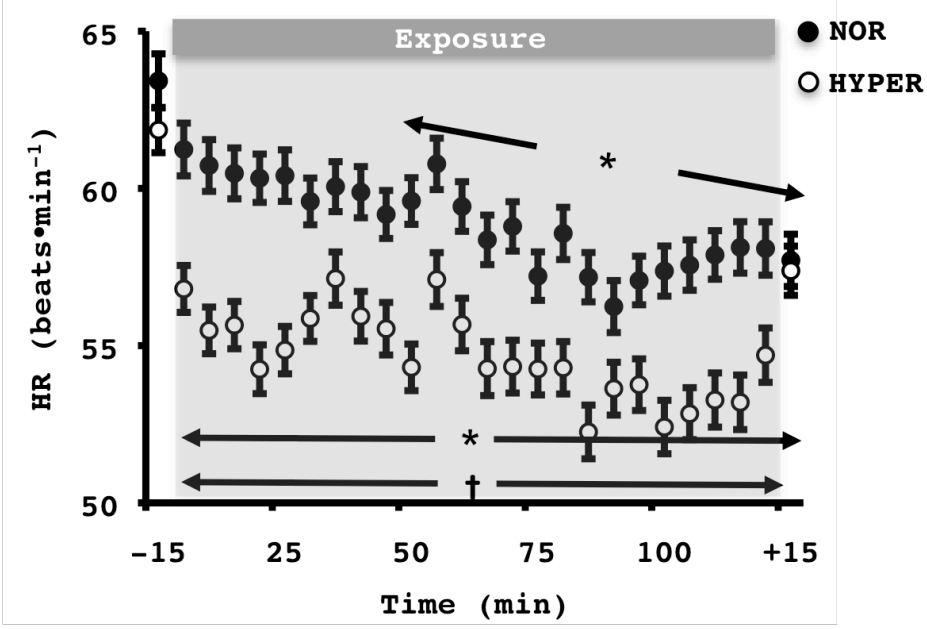

Figure 6.5: *Heart rate (HR) during air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are means ± SE. * Significant differences from baseline values; † Significant differences between NOR and HYPER; ($P \leq 0.05$).

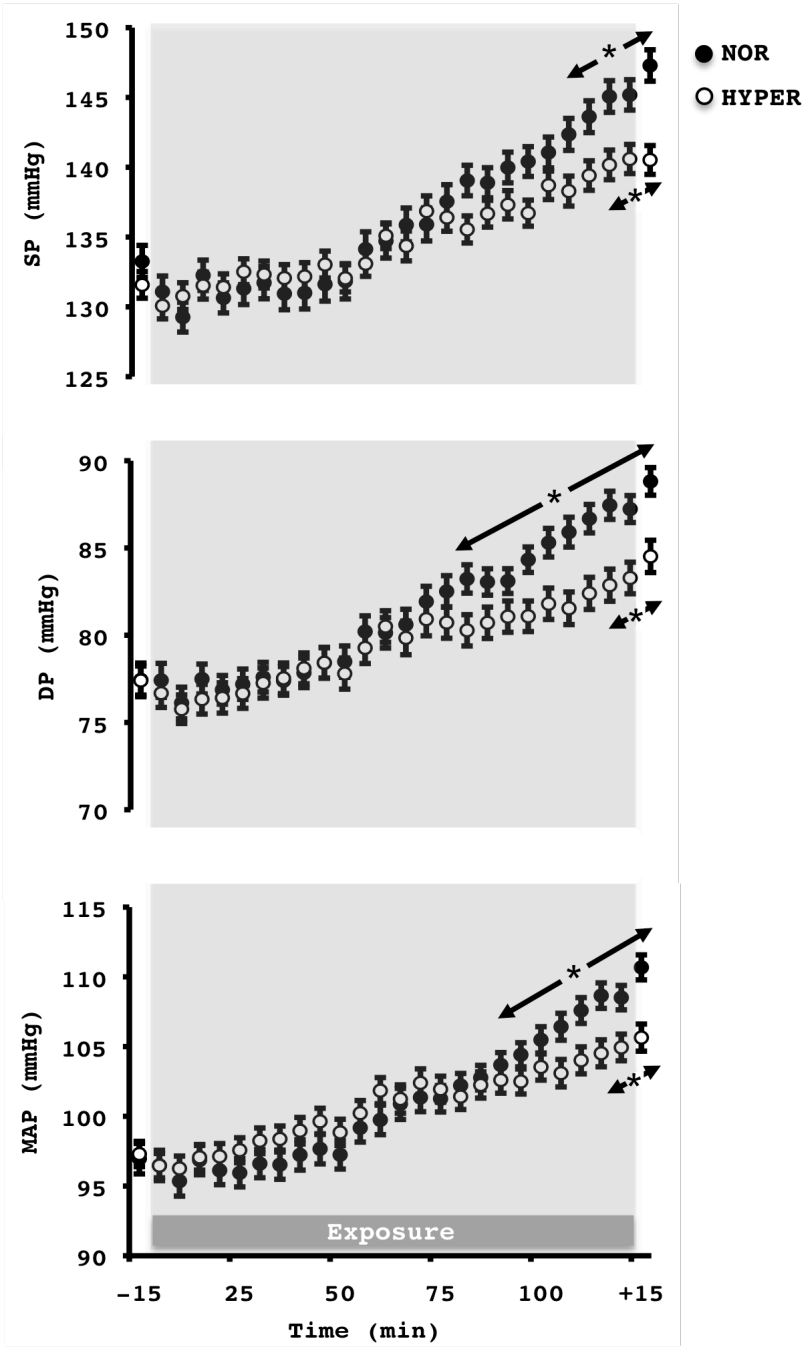

Figure 6.6: *Systolic (SAP), diastolic (DAP) and mean arterial blood pressure (MAP) during air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are means ± SE. * Significant differences from baseline values; ($P \leq 0.05$).

During the HYPER trial, $\Delta T_{calf\text{-}toe}$ remained unchanged (-0.3 ± 0.5°C), whereas during the NOR trial it slightly decreased (-2.8 ± 0.6°C), indicating a peripheral vasodilatation; and it was significantly lower in the NOR than in the HYPER trial after the 20th min of the exposure ($P = 0.02$) (Figure 6.7).

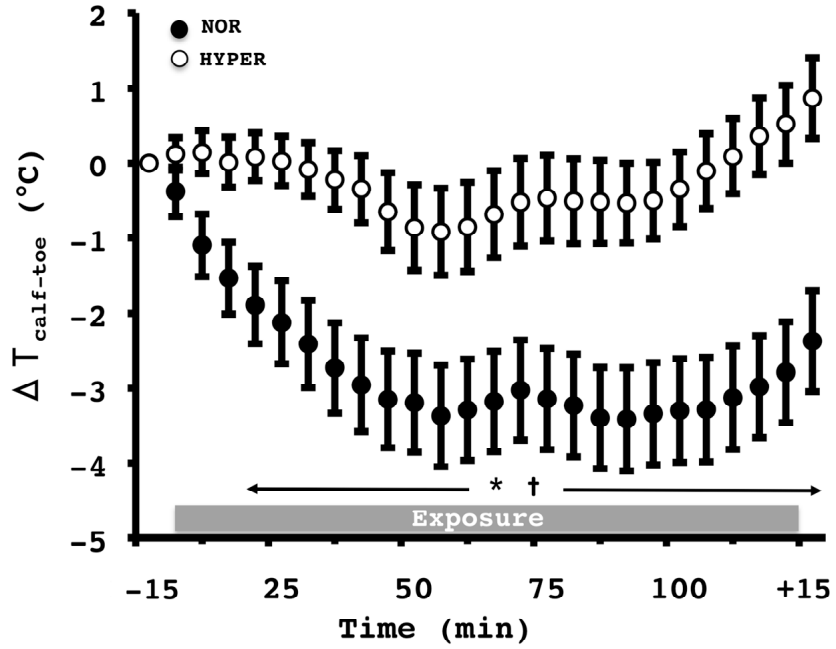

Figure 6.7: *Changes from baseline values in the calf-toe skin temperature gradient ($\Delta T_{calf\text{-}toe}$) of the left leg during air (NOR) and hyperoxic (HYPER) breathing intervention.* Values are means ± SE. * Significant differences from baseline values; † Significant differences between NOR and HYPER; ($P \leq 0.05$).

6.2.3 Psychometric response scales

There were no differences in D-RPE or in FS between the trials (Table 4.2). Subjects experienced mild discomfort after the 60th min of both trials, as revealed by the low values in FS (Table 6.1).

Table 6.1: *Median (range) values of perceived exertion of dyspnoea (D-RPE) and feeling (FS) during air (NOR) and hyperoxic (HYPER) breathing intervention.* * Significant different from baseline values; ($P \leq 0.05$).

	NOR			HYPER		
	Baseline	60-min	120-min	Baseline	60-min	120-min
D-RPE	0	0	0	0	0.5	1
(0 to 10)	(0 to 2)	(0 to 3)	(0 to 1)	(0 to 1)	(0 to 3)	(0 to 3)
FS	2	-0.5	-0.5	0.5	0	0
(-5 to 5)	(-1 to 4)	(-2 to 3)*	(-3 to 4)*	(-1 to 4)	(-2 to 4)*	(-3 to 4)*

6.3 Discussion

The principal finding of the present study was that an acute exposure to normobaric O_2 at rest induced heterogeneous responses between cerebral and muscle oxygenation. Namely, hyperoxia promptly increased $\Delta[O_2Hb]$ and decreased $\Delta[HHb]$ in the prefrontal cortical region. These O_2-induced responses were immediately and fully countered upon return to normoxic conditions. By contrast, in the leg and respiratory muscle regions only $\Delta[HHb]$ was affected by the hyperoxic intervention; the decrements transpired slower than those in the cerebral area and were only partially reversed during the 15-min normoxic recovery period.

6.3.1 Cerebral and muscle oxygenation

A marked drop in cerebral O_2 extraction, as indicated by the lower values of Δ[HHb] (cf. Grassi et al., 1996), was detected during the O_2 breathing. This seemingly paradoxical response has been confirmed by others (McLeod et al. 2003; Tisdall et al. 2009), and has been attributed to maldistribution of blood flow in the cerebral microcirculation (Boakye et al., 2001; Reinhart et al., 1991). Δ[tHb], which is an index of regional blood volume (Van Beekvelt et al. 2001), was significantly reduced during the first 20 min of the HYPER trial, but thereafter did not differ significantly between trials. This might be explained by the wide inter-individual variability in the Δ[O_2Hb] response. Thus, despite that all subjects exhibited a reduction in Δ[HHb] during the course of the HYPER trial, Δ[tHb] was reduced only in the four subjects in which Δ[O_2Hb] remained unchanged. There are however several accounts in the literature that O_2 breathing not only induces a maldistribution of cerebral perfusion at the microvascular level, but also brings about substantial and sustained reductions in regional cerebral blood flow. McLeod et al., (2003) recently demonstrated significantly reduction (~2.3 mM) of cerebral NIRS Δ[tHb] signal in eight adult patients being treated for traumatic brain injury. The drop was due to the O_2-induced cerebral vasoconstriction that sequentially reduces the cerebral blood volume (Bulte et al., 2007; Floyd et al., 2003; McLeod et al., 2003; Tisdall et al., 2009; Watson et al., 2000). The mechanism underlying such O_2-induced cerebral vasoconstriction has been proposed to be either hypocapnia (Becker et al., 1996) resulting from the O_2-induced hyperventilation (Dean et al., 2004; Ogoh et al., 2010) or the increase in PaO_2 *per se*, which has been shown to excert a direct vasoconstrictive effect (Floyd et al., 2003; Kolbitsch et al., 2002).

The leg and respiratory muscles did not respond to O_2 in a similar manner as did the cerebral cortex. Specifically, in both muscles a marked reduction in Δ[HHb] was noted during the HYPER exposure, a drop which was not accompanied by any change in Δ[O_2Hb] nor in Δ[tHb]. Although most studies regarding O_2 effects on muscle blood flow have observed a significant decrement in muscle blood flow and volume during normobaric hyperoxia at rest (Bredle et al., 1988; Rousseau et al. 2005), a few studies have not detected any such changes (Dufour et al., 2010; Plewes and Farhi, 1983). This discrepancy between results from different studies could be due to different methodologies used for monitoring muscle blood flow, to different muscle groups being investigated, and to the wide range of inter-individual variation with respect to the effect of O_2 on muscle perfusion.

Present results suggest that the oxygenation responses to O_2 breathing differ considerably between the cerebral and muscle tissues. Thus, following the O_2 administration, cerebral Δ[HHb] decreased immediately, whereas leg and respiratory muscle Δ[HHb] dropped after a delay of ~15 and ~65 minutes, respectively. Similarly, after the cessation of the O_2 exposure, Δ[HHb] returned to baseline values promptly in the cerebrum, but did not return to baseline values within the recovery period in the muscles. That the cerebral and muscle tissues responded differently to the offset of O_2 exposure is in agreement with the conclusions by Larsson et al., (2010), who compared the levels of oxygenation upon a transition from hyperoxia to normoxia in muscle tissue from values obtained in their own study, with values for cerebral tissue, derived in a different study (Litscher et al., 1997). Bergofsky and Bertun (1966) observed, in anaesthesized dogs, that hyperoxia decreased cerebral, bowel and hindlimb blood flow to varying degrees, the effect being most marked in the brain, less in the bowel and least obvious in the limb. Nielsen et al., (1999) observed that supplementation of hyperoxic breathing gas during a maximal exercise test diminished the exercise-induced drop in cerebral, but not in muscle oxygenation. Similarly, Amann et al., (2007) and Subudhi et al., (2008) observed that the

switch to hyperoxia at the task failure point during maximal exercise increased rapidly and substantially cerebral oxygenation, but only slightly muscle oxygenation; the increment in cerebral tissue oxygenation was especially pronounced under severe hypoxic exercise conditions.

6.3.2 Haemodynamic responses

One of the most consistent physiological responses to normobaric hyperoxia is the drop in HR (Daly and Bondurant, 1962; Gole et al., 2011; Larsson et al., 2010; Waring et al., 2003; Rousseau et al., 2005), which was also observed in the present study (i.e. ~12%). The O_2-induced bradycardia is usually attributed to a combination of reduced sympathetic tone (Seals et al., 1991) and enhanced vagal efferent discharge that increases the parasympathetic activity in the heart (Gole et al., 2011; Lund et al., 1999). However, the underlying mechanisms including the specific afferent pathways, evoking these responses are not fully understood. It has been postulated that the arterial baroreflex plays a role in the initiation of the hyperoxia-induced bradycardia (Rousseau et al., 2005), although its role in this context has been challenged since some studies have shown a slight rise in both SAP and DAP in response to hyperoxia (Daly and Bondurant, 1962; Waring et al., 2003), a finding which, however, is not consistent (Dufour et al., 2010; Gole et al., 2011; Larsson et al., 2010; Rousseau et al., 2005; Yamazaki et al., 2007). In the current study, the arterial pressure response was similar in the two trials; the small increases in SAP, DAP and MAP that were detected during the last 30 min in both trials may have been due to the mild discomfort experienced by the subjects after the prolonged period of immobility.

An alternative mechanism of the hyperoxic bradycardia is an activation of the arterial chemoreceptors (Daly and Bondurant, 1962; Gole et al., 2011). However, Lodato and Jubran, (1993) argued that the response time of the arterial chemoreflex acting through cardiac vagal efferent nerves is too fast - it is typically fully activated within a few seconds - to fully explain the bradycardic response to hyperoxia, which takes approximately 5 min to develop.

Present reults that hyperoxia diminished skin vasodilatation ($\Delta T_{calf\text{-}toe}$) is in keeping with induced a cutaneous vasoconstriction, the well established fact that hyperoxia induces peripheral vasoconstriction, including in cutaneous vessels (Rousseau et al., 2007; Yamazaki et al., 2007). It has been suggested that a peripheral mechanism at the microvascular level might mediate the O_2-dependent vasoconstriction by either an endothelial release of vasoconstrictors (O_2 radicals) (Rubanyi and Vanhoutte, 1986) or a diminished effect of vasodilators (prostaglandin, nitric oxide-NO) (Messina et al., 1994). It has been hypothesized that the hyperoxia-dependent bradycardia is secondary to the peripheral vasoconstriction tending to raise the MAP, an effect that is countered by activation of the arterial baroreceptors (Rousseau et al., 2005).

7 Carbon monoxide exposure during exercise performance: muscle and cerebral oxygenation

Despite the fact that atmospheric conditions have been improved in recent years, a number of air pollutants in the atmosphere of modern urban areas may have a detrimental effect on health and exercise performance (Carlisle and Sharp, 2001). Carbon monoxide (CO) is one of the most common air pollutants, and is a product of internal combustion engines in motor vehicles, especially those with petrol engines. Hence, considering the amount of people exercising daily in urban areas and that the majority of sporting events take place in megalopolises, it might be necessary to examine the effect of CO in the inspired air as recorded currently during peak hours of traffic in highly polluted areas on exercise performance.

It is widely accepted that decreases in arterial O_2 content (CaO_2) through a reduction in either inspired O_2 fraction (hypoxia) and/or haemoglobin (Hb) concentration diminish endurance exercise performance in healthy individuals (Amann et al., 2007; Koskolou and McKenzie, 1994; Subudhi et al., 2007). Likewise, hypoxia induced by CO inhalation inhibits O_2 utilization, and thus reduces the aerobic capacity (Aronow and Cassidy, 1975; Ekblom and Huot, 1972; Horvath et al., 1975; Nielsen, 1971; Pirnay et al., 1971; Vogel and Gleser, 1972). The main proposed mechanisms for such a CO-induced reduction in exercise performance include a binding of CO to Hb and formation of carboxyhaemoglobin (COHb), resulting in a reduction in the O_2-carrying capacity of the blood (Asmussen and Chiodi, 1941); a leftward shift of the Hb–O_2 dissociation curve and the consequent decreased O_2 delivery to the tissues (Roughton and Darling, 1944). It is suggested that a rise of COHb above the critical threshold of 2.7% results in a marked drop of aerobic performance (Raven et al., 1974).

A reduction in CaO_2 during hypoxic exercise (Amann et al., 2007, Subudhi et al., 2007) causes a significant drop in locomotor muscle oxygenation, as measured by near-infrared spectroscopy (NIRS). Likewise, Maehara et al., (1997) reported a greater leg muscle deoxygenation after a brief period of CO inhalation (~1000 ppm) that raised the COHb to ~15% during a constant-work test. Yet, it is unclear if, or to what extent, CO-induced deoxygenation might occur also at lower levels of CO, commonly recorded in modern urban areas (Carlisle and Sharp, 2001; Crown, 2010). In addition, considering that during strenuous dynamic leg exercise, the oxygenation in the intercostal muscles appears to co-vary with that of the exercising leg muscles (Keramidas et al., 2011a, Legrand et al., 2007), most likely due to the inability of the circulatory system to meet the increasing energy demands of both working regions (Vogiatzis et al., 2009), the effect of CO on respiratory muscle oxygenation during exercise should be investigated.

In addition to muscle oxygenation, the oxygenation in prefrontal cortical regions has been shown to play a dominant role in determining exercise performance in hypoxic conditions (Amann et al., 2007; Rupp and Perrey, 2009; Subudhi et al., 2007). Namely, it has been suggested that cerebral hypoxia limits aerobic performance once arterial PO_2 falls below a critical level (Amann et al., 2007; Subudhi et al., 2008). In contrast to hypoxic hypoxia, CO-induced hypoxia reduces CaO_2 without altering the arterial PO_2, and the resultant effect on cerebral oxygenation remains unresolved.

Based on clinical evidence that normobaric O_2 administration can be used to treat CO

poisoning (Weaver, 2009) since it hastens the elimination of CO from the blood (Weaver et al., 2000), it has been suggested that a brief period of O_2 breathing prior to an athletic event might improve exercise performance under conditions of detrimentally elevated ambient CO levels (Shephard, 1984). However, although it has been confirmed that during exhaustive exercise, administration of supplemental O_2 at the task-failure point enhances cerebral and muscle oxygenation and thus work capacity (Amann et al., 2007; Nielsen et al., 1999; Subudhi et al., 2008), whether breathing O_2 following exposure to CO improves the subsequent exercise performance is doubtful (Sperlich et al., 2010; Webster et al., 1998).

Accordingly, the main purpose of the present study was to investigate the effect of low levels of CO in the inspired air on cerebral, respiratory and leg muscle oxygenation during strenuous constant-power cycle ergometry. A secondary purpose was to examine the effect of O_2 breathing on cerebral and muscle oxygenation during such constant-power exercise under CO conditions. The administered levels of CO was 18.9 ppm, based on the recorded peak values in outdoor environments of urban areas (Carlisle and Sharp, 2001; Crown, 2010). We hypothesized that the 18.9 ppm CO administration: (a) will reduce exercise performance (b) will decrease leg and respiratory muscle oxygenation via formation of COHb and a leftward shift of the O_2 dissociation curve, but (c) will not influence the cerebral oxygenation due to the unaltered levels of PO_2. In addition, we hypothesized that a brief period of O_2 breathing will not increase exercise performance, but will improve muscle oxygenation as a result of regional elimination of CO.

7.1 Materials and Methods

7.1.1 Subjects

Nine healthy males participated in the study (Table 7.1). All were physically active on a recreational basis (no more than 2-4 hours of exercise per week); however none of them were engaged in a formal sport-training program. They were non-smokers and free of heart and lung diseases and had normal resting pulmonary function, as assessed by a standard pulmonary function test (PFT). The subjects were informed regarding the experimental procedures and risks involved, and gave their written consent. They were instructed not to engage in any strenuous activity and to refrain from consuming alcohol or any caffeinated product on testing days. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia and conformed to the Declaration of Helsinki.

Table 7.1: *Anthropometric characteristics of the subjects that paricipated in study IV*. Values are mean ± SD. FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; PEF: peak expiratory flow; SVC: slow vital capacity; $\dot{V}O_{2max}$: maximal O_2 uptake; PPO: peak power output.

Variables	
Age (years)	23.6 ± 3.0
Body mass (kg)	74.4 ± 3.1
Stature (cm)	179.9 ± 5.5
Body fat (%)	9.7 ± 2.7
FVC (L)	5.8 ± 0.7
FEV_1 (L)	4.9 ± 0.7
PEF ($L \cdot s^{-1}$)	10.4 ± 1.0
SVC (L)	5.3 ± 0.8
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	51.3 ± 2.2
PPO (W)	357 ± 29

7.1.2 Experimental Protocol

On a first visit to the laboratory, subjects were thoroughly familiarized with the equipment and experimental procedure. During a preliminary session, they performed a pulmonary function test and an incremental exercise test to exhaustion to determine their $\dot{V}O_{2max}$ and PPO. Commencing five days later, they participated in three trials that were conducted in a counter-balanced order, at the same time of the day and separated by at least 48 hours (Figure 7.1): (a) a 3-hour air (O_2: 20.9%) exposure (EXP_{NOR}) followed by a control constant-power test (CPT), (b) a 1-hour air and a 2-hour CO exposure (EXP_{CO}) succeeded by a constant-power test under CO conditions (CPT_{CO_A}), and (c) a 2-hour CO and a 1-hour 100% normobaric O_2 (EXP_{CO+O_2}) exposure followed by a constant power test under CO conditions (CPT_{CO_B}).

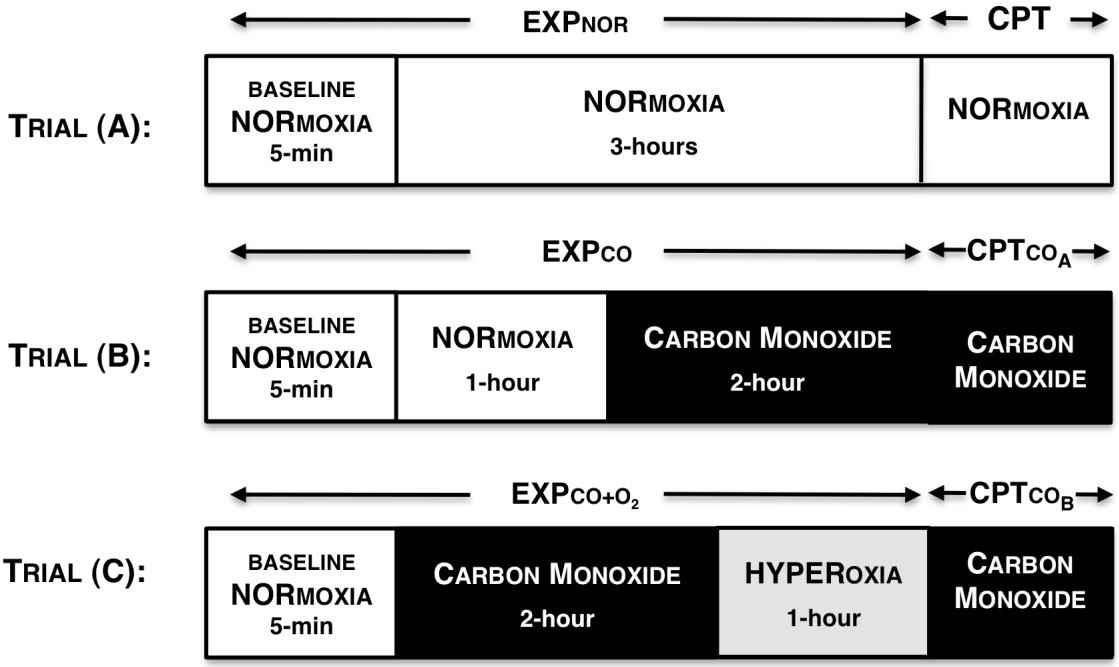

Figure 7.1: *Schematic representation of the overall study protocol.* EXP_{NOR}: 3-hour air exposure, EXP_{CO}: 1-hour air and 2-hour carbon monoxide exposure, EXP_{CO+O_2}: 2-hour carbon monoxide and 1-hour 100% O_2 exposure, CPT: control constant-power test, CPT_{CO_A}: constant-power test in carbon monoxide conditions, CPT_{CO_B}: constant-power test in carbon monoxide conditions preceded by pure O_2 breathing.

During all resting and exercise CO conditions, subjects breathed 18.9 ppm of CO. In all trials, participants were naïve regarding the breathing gas. The 3-hour pre-exercise breathing interventions were carried out with the subject in a resting semi-reclining position (25° head-up tilt), and the exercise tests were performed on an electrically braked cycle-ergometer (Daun Electronic, Furth, Germany). During all testing sessions, the environmental conditions were kept constant and thermoneutral: the mean ambient temperature, relative humidity and barometric pressure were 21.0 ± 0.9°C, 34.9 ± 3.7% and 978 ± 7 mb, respectively.

7.1.2.1 Preliminary session

7.1.2.1.1 Pulmonary function test

Pulmonary function was assessed using a Cardiovit AT-2 plus (Schiller, Baar,

Switzerland) spirometer, according to the criteria by Miller et al., (2005). The spirometer was calibrated before every test with a 2 L syringe (Schiller, Baar, Switzerland). Each subject performed each test three times and the highest of the three values was used for subsequent analysis. The PFT was used to obtain measures of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), peak expiratory flow (PEF), slow vital capacity (SVC) and maximum voluntary ventilation (MVV).

7.1.2.1.2 $\dot{V}O_{2max}$ testing

The $\dot{V}O_{2max}$ test commenced with a 3-min rest period, followed by a 5-min warm up on a cycle-ergometer at a work rate of 60 W. Thereafter, the load was increased by 25 $W \cdot min^{-1}$ until exhaustion. Attainment of $\dot{V}O_{2max}$, defined as the highest $\dot{V}O_2$ averaged over 60 s, was confirmed according to the following criteria, listed in priority order: (a) severe fatigue or exhaustion resulting in an inability to maintain exercise at a given work rate (cycling cadence lower than 60 rpm), (b) a plateau in $\dot{V}O_2$, and/or (c) a subjective rating of perception of effort at or near maximal.

7.1.2.2 Pre-exercise exposures

All the pre-exercise resting periods commenced with a 5-min baseline period in normoxia that was followed by the 3-hour exposure. Subjects breathed through a low resistance two-way respiratory valve (Model 2, 700 T-Shape, Hans Rudolph, Inc. Shawnee, USA). The inspiratory side of the respiratory valve was connected via respiratory corrugated tubing to a 200 L Douglas bag, which was continuously filled with the pre-mixed humidified breathing mixture. During the exposures, subjects were requested to relax and reduce their body movements.

7.1.2.3 Constant-power tests

There was a 8-10-min interval between the pre-exercise exposure and the subsequent constant-power test. During that period, subjects relocated to the cycle-ergometer, while they continuously breathed via the facemask either air in CPT or the gas mixture containing CO in CPT_{CO_A} and CPT_{CO_B}. They continued breathing via the Douglas bag throughout the interval period and the exercise tests.

Each constant-power test began with a 3-min resting period on the ergometer to record baseline values. Thereafter, the subjects were asked to complete a 5-min warm-up at an individualized work rate of 1.5 $W \cdot kg^{-1}$ body weight (mean power output = 112 ± 5 W). Subsequently, they cycled at 85% of PPO (mean power output = 303 ± 24 W). The subjects selected their preferred pedal cadence (between 60 to 90 rpm), which they maintained via visual and verbal feedback throughout the trial. The investigator terminated the test when the pedal cadence dropped below 70% of the self-selected cadence for ≥5 sec (task failure). Throughout each test, subjects remained seated on the cycle-ergometer to minimize changes in muscle recruitment; and received verbal encouragement always by the same investigator.

7.1.3 Instrumentation

7.1.3.1 Respiratory measurements

During the resting exposures and the exercise tests, $\dot{V}O_2$, ventilation ($\dot{V}E$), carbon

dioxide production ($\dot{V}CO_2$), partial pressure of end-tidal oxygen ($P_{ET}O_2$) and partial pressure of end-tidal carbon dioxide ($P_{ET}CO_2$) were measured on-line with a metabolic cart (Quark CPET, Cosmed, Rome, Italy). The gas analyzers and pneumotachograph were calibrated before each test with two different gas mixtures and a 3 L syringe (Cosmed, Rome, Italy), respectively. Due to the technical limitations of the metabolic card, the O_2 analyzer did not measure $\dot{V}O_2$ and $P_{ET}O_2$ responses during the 100% O_2 breathing in EXP_{CO+O_2}.

7.1.3.2 Heart rate and peak power output

HR was measured and recorded using a HR monitor (S800CX, Polar, Kempele, Finland). PPO was calculated by the equation (Kuipers et al., 1985):

$$PPO = PO_{FINAL} + (t / 60 \times 25\ W), \quad (1)$$

where PO_{FINAL} refers to the last workload completed, and t is the number of secods for which the final, uncompleted workload was sustained.

7.1.3.3 Capillary oxyhaemoglobin saturation

Throughout the resting exposures and constant-power tests, SpO_2 was monitored with a finger pulse oxymeter (BCI 3301, Wisconsin, USA), with an accuracy of ±2 units across the range of 70-100% and an acceptable resilience to motion artifacts (Langton and Hanning, 1990).

7.1.3.4 Ratings of perceived exertion

During the resting exposures, subjects were requested to provide ratings of perceived exertion for D-RPE every 15 min. During the exercise trials, subjects provided ratings for D-RPE and leg effort (L-RPE) at 1-min intervals. The modified Borg scale (scale 0-10) was explained to the participants by the investigator during the familiarization session and prior to the trials.

7.1.3.5 Arterial pressure

SAP and DAP pressures were measured with a noninvasive oscillometric automated sphygnomanometer (Omron M6, Kyoto, Japan) on the left hand by the same investigator at 30-min intervals during the resting exposures. MAP was calculated by the equation:

$$DAP + 1/3\ (SAP - DAP) \quad (2)$$

7.1.3.6 Blood lactate concentration

At the 3rd-min of recovery of the constant-power tests, two blood samples were taken from the tip of the left index finger to measure the [La]. Before every collection, the finger was cleaned and dried in order to avoid contamination from sweat and dirt. The skin was punctured with a lancet (Accu-Chek, Scoftclix Pro, Basel, Switzerland); the second drop of blood was placed on a strip (BM-lactate, Roche, Basel, Switzerland) and immediately analyzed with a portable analyzer (Accutrend Lactate, Roche, Basel, Switzerland).

7.1.3.7 Near-infrared spectroscopy

During the constant-power tests, the cerebral, intercostal and leg muscle oxygenation was monitored by three pairs of continuous-wave infrared spectroscopy (NIRS) probes (Artinins Medical System, Oxymon MKIII, Zetten, the Netherlands). The theory,

limitations and reliability of the cerebral and muscle measurements obtained with the NIRS device during exercise have been detailed previously (Ferrari et al., 2004; Perrey, 2008). In particular, the cerebral probe was positioned over the left prefrontal cortex between Fp1 and F3, according to the modified international EEG 10-20 system; the respiratory muscle probe was positioned over the left 7th intercostal space of the serratus anterior muscle and the leg muscle probe above the vastus lateralis, ~15 cm above the proximal line of the patella and ~5 cm lateral to the midline of the thigh. The probes consisted of one emitter and one detector housed in a black, plastic holder that was stabilized on the shaved and cleaned skin with double-sided adhesive tape. A bandage covered and stabilized each probe holder in order to reduce the intrusion of external light and the loss of transmitted NIR light from the measuring area. The inter-optode distance was kept at 4.5 cm to minimize the influence of skin blood flow.

The NIR light consisted of two wavelengths (780 and 850 nm), and the micromolar changes in tissue oxygenation - oxygenated ($\Delta[O_2Hb]$) and deoxygenated ($\Delta[HHb]$) haemoglobin - were calculated from the age-dependent differential path-length factors (DPF; range: 4.95-6.12) (Duncan et al., 1995). Although COHb also absorbs variation in the near infrared region, the absorbtion is negligible monitored by NIRS for Hb and O_2Hb (Zijlstra et al., 2000). In addition, total haemoglobin ($\Delta[tHb]$), which is the sum of $\Delta[O_2Hb]$ and $\Delta[HHb]$, was used as an index of change in regional blood volume (Van Beekvelt et al., 2001).

NIRS data were recorded at 50 Hz and stored in a PC for further analysis. Because the exact DPF was unknown, cerebral and muscle measurements were normalized to reflect the magnitude of changes from the baseline period prior the resting exposure of each trial (arbitrarily defined as 0 μM).

7.1.4 Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft, Inc., Tulsa, USA). All data are reported as mean (SD), unless otherwise indicated. Statistical significance of the cardiorespiratory variables throughout the pre-exercise resting exposures was assessed with a one-way analysis of variance (ANOVA) for repeated measures. Likewise, a one-way ANOVA for repeated measures was used for the analysis of the performance time and [La] attained during the exercise tests. A two-way ANOVA for repeated measures was used for the cardiorespiratory and NIRS data obtained during the constant-power tests (condition × overtime). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVAs revealed significant F-ratio for main effects. Differences in D-RPE and L-RPE were evaluated with a Friedman, followed by a Wilcoxon matched pairs non-parametric tests. The alpha level of significance was set *a priori* at 0.05.

7.2 Results

7.2.1 Preliminary session

7.2.1.1 Pulmonary function test

The mean ± SD values (% of predicted value) of FVC, FEV_1, PEF, SVC and MVV were 5.77 ± 0.68 L (109.1%), 4.86 ± 0.66 L (111.8%), 10.41 ± 0.99 $L \cdot sec^{-1}$ (105.4%), 5.39 ± 0.79 L (101.2%) and 196.33 ± 36.54 $L \cdot min^{-1}$, respectively.

7.2.1.2 $\dot{V}O_{2max}$ testing.

The average value of $\dot{V}O_{2max}$ and PPO are presented in Table 7.1. Peak values of HR, $\dot{V}E$ and SpO_2 were 180 ± 11 beats·min^{-1}, 144.5 ± 15.1 L·min^{-1} and 92 ± 3%, respectively; and the median (range) L-RPE and D-RPE were 10 (7 - 10) and 10 (7 - 10), respectively.

7.2.2 Pre-exercise exposures

7.2.2.1 Cardiorespiratory responses.

Mean values of the cardiorespiratory variables during the EXP_{NOR}, EXP_{CO} and EXP_{CO+O_2} are summarized in Table 7.2. $\dot{V}O_2$, $\dot{V}E$, SAP, DAP and MAP did not alter throughout the three resting exposures. Moreover, there were no differences in $P_{ET}CO_2$ or HR in EXP_{NOR} and EXP_{CO}. However, $P_{ET}CO_2$ and HR were significantly lower, and SpO_2 was significantly higher during the 1-hour O_2 breathing period compared to baseline and CO exposure in EXP_{CO+O_2} ($P < 0.001$). There were no differences in D-RPE during the exposures [EXP_{NOR}: 0 (0-2), EXP_{CO}: 0 (0-1), EXP_{CO+O_2}: 0 (0-1)].

Table 7.2: *Cardiorespiratory values during the 3-hourr air exposure (EXP_{NOR}), the 1-hour air and 2-hour carbon monoxide exposure (EXP_{CO}) and the 2-hour carbon monoxide and 1-hour 100% O_2 exposure (EXP_{CO+O_2})*. Values are mean ± SD. $\dot{V}O_2$: oxygen uptake, $\dot{V}E$: expired ventilation, $P_{ET}CO_2$: partial pressure of end-tidal carbon dioxide, HR: heart rate, SpO_2: arterial oxygen saturation, SAP: systolic blood pressure, DAP: diastolic blood pressure, MAP: mean arterial blood pressure.* Statistically significant different from baseline ($P < 0.001$).† Statistically significant different from carbon monoxide exposure ($P < 0.001$).

	EXP_{NOR}		EXP_{CO}			EXP_{CO+O_2}		
	Rest	NOR	Rest	NOR	CO	Rest	CO	100% O_2
$\dot{V}O_2$ (L·min^{-1})	0.33± 0.13	0.32± 0.13	0.35± 0.15	0.32± 0.12	0.31± 0.12	0.39± 0.15	0.34± 0.13	-
$\dot{V}E$ (L·min^{-1})	10.0± 1.6	9.3± 1.7	10.2± 1.9	9.2± 1.5	8.8± 1.7	11.0± 1.7	9.4± 1.6	10.0± 2.3
$P_{ET}CO_2$ (mmHg)	36.7± 2.3	37.3± 2.0	37.4± 1.2	37.9± 1.5	36.7± 1.6	37.8± 1.3	37.8± 2.1	32.6± 1.4*†
HR (beats·min^{-1})	58±9	55±7	59±8	56±7	55±8	59±7	56±6	50±6*†
SpO_2 (%)	96±1	96 ±1	96±1	96±1	96±1	96±1	96±1	98±0*†
SAP (mmHg)	125±6	125±8	123±5	122±5	126±6	124±8	123±8	125±7
DAP (mmHg)	67±5	68 ±5	65±3	65±5	70±6	67±4	65±5	70±5
MAP (mmHg)	86±5	87±6	85±3	84±4	89±5	86±4	85±5	88±5

7.2.3 Constant-power tests

7.2.3.1 Performance time

There were no differences in performance time between the constant-power tests (CPT = 6:51 ± 1:28 min, CPT_{CO_A} = 6:41 ± 1:39 min, CPT_{CO_B} = 6:52 ± 2:05 min; $P > 0.05$).

7.2.3.2 Cardiorespiratory responses

Cardiorespiratory variables during the CPT, CPT_{CO_A} and CPT_{CO_B} are summarized in Table 7.3. $\dot{V}O_2$, $\dot{V}E$ and HR were similar in the three performance tests ($P > 0.05$). However, during the resting period, $P_{ET}O_2$ was significantly higher in the CPT_{CO_B} than in the CPT and CPT_{CO_A}, and $P_{ET}CO_2$ was significantly lower in the CPT_{CO_B} than in the CPT. These differences disappeared after the initiation of the exercise task. There were no differences in SpO_2 at the maximal intensity between the trials (CPT: 93 ± 1%, CPT_{CO_A}: 93 ± 1%, CPT_{CO_B}: 93 ± 2%; $P > 0.05$). However, [La] at the termination of exercise was significantly higher in the CPT_{CO_A} than in the CPT and CPT_{CO_B} (CPT: 12.8 ± 1.3 mmol·l^{-1}, CPT_{CO_A}: 13.5 ± 1.6 mmol·l^{-1}, CPT_{CO_B}: 12.2 ± 1.3 mmol·l^{-1}; $P < 0.001$). There were no differences in D-RPE [CPT: 10 (7-10), CPT_{CO_A}: 10 (6-10), CPT_{CO_B}: 10 (6-10); $P > 0.05$] or L-RPE [CPT: 10 (8-10), CPT_{CO_A}: 9 (7-10), CPT_{CO_B}: 9 (7-10); $P > 0.05$] between the trials.

Table 7.3: *Cardiorespiratory values during the control constant-power test (CPT), the constant power-test in carbon monoxide conditions (CPT_{CO_A}) and the constant-power test in carbon monoxide conditions preceded by pure O_2 breathing (CPT_{CO_B}) at the same relative performance time.* Values are mean ± SD. $\dot{V}O_2$: oxygen uptake, $\dot{V}E$: expired ventilation, $P_{ET}O_2$: partial pressure of end-tidal oxygen, $P_{ET}CO_2$: partial pressure of end-tidal carbon dioxide, HR: heart rate. Data in three conditions were significantly different over time. ** Statistically significant difference between CPT and CPT_{CO_B}. † Statistically significant difference between CPT_{CO_A} and CPT_{CO_B}; ($P \leq 0.05$).

	CPT		$CPTCO_A$		$CPTCO_B$	
	Rest	Exercise	Rest	Exercise	Rest	Exercise
$\dot{V}O_2$ ($L \cdot min^{-1}$)	0.34± 0.12	3.78± 0.65	0.39± 0.09	3.85± 0.69	0.32± 0.09	3.79± 0.70
$\dot{V}E$ ($L \cdot min^{-1}$)	9.2± 3.3	114.7± 33.7	10.3± 2.1	116.0± 34.6	8.8± 2.7	113.5± 35.1
$P_{ET}O_2$ (mmHg)	102.3± 3.0	106.6± 6.0	104.2± 2.5	107.6± 6.4	115.8± 6.7**†	106.9± 6.1
$P_{ET}CO_2$ (mmHg)	33.2± 2.4	38.9± 4.4	32.9± 1.0	38.6± 4.4	31.4± 2.0**	39.4± 4.5
HR (beats·min^{-1})	72±7	166±18	71±7	165±15	71±7	165±16

7.2.3.3 Vastus lateralis oxygenation

$\Delta[O_2Hb]$ of the vastus lateralis decreased progressively during exercise up to 20% of exercise performance time, and thereafter plateaued through the end of the tests (Figure 7.2a). However, $\Delta[O_2Hb]$ was significantly lower in the CPT_{CO_B} (~25%) and CPT_{CO_A} (~30%) than in the CPT from the 20 and 40% of performance time, respectively, until the end of the trials ($P \leq 0.05$). Δ[HHb] did not differ between the CPT and CPT_{CO_A}; but was significantly higher in the CPT_{CO_B} than in the CPT and CPT_{CO_A} at 40 and 60% of performance time ($P \leq 0.05$) (Figure 7.2b). Δ[tHb] was significantly lower in the CPT_{CO_A}

than in the CPT at 80 and 100% of the performance time ($P \leq 0.05$). There were no differences in Δ[tHb] between the CPT and $CPTco_B$ (Figure 7.2c).

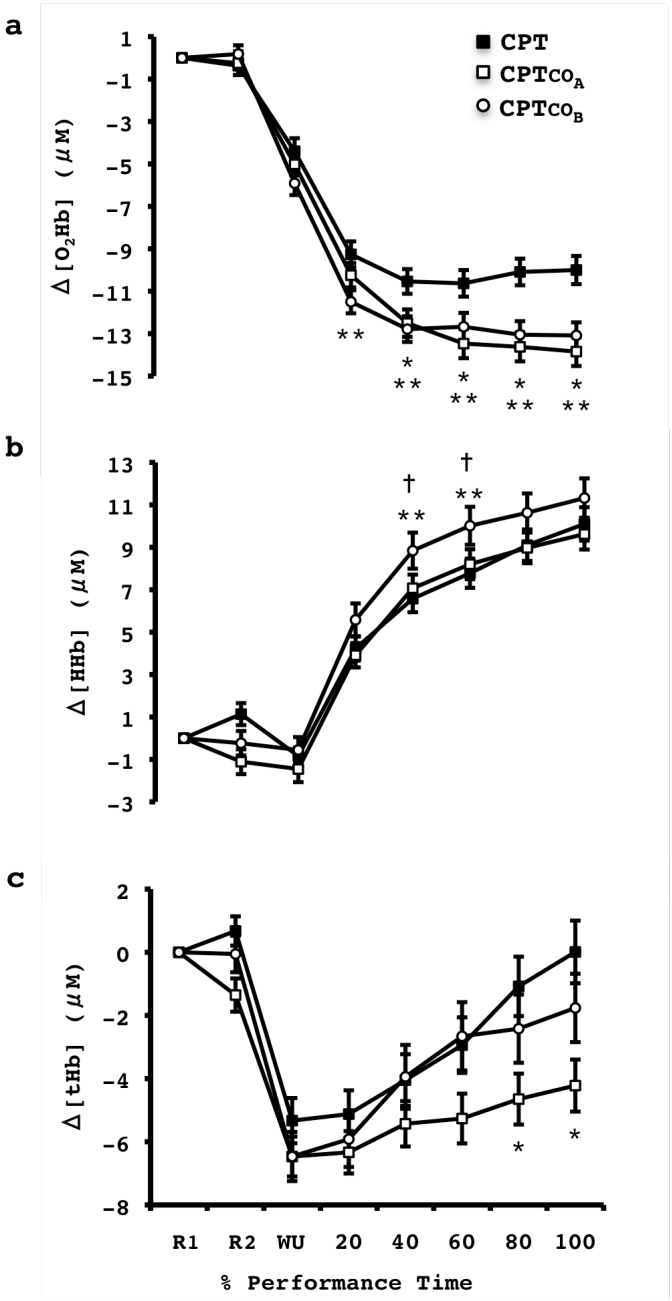

Figure 7.2: *Changes from resting values in vastus lateralis (a) oxyhaemoglobin (Δ[O_2Hb]), (b) deoxyhaemoglobin (Δ[HHb]) and (c) total haemoglobin (Δ[tHb]) during the control constant-power test (CPT), the constant power-test in carbon monoxide conditions ($CPTco_A$) and the constant-power test in carbon monoxide conditions preceded by pure O_2 breathing ($CPTco_B$) at the same relative performance time*. (means ± SE). Data in all conditions were significantly over time; * Significant difference between CPT and $CPTco_A$, ** Significant difference between CPT and $CPTco_B$, † Significant difference between $CPTco_A$ and $CPTco_B$; ($P \leq 0.05$).

7.2.3.4 Serratus anterior oxygenation

In all three trials, Δ[O_2Hb] of the serratus anterior declined until the end of exercise, but the drop was more pronounced in the $CPTco_A$ than in the CPT and $CPTco_B$ at 60, 80 and 100% of the exercise performance time ($P \leq 0.05$) (Figure 7.3a). There were no

differences in Δ[HHb] among the trials ($P > 0.05$) (Figure 7.3b). Δ[tHb] was significantly reduced in the $CPTco_A$ compared to in the CPT at 60 and 100% of performance time; and compared to in the $CPTco_B$ at 100% ($P \leq 0.05$) (Figure 7.3c).

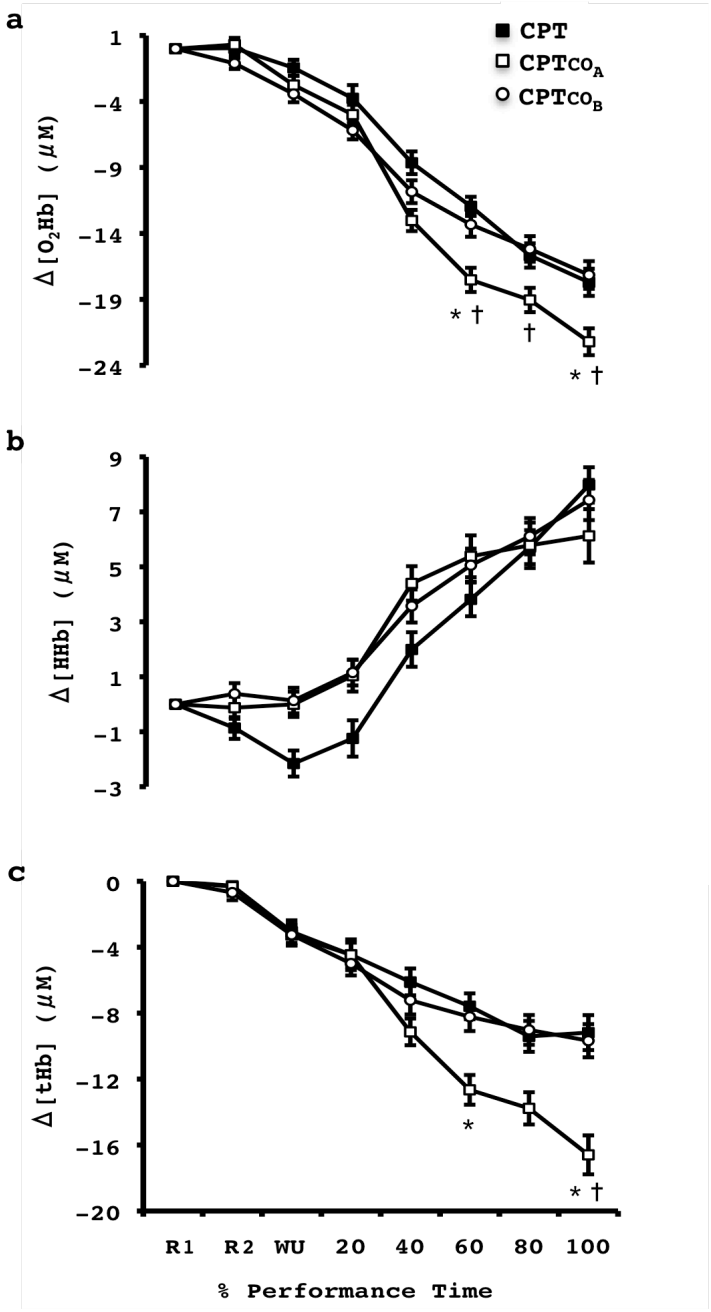

Figure 7.3: *Changes from resting values in serratus anterior (a) oxyhaemoglobin (Δ[O_2Hb]), (b) deoxyhaemoglobin (Δ[HHb]) and (c) total haemoglobin (Δ[tHb]) during the control constant-power test (CPT), the constant power-test in carbon monoxide conditions ($CPTco_A$) and the constant-power test in carbon monoxide conditions preceded by O_2 breathing ($CPTco_B$) at the same relative performance time.* (means ± SE). Data in all conditions were significantly over time; * Significant difference between CPT and $CPTco_A$, † significant differences between $CPTco_A$ and $CPTco_B$; ($P \leq 0.05$).

7.2.3.5 Cerebral oxygenation

In all tests, Δ[O_2Hb] and Δ[tHb] significantly increased after 80% of the exercise

performance time, but there were no differences between trials at any time point ($P > 0.05$) (Figure 7.4a and c). Δ[HHb] was significantly lower in the CPT_{CO_A} and CPT_{CO_B} than in the CPT (~42%) at peak exercise intensity ($P \leq 0.05$) (Figure 7.4b).

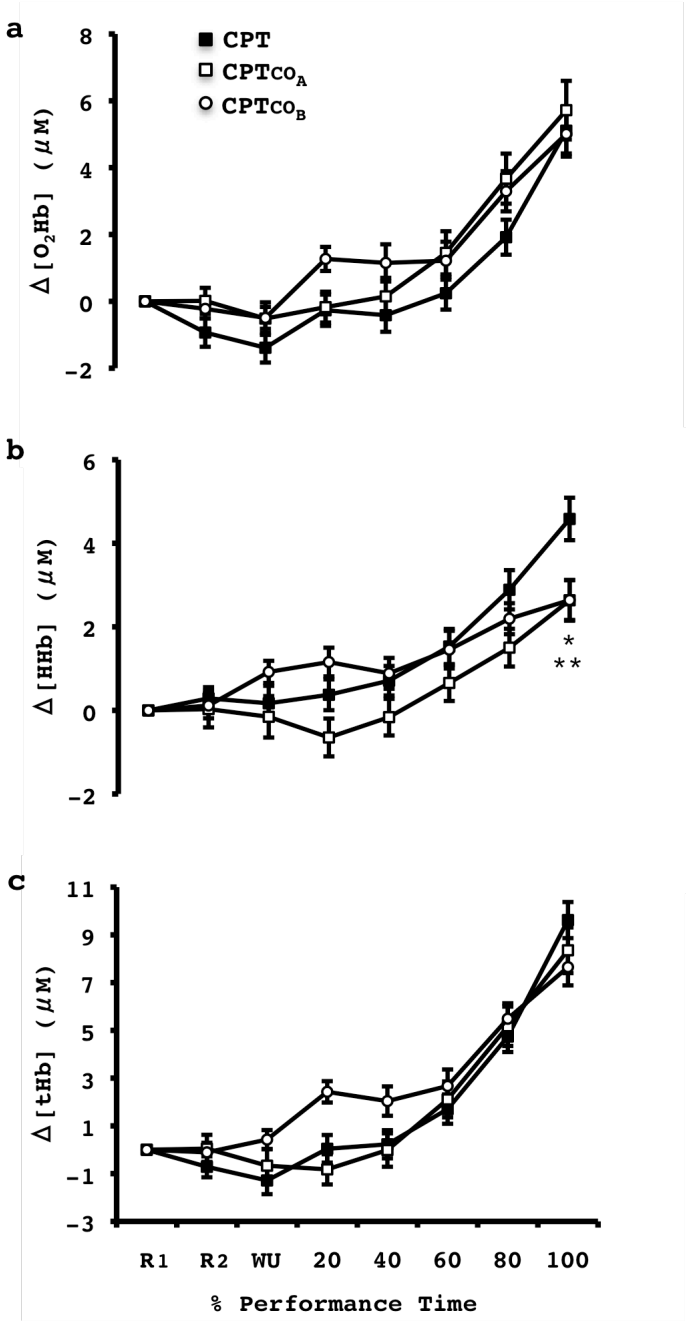

Figure 7.4: *Changes from resting values in cerebral (a) oxyhaemoglobin (Δ[O_2Hb]), (b) deoxyhaemoglobin (Δ[HHb]) and (c) total haemoglobin (Δ[tHb]) during the control constant-power test (CPT), the constant power-test in carbon monoxide conditions (CPT_{CO_A}) and the constant-power test in carbon monoxide conditions preceded by O_2 breathing (CPT_{CO_B}) at the same relative performance time.* (means ± SE). Data in all conditions were significantly over time; * Significant difference between CPT and CPT_{CO_A}, ** Significant differences between CPT and CPT_{CO_B}; ($P \leq 0.05$).

7.3 Discussion

The principal finding of the present study is that, during a constant-power test to exhaustion, exposure to low levels of CO decreased the oxygenation in the vastus lateralis and serratus anterior muscles, but did not affect the balance between O_2 delivery and utilization in the frontal cortex area. The CO-induced decrements in the oxygenation of leg and respiratory muscles did not affect exercise performance time. Moreover, a brief period of breathing normobaric O_2 moderated the CO-induced reductions in muscle oxygenation, mainly in the intercostals, whereas it did not affect cerebral oxygenation nor exercise endurance.

7.3.1 Exercise performance

7.3.1.1 Carbon monoxide exposure

CO has been considered a critical environmental air pollutant in determining exercise performance in healthy individuals. Thus, several studies have observed that moderate levels of CO inhalation during exercise conditions markedly reduce aerobic capacity and endurance performance (Aronow and Cassidy, 1975; Ekblom and Huot, 1972; Horvath et al., 1975; Pirnay et al., 1971; Vogel and Gleser, 1972), due to: (a) the binding of CO to Hb, the formation of COHb and the reduction in O_2 carrying capacity (Asmussen and Chiodi, 1941), (b) the leftward shift of the Hb-O_2 dissociation curve and the decreased O_2 delivery to the tissues (Roughton and Darling, 1944), (c) the binding of CO to myoglobin (Mb) and the formation of COMb (King et al., 1987), and (d) the interaction of CO with cytochrome *c* oxidase (Piantadosi et al., 1987).

However, the continual monitoring of CO levels in modern megalopolises reveals considerably lower CO concentrations in the atmosphere (Carlisle and Sharp, 2001; Crown, 2010), than those used in the aforementioned investigations on exercise endurance. A number of recent studies, which have analyzed individuals' performance times attained in races in association with the recorded levels of air pollutants of the area in which the competitions were held (i.e. Athens, Beijing), have suggested that the level of inspired CO is commonly not the most detrimental factor in limiting exercise performance, even in the most polluted areas (Flouris, 2006; Lippi et al., 2008; Marr and Ely, 2010; Nassis and Geladas, 2002). Indeed, a raise of the COHb above 2.7% has been suggested to be the critical threshold resulting in a significant drop of aerobic performance in healthy individuals (Raven et al., 1974). In the present study, COHb was not recorded, but it has previously been shown that a heavily exercising subject might increase COHb up to ~1.6% after inhaling a gas mixture containing 20 ppm CO for an hour (Carlisle and Sharp, 2001). Hence, the current administration of ~19 ppm CO presumably did not augment COHb of the subjects above the threshold of 2.7%, which in turn could explain their unaltered performance during the CPT_{CO_A}.

7.3.1.2 O_2 breathing

Although the beneficial effects of hyperoxic gas administration during exercise are well documented (Prieur et al., 2002; Tucker et al., 2007; Wilson and Welch, 1975), the potential ergogenic action of pre-exposure to O_2 to subsequent physical performance is doubtful (Eynan et al., 2010; Kawada et al., 2008; Sperlich et al., 2010). Still, considering that pure O_2 hastens the elimination of CO from the blood in conditions of CO poisoning (Weaver et al., 2000), it has been proposed that breathing normobaric O_2 might improve performance in CO-induced hypoxia (Shephard, 1984). That, in the present study, the 1-hour exposure to pure O_2 prior to CPT_{CO_B} did not increase performance is not surprising considering that the CO administration did in fact not reduce performance. That the O_2-

induced alterations in SpO_2, $P_{ET}O_2$ and $P_{ET}CO_2$ abated rapidly upon initiation of the CPT_{CO_B} confirm that the additional amount of O_2 dissolved in the blood during the EXP_{CO+O_2} was promptly washed out upon cessation of O_2 breathing (Sperlich et al., 2010), and thus did not significantly affect energy expenditure during the CPT_{CO_B}.

7.3.2 Muscle and cerebral oxygenation

7.3.2.1 Muscle oxygenation

Even though inhalation of 18.9 ppm CO did not alter performance time, leg muscle oxygenation was significantly reduced during the CPT_{CO_A}. This drop is in agreement with that previously reported by Maehara et al., (1997), who detected a greater vastus lateralis deoxygenation during exercise when the exercise bout was preceded by a brief CO administration inducing a 15% increase in COHb. Also, these CO-induced locomotor muscle responses are in agreement with the leg muscle deoxygenation observed during exercise in hypoxic hypoxia (Costes et al., 1996; Heubert et al., 2005; Maehara et al., 1997; Subudhi et al., 2007). In the present CO trials, the respiratory muscle oxygenation showed a similar course to that of the leg muscle, as has also been noted in previous studies (Keramidas et al., 2011a, Legrand et al., 2007). Most likely the CO-induced reductions of oxygenation of leg and respiratory muscles reflect limited capacity of the circulatory system to supply O_2 to the working muscles (Calbet et al., 2004, Vogiatzis et al., 2009).

The period of normobaric hyperoxia seemed to moderate the CO-induced muscle deoxygenation, most likely by facilitating elimination of CO from the muscles. That is, O_2 breathing did not influence leg $\Delta[O_2Hb]$ during the subsequent CO inhalation, whereas it appeared to preserve oxygenation in intercostal muscles at the same levels as in the CPT throughout exercise. The cause of the different responses in the vastus lateralis and intercostal muscles remains to be elucidated. Yet, the results support the notion that oxygenation of the intercostals does not limit exercise performance during constant-power pedalling in normoxia considering that, despite the O_2-induced increment of oxygenation in the serratus anterior, performance time remained unchanged between the trials performed in CO conditions.

The physiological mechanism by which CO hypoxia causes such marked muscle deoxygenation, similar to that observed during moderate hypoxic hypoxia, is unclear; in view of the fact that CO reduces CaO_2 without altering arterial PO_2. It has commonly been proposed that the CO-induced leftward shift of the O_2 dissociation curve disturbs the balance between delivery and utilization of O_2 in the muscle (King et al., 1987). Judging from the present study, it appears that even a small shift of the curve may significantly hinder tissue O_2 extraction, as reflected by the similar values of $\Delta[HHb]$ (Grassi et al., 1996) in the two trials.

CO-induced deoxygenation in the working muscles might conceivably also explain the slightly elevated [La] during the CPT_{CO_A} (Stringer et al., 1994), reflecting a higher contribution of anaerobic metabolism to the total energy requirements during the CPT_{CO_A} than the CPT. It would be expected that the [La] in CPT_{CO_B} would be similar to that in CPT_{CO_A}, considering the comparable drops in leg $\Delta[O_2Hb]$ in the two trials. However, the O_2 breathing did in fact slightly reduce the [La] in the CPT_{CO_B} compared to in the CPT_{CO_A}; and it reached similar values as in the CPT. It should be noted though that the small detected inter-trial differences in [La], amounting to about 6% do not allow us to draw any firm conclusions regarding the relationship between [La] and muscle tissue oxygenation.

Irrespective of the underlying mechanisms, the findings that subjects were able to

continue cycling at lower levels of muscle oxygenation and to attain equivalent performance times in all conditions, support the notion that muscle tissues are able to maintain adequate function under a wide range of oxygenation (Andersen and Saltin, 1985; Subudhi et al., 2007), and hence that oxygenation of the working leg muscles or the respiratory muscles does not limit exercise endurance during constant-power cycling in normoxic conditions.

7.3.2.2 Cerebral oxygenation.

The course of the frontal cortex oxygenation during the CPT_{CO_A} and CPT_{CO_B} did not differ from that during the CPT. In particular, a fall of the cerebral oxygenation at the beginning of the exercise was followed by a progressive increment until the termination of the task in all trials. This pattern is in agreement with findings in previous studies that have monitored cerebral oxygenation by NIRS technology during a normoxic constant-power test (Ide et al, 1999; Keramidas et al., 2011a). Accordingly, it appears that cortical oxygenation in the prefrontal lobe is not affected by CO-induced hypoxia of the current magnitude. This contrasts not only to the CO-induced muscle responses, but also to cerebral responses observed during exercise in hypoxic hypoxia (Subudhi et al., 2007); which might be explained by the similar levels of PO_2 in the three trials (Amann et al., 2007; Subudhi et al., 2008).

The pre-exposure to normobaric hyperoxia did not change the pattern of the cerebral oxygenation during exercise. Previous studies (Amann et al., 2007; Nielsen et al., 1999; Subudhi et al., 2008) have shown that administration of supplemental O_2 at the task failure point enhances cerebral oxygenation and work capacity. However, the present results confirm (Sperlich et al., 2010; Webster et al., 1998) that pre-exposure to normobaric O_2 does not have any ergogenic effect on exercise performance due to the fact that the additional amount of O_2 dissolved in the blood is minute.

7.3.3 Methodological considerations

The NIRS technology provides reproducible and non-invasive assessment of local differences in the balance between $\dot{V}O_2$ and delivery (Van Beekvelt et al., 2001), yet it is important to carefully consider the limitations of the technique in order to appropriately interpret the current findings. In particular, the NIR light is absorbed by heme groups both within Hb and Mb, and thence the exact contribution of the two compounds to the total signal cannot be separated. However, the relative contribution of Hb seems to be substantially greater than that of Mb, since the Hb is tetrameric and exists in appreciably greater concentrations than the monomeric Mb pigment (Wilson et al., 1989). Moreover, it has been shown that Mb desaturation occurs at low work intensities (<50% PPO) (Richardson et al., 1995), suggesting that changes in the NIRS signal occurring at the present intensity of 85% of PPO originate mainly from changes in Hb absorption. Furthermore, the thickness of the subcutaneous adipose layer may influence the NIRS signal (McCully and Hamaoka, 2000), potentially biasing comparison of signal magnitudes between subjects, which though in the present study was minimized by the low body fat of the subjects and by the counterbalanced crossover design. Lastly, it should be taken into account that the NIRS data represent regional and not global changes in oxygenation, and thus we are not able to ascertain the overall responses during the constant-power tests.

8 Conclusions

The main findings and conclusions of the present thesis were:

- **Study I**. A relatively brief exposure to normobaric hyperoxia does not increase the production of EPO in aerobically fit and healthy males. On the contrary, the increased O_2 tension suppresses the production of EPO 3 to 5 hours after the hyperoxic breathing intervention after which the [EPO] seems to recommence a natural circadian rhythm. Thus, the present results do not support the notion of a "normobaric oxygen paradox".
- **Study II**. Daily short-term exposures to normobaric hyperoxia do not increase the [EPO] in healthy male subjects. Hence, the present findings do not support the theory of a "normobaric oxygen paradox" in healthy populations.
- **Study III**. The acute normobaric hyperoxic exposure at rest influences the cerebral, leg and respiratory muscle oxygenation of healthy individuals, but not in the same manner. Namely, the cerebral tissue appears to be more sensitive to the O_2-induced changes compared to the muscle region; a fact which is characterized by the heterogeneous vasoconstrictive response to pure O_2 and the dissimilar return to baseline levels upon the transition to normoxic environment.
- **Study IV**. The substantial drop in leg and respiratory muscle oxygenation induced by inhalation of 18.9 ppm CO during a constant-power cycle-ergometry test does not affect exercise endurance. That oxygenation of locomotor and respiratory muscles is not critical for constant-power endurance is further supported by the finding that breathing normobaric O_2 moderates the CO-induced reduction in muscle oxygenation, mainly in the intercostals, but does not have any beneficial effect on performance. Oxygenation in cortical prefrontal regions does not seem to be influenced by inhaling CO levels similar to those encountered in highly polluted urban areas. However, it should be noted that the current study investigated the effects of a singular air pollutant, which in real conditions is not independent, but rather an element of a coalescent mixture of pollutants with a potential intoxicating synergy to exercise performance of healthy individuals.

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Have passion!

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Publications and Conference Presentations

Publications and conference presentations related to this thesis

The Chapters 4-7 of this thesis represent individual research papers that have been published or submitted to peer-review journals for publication. These papers are listed below:

[1] **Keramidas ME**, Kounalakis SN, Debevec T, Norman B, Gustafsson T, Eiken O, and Mekjavic IB. Acute normobaric hyperoxia transiently attenuates plasma erythropoietin concentration in healthy males: Evidence against the "normobaric oxygen paradox" theory. *Acta Physiologica* **202**, 91-98. (2011). (***Appendix A***)

[2] **Keramidas ME**, Norman B, Gustafsson T, Eiken O, and Mekjavic IB. Long-term intermittent hyperoxic exposures do not enhance erythropoiesis. *European Journal of Clinical Investigation*. (2011). doi: 10.1111/j.1365-2362.2011.02578.x. (***Appendix B***).

[3] **Keramidas ME**, Kounalakis SN, Eiken O, and Mekjavic IB. Carbon monoxide exposure during exercise performance: muscle and cerebral oxygenation. (*Under review*).

[4] **Keramidas ME**, Kounalakis SN, Geladas ND, Eiken O, and Mekjavic IB. Cerebral and muscle oxygenation during acute normobaric hyperoxic exposure at rest in healthy individuals. (*In preparation*).

Some of the aforementioned papers have been also presented in international conferences that are listed below:

[1] **Keramidas ME**, Kounalakis SN, Debevec T, Norman B, Gustafsson T, Eiken O, and Mekjavic IB. The effect of acute normobaric hyperoxia on EPO concentration in healthy males. *36th Annual Meeting of the European Underwater and Baromedical Society (EUBS)*, Istanbul, Turkey, 14-18/9/2010.

[2] **Keramidas ME**, Barbara N, Gustafsson T, Eiken O, and Mekjavic IB. Enhancement of erythropoiesis with normobaric hyperoxia: a promising treatment turns *anaemic*? *XIV International Conference on Environmental Ergonomics*, Nafplio, Greece, 10-15/7/2011.

Other publications during my PhD studies

During my PhD studies, I have published the following articles as a first author or a co-author (in chronological order):

[1] Geladas ND, Anastassopoulos S, **Keramidas ME**, and Koskolou MD. Maximal oxygen uptake may be limited by sensation of muscle oxygenation. *The Open Sports Medicine Journal*. 4: 9-16: 2010.

[2] **Keramidas ME**, Debevec T, Amon M, Kounalakis SN, Simunic B, and Mekjavic IB. Respiratory muscle endurance training: Effect on normoxic and hypoxic exercise performance. *European Journal of Applied Physiology*. 108:

759-769, 2010.

[3] Debevec T, Amon M, **Keramidas ME**, Kounalakis SN, Pisot R, and Mekjavic IB. Normoxic and hypoxic performance following four weeks of normobaric hypoxic training. *Aviation, Space and Environmental Medicine*. 81 (4): 387-393, 2010.

[4] **Keramidas ME**, Musizza B, Kounalakis SN, and Mekjavic IB. Enhancement of the finger cold-induced vasodilatation response with exercise training. *European Journal of Applied Physiology*. 109: 133-140, 2010.

[5] **Keramidas ME**, Kounalakis SN, Eiken O, and Mekjavic IB. Muscle and cerebral oxygenation after short-term respiratory work. *Respiratory Physiology and Neurobiology*. 175: 247-254, 2011. (***Appendix C***)

[6] **Keramidas ME**, Kounalakis SN, and Mekjavic IB. Aerobic exercise training preceded by respiratory muscle endurance training: a synergistic action enhances the hypoxic aerobic capacity. *European Journal of Applied Physiology*. 2011. doi: 10.1007/s00421-011-1887-2.

[7] Debevec T, **Keramidas ME**, Norman B, Gustafsson T, Eiken O, and Mekjavic IB. Acute short-term hyperoxia followed by mild hypoxia does not increase EPO production: Resolving the "normobaric oxygen paradox". *European Journal of Applied Physiology*. 2011. doi: 10.1007/s00421-011-2060-7.

Appendix A

KOMISIJA REPUBLIKE SLOVENIJE ZA MEDICINSKO ETIKO

Prof. dr. Igor B. Mekjavič
Odsek za avtomatizacijo, biokibernetiko in robotiko
Institut Jožef Stefan, Jamova 39, 1000 Ljubljana

Štev.: 129/07/11
Datum: 10. 8. 2011

Spoštovani gospod prof. dr. Mekjavič,

Komisiji za medicinsko etiko (KME) ste 6. 7. 2011 (osebna dostava, brez spremnega pisma) poslali v oceno predlog raziskave z naslovom:

"Izpostavljenost ogljikovemu monoksidu med telesno zmogljivostjo: mišična in možganska oksigenacija." "Carbon monoxide exposure during exercise performance: muscle and cerebral oxygenation."

KME je na seji 12. julija 2011 ocenila, da je raziskava etično sprejemljiva, in Vam s tem izdaja svoje soglasje.

Lep pozdrav,

prof. dr. Jože Trontelj
predsednik Komisije RS za medicinsko etiko

Naslov:
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Appendix B

ACTA PHYSIOLOGICA

Acta Physiol 2011, **202**, 91–98

Acute normobaric hyperoxia transiently attenuates plasma erythropoietin concentration in healthy males: evidence against the 'normobaric oxygen paradox' theory

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Abstract

Aim: The purpose of the present study was to evaluate the 'normobaric oxygen paradox' theory by investigating the effect of a 2-h normobaric O_2 exposure on the concentration of plasma erythropoietin (EPO).

Methods: Ten healthy males were studied twice in a single-blinded counterbalanced crossover study protocol. On one occasion they breathed air (NOR) and on the other 100% normobaric O_2 (HYPER). Blood samples were collected Pre, Mid and Post exposure; and thereafter, 3, 5, 8, 24, 32, 48, 72 and 96 h, and 1 and 2 weeks after the exposure to determine EPO concentration.

Results: The concentration of plasma erythropoietin increased markedly 8 and 32 h after the NOR exposure (approx. 58% and approx. 52%, respectively, $P \leq 0.05$) as a consequence of its natural diurnal variation. Conversely, the O_2 breathing was followed by approx. 36% decrement of EPO 3 h after the exposure ($P \leq 0.05$). Moreover, EPO concentration was significantly lower in HYPER than in the NOR condition 3, 5 and 8 h after the breathing intervention ($P \leq 0.05$).

Conclusion: In contrast to the 'normobaric oxygen paradox' theory, the present results indicate that a short period of normobaric O_2 breathing does not increase the EPO concentration in aerobically fit healthy males. Increased O_2 tension suppresses the EPO concentration 3 and 5 h after the exposure; thereafter EPO seems to change in a manner consistent with natural diurnal variation.

Keywords diurnal variation, erythropoiesis, hyperoxaemia, individual variability, oxygen therapy.

Erythropoietin (EPO) is a glycoprotein hormone that is produced primarily by the adult kidney. It stimulates the proliferation, differentiation and maturation of the bone marrow erythroid progenitor cells and accordingly regulates the production rate of red blood cells (Jelkmann 1992, 2010, Gunga *et al.* 2007). The secretion of EPO is regulated by the relative amount of O_2 availability to the tissues, and it is broadly accepted that acute (Friedmann *et al.* 2005, Mackenzie *et al.* 2008) and chronic (Gunga *et al.* 1994, Chapman *et al.* 1998, Berglund *et al.* 2002, Ge *et al.* 2002) hypoxia lead to an enhancement of EPO formation. In particular, it has been shown that either a short period (approx. 70–120 min) of continuous hypoxic exposure

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(Eckardt *et al.* 1989, Knaupp *et al.* 1992, Rodriguez *et al.* 2000, Mackenzie *et al.* 2008) or intermittent hypoxia induced by repeated voluntary maximal duration apnoeas (de Bruijn *et al.* 2008) markedly increase the levels of EPO concentration.

In contrast, an erythropoietic suppression following hyperoxic exposure in animals (Fletcher *et al.* 1973, Morshchakova *et al.* 1980) and humans (Kokot *et al.* 1994a,b) has been reported, although the underlying physiological mechanisms are still unclear. Recent studies have reported that the initial decrease of serum EPO concentration is followed by a significant increase 24 and 36 h after the cessation of a 2-h normobaric O_2 breathing (Balestra *et al.* 2004, 2006). The authors suggested that such hyperoxia-induced EPO production, which they term the 'normobaric O_2 paradox', is because of the sudden and sustained decrease in tissue O_2 level ('relative hypoxia') upon the transition from a hyperoxic to a normoxic breathing mixture. These findings were not confirmed by a similar study conducted by McGuire *et al.* (2006), who did not detect any significant differences compared with pre-exposure values after the O_2 breathing intervention.

One factor that might contribute to the aforementioned discrepancy is the wide inter-individual variability of the EPO response (Friedmann *et al.* 2005, Mackenzie *et al.* 2008). Moreover, the diurnal variation of EPO concentration may have contributed to the observation of the 'normobaric O_2 paradox', though the results from studies investigating the diurnal variation of EPO concentration are inconsistent (Wide *et al.* 1989, Klausen *et al.* 1993, Roberts & Smith 1996).

Accordingly, the purpose of the present study was to investigate the effect of a 2-h normobaric hyperoxic exposure on the EPO concentration. To minimize the inter-individual variability of the EPO response, a homogenous group of healthy aerobically well-trained males participated in this single-blinded counterbalanced crossover study. To account for the contribution of diurnal rhythm, we monitored the EPO concentration at regular intervals for 2 weeks following a 2-h period of breathing either air or 100% normobaric O_2. We hypothesized that an initial phase of suppressed EPO production will transpire few hours after the normobaric hyperoxic intervention and that such hyperoxia-induced drop in EPO concentration will not be followed by any further increment compared with the control condition.

Materials and methods

Subjects

Ten healthy males (mean ± SD; age 25.5 ± 3.0 years, body mass 74.3 ± 6.5 kg, stature 180.4 ± 6.9 cm, body fat 9.3 ± 4.2%) participated in the present study. All were physically active on a recreational basis; however, none of them was engaged in a formal sport-training programme. They were non-smokers, had no history of any renal, haematological, heart or lung disease and had not used any drugs acting as prostaglandin inhibitors during the month preceding the experiments. The subjects were informed in detail about the experimental procedures and risks involved and gave their written consent. They were instructed to abstain from consuming alcohol or any caffeinated product prior to, and during the study. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of Republic of Slovenia and conformed to the Declaration of Helsinki.

The sample size for the present study was determined using the reported mean (SD) responses of EPO reported by Balestra *et al.* (2006) and setting the level of statistical significance at 0.05. The analysis assumed that a 15% difference in the mean (SD) values between the two experimental conditions would be statistically significant and that the power of the test would be 0.85. *Post hoc* analysis revealed that the power of the statistical test performed was 0.95 (Cohen 1988).

Experimental protocol

On the first visit to the laboratory, the participants were thoroughly familiarized with the equipment and the experimental procedures. The experimental protocol consisted of a preliminary session during which an incremental exercise test to exhaustion was performed, followed after 1 week by two experimental sessions: a 2-h 100% normobaric O_2 exposure (HYPER) and a 2-h air exposure (NOR) (Fig. 1a). The order of the two experimental sessions was randomized.

Preliminary session. To ensure that subjects had similar levels of aerobic fitness, they performed an incremental exercise test to exhaustion on an electrically braked cycle-ergometer (Daun Electronic, Furth, Germany) to determine their maximal oxygen uptake ($\dot{V}O_{2max}$) and peak power output (PPO). Oxygen uptake ($\dot{V}O_2$) and ventilation ($\dot{V}E$) were measured online with a metabolic cart (Quark CPET; Cosmed, Rome, Italy). The heart rate (HR) was measured continuously using a HR monitor (S810i; Polar, Kempele, Finland). The blood lactate concentration (La) was measured from the tip of the left index finger at the third minute of recovery (Accutrend Lactate; Roche, Basel, Switzerland).

Air and hyperoxic exposure. One week after the preliminary session, the participants conducted the

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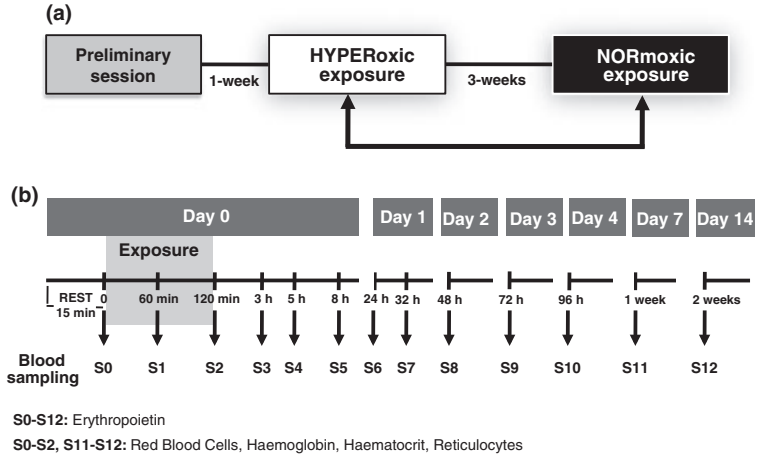

Figure 1 Schematic representation of (a) the overall study protocol and (b) the blood sampling in the hyperoxic (HYPER) and control (NOR) phases.

main experimental trials in a single-blind manner. Five subjects breathed 100% normobaric O_2 for 2 h, and the rest of the participants breathed air (O_2: 20.93%) for the identical time. After a 22-day washout period, the experiments were repeated with the subjects crossed in conditions.

During both exposures, the participants were in a supine position for 2 h and breathed through a low resistance two-way respiratory valve (Model 2, 700 T-Shape; Hans Rudolph, Shawnee, KS, USA). The inspiratory side of the respiratory valve was connected via respiratory corrugated tubing to a 200 L Douglas bag filled with the premixed humidified breathing mixture. Throughout the 2-h period, HR was recorded continuously using a HR monitor (Polar); and ratings of perceived exertion (RPE; scale 0–10) for dyspnoea-respiratory discomfort (D-RPE) were requested every 15 min.

All tests were conducted at the same time of the day (beginning of the exposure at 8:00–8:30 hours) to ensure that the effect of diurnal variations was similar in both trials. The environmental conditions were kept constant and thermoneutral during both exposures: the mean ambient temperature, relative humidity and barometric pressure were 21.5 ± 1.0 °C, 41.0 ± 1.9% and 978 ± 9.3 mmHg respectively. The participants were instructed not to engage in any strenuous activity a day before and throughout the first week after the exposure. Apart from that, they followed their normal daily routines (no more than 3–5 h of exercise per week) during the next 2 weeks. During the entire experimental period, they were asked to record their physical activity in individual diaries.

Blood analyses

Erythropoietin. The participants reported to the laboratory at 7:30 in the morning, after an overnight fast. Venous blood samples were collected in EDTA tubes from an antecubital vein immediately before (Pre), in the middle (Mid) and at the end (Post) of the breathing interventions (Fig. 1b). Thereafter, blood samples were collected 3, 5, 8, 24, 32, 48, 72 and 96 h, and 1 and 2 weeks after the cessation of O_2- or air-breathing intervention. The blood was immediately centrifuged and the plasma was frozen to −80 °C for the subsequent analysis.

The concentration of EPO was determined by sandwich enzyme-linked immunoassay (Quantikine IVD EPO ELISA; R&D Systems, Minneapolis, MN, USA) using 100 μL of plasma. Optical density was quantified on a microplate reader Quant (Bio-Tek Instruments, Winooski, VT, USA) set at 450 nm and corrected at 600 nm. The current method has been validated before (Sakata *et al.* 1995). All techniques and materials were in accordance with the protocol provided by the company. All samples were assayed in triplicate and one microplate was used for each subject. The estimated coefficient of variation of the analysis was 3.1% and the sensitivity of the measurement was 0.6 mU mL^{-1}.

Complete blood count and reticulocyte count. Venous blood samples (500 μL) were collected for a complete blood count and reticulocyte count Pre, Mid, Post and 1 and 2 weeks after the breathing interventions (Fig. 1b). The complete blood count including analysis of total red blood cells (RBC), haemoglobin concentration (Hb) and haematocrit (Hct), and the reticulocyte count were obtained with an automated laser-based haematology analyser (Advia 120; Siemens, Munich, Germany) within 6 h after the blood sampling. The apparatus was calibrated before each measurement. All samples were assayed in duplicate. The estimated coefficient of variation for the RBC, Hb, Hct and reticulocyte count was 1.1, 0.8, 1.3 and 8.1% respectively.

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Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft, Tulsa, OK, USA). All data are reported as mean (SD), unless otherwise indicated. A two-way analysis of variance (ANOVA) for repeated measures was used for the haematological variables (condition × time). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVA revealed significant *F*-ratio for main effects. Moreover, Pearson's correlation analysis was used for selected variables. The alpha level of significance was set *a priori* at 0.05.

Results

$\dot{V}O_{2max}$ testing

The average value of $\dot{V}O_{2max}$ was 55.4 ± 5.1 mL kg^{-1} min^{-1} and PPO was 348 ± 30 W. Moreover, the maximal HR, $\dot{V}_E$ and La were 185 ± 11 beats min^{-1}, 152.9 ± 16.6 L min^{-1} and 14. 6 ± 3.1 mmol l^{-1} respectively.

Air and hyperoxic exposure

HR and D-RPE. The mean HR was significantly lower during the HYPER than during the NOR condition (NOR: 59 ± 6 beats min^{-1}; HYPER: 55 ± 6 beats min^{-1}; $P \leq 0.05$). There was no difference in D-RPE between the exposures [NOR: 0 (0–3); HYPER 0.5 (0–5); $P > 0.05$].

Erythropoietin. The mean absolute values of EPO concentration throughout the experimental period are presented in Figure 2, which also reveals the wide individual variability of plasma EPO concentration. Figure 3 shows the relative changes of EPO concentration in both conditions up to the blood sample 48 h after the breathing interventions. During NOR, EPO concentration showed an initial increase of approx. 35% 5 h after the cessation of the breathing intervention; the increase was more pronounced 8 and 32 h after the exposure (approx. 58% and approx. 52%, respectively; $P \leq 0.05$). Conversely, the 100% normobaric O_2 breathing was followed by approx. 36% decrement of EPO concentration 3 h after the intervention ($P \leq 0.05$); EPO returned to pre-exposure values 8 h after the exposure. Furthermore, the EPO levels were significantly lower in the HYPER than in the NOR condition 3, 5 and 8 h after the intervention ($P \leq 0.05$); no more differences were observed between the two conditions at any other time-point.

Complete blood count and reticulocytes. The mean values of Hb, Hct, reticulocyte count and RBC are summarized in Table 1. In both conditions, Hct and reticulocyte count did not alter throughout the experimental period ($P > 0.05$). Hb and RBC increased slightly 1 and 2 weeks after the breathing intervention ($P \leq 0.05$). However, there were no differences in any of the measured variables between the two conditions.

Discussion

The principal finding of the present study is that a short period of 100% normobaric O_2 breathing caused an initial erythropoietic suppression that was not followed by a marked increase of EPO concentration. Moreover, besides the decrement 3 and 5 h after the normobaric O_2 exposure, the concentration of EPO seemed to change in a manner consistent with natural diurnal variation. The present results are contrary to those previously reported by Balestra *et al.* (2006), who detected a marked increase in EPO 32 h after the exposure, but in agreement with the results of McGuire *et al.* (2006), who did not observe any significant

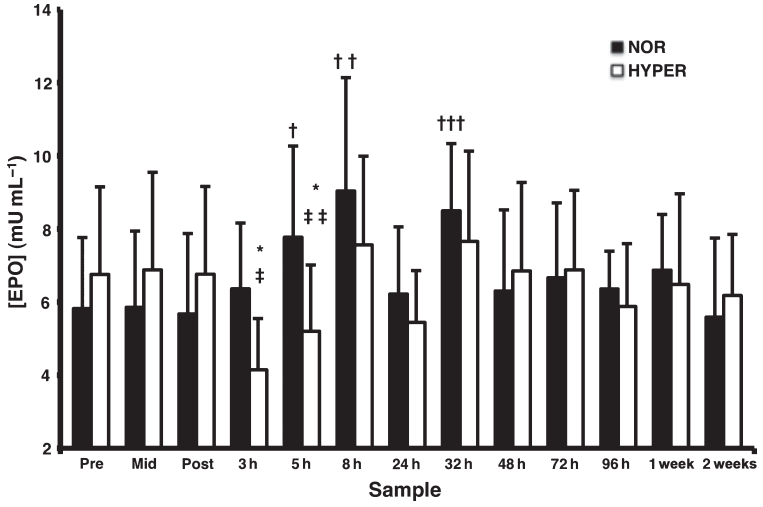

Figure 2 Absolute values of erythropoietin concentration Pre, Mid, Post 3, 5, 8, 24, 32, 48, 72, 96 h, 1 and 2 weeks after air (NOR) and hyperoxic (HYPER) breathing intervention. Values are means ± SD. Significantly different: †from Pre, Mid, Post and 1 week; ††from Pre, Mid, Post, 3, 24, 48, 72, 96 h and 1 week; †††from Pre, Mid, Post 3, 24, 48, 96 h and 2 weeks; ‡from Pre, Mid, Post, 8, 32, 48, 72 h, 1 and 2 weeks; ‡‡from 8 and 32 h. *Significant difference between normoxia and hyperoxia ($P \leq 0.05$).

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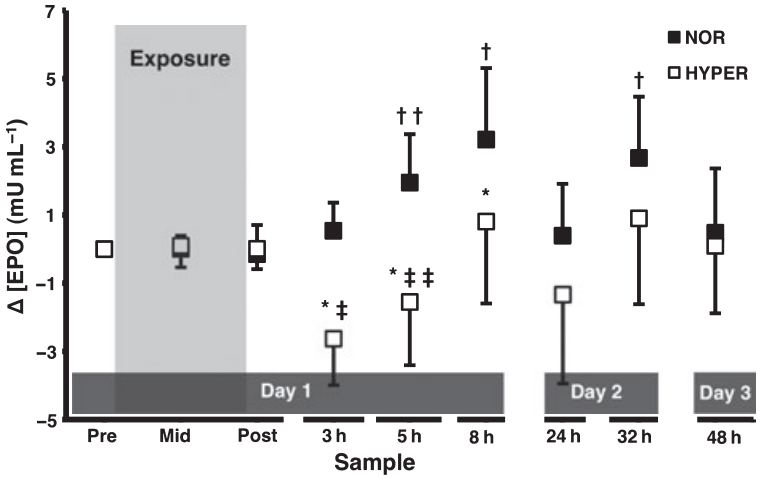

Figure 3 Changes from Pre values of erythropoietin concentration Mid, Post, 3, 5, 8, 24, 32 and 48 h after air (NOR) and hyperoxic (HYPER) breathing intervention. Values are means ± SD. Significantly different: †from Pre, Mid, Post, 3, 24 and 48 h; ††from Pre, Mid and Post; ‡from Pre, Mid, Post, 8, 24, 32 and 48 h; ‡‡from 8 and 32 h. *Significant difference between normoxia and hyperoxia ($P \leq 0.05$).

changes in EPO concentration after a period of breathing normobaric O_2.

It is widely accepted that tissue hypoxia is the primary stimulus of EPO production; and it is assumed that the O_2-sensitive sensor triggering the synthesis of EPO is located in the renal cortex (Bauer & Kurtz 1989). In particular, the cells controlling the synthesis of EPO appear to respond to changes in the O_2 capacity, the O_2 tension and the O_2 affinity of the blood, and to the renal blood flow (Jelkmann 1992). In the present study, a general decline of EPO concentration was observed 3 and 5 h after the end of O_2 breathing intervention, which confirms the findings of others (Fletcher *et al.* 1973, Morshchakova *et al.* 1980, Kokot *et al.* 1994a,b). This decrement in EPO concentration is of interest in view of the shape of the haemoglobin O_2 binding curve, because the O_2 content of fully oxygenated arterial blood increases very little when the O_2 tension is enhanced above normal. Furthermore, renal blood flow does not appear to be affected by normobaric hyperoxia as suggested by studies in conscious rats (Torbati *et al.* 1979, Flemming *et al.* 2000) and dogs (Berry *et al.* 1998). Thus, it is questionable whether the renal cortex is the principal origin of diminished EPO secretion or increased EPO elimination. Recent studies have suggested the presence of humoral factor(s) stemming from the hypothalamic–hypophyseal system that, at least in part, has a regulating effect on renal EPO secretion (Pagel *et al.* 1989, von Wussow *et al.* 2005). The hypophysial influence on EPO production is likely mediated by the concerted action of several hormones, including adrenocorticitropic hormone, growth hormone, thyroid hormones and sex steroid hormones (Jelkmann 1992), but the exact mechanisms are still undefined. As it has been demonstrated that the brain comprises sensors for the detection of changes in arterial PO_2 (Lahiri *et al.* 2006), the prospect that high PO_2 suppresses EPO secretion via the central nervous system should also be considered. Thus, the mechanisms underlying the O_2-induced suppression of EPO remain speculative and need to be further investigated.

'Normobaric oxygen paradox'

The suppression of EPO was diminished 5 h after the cessation of the O_2 breathing intervention, and it was reversed to the basal values 8 h later. Despite this, the EPO concentration remained lower than in NOR; and the difference disappeared 1 day later, in contrast to Balestra *et al.* (2006), who observed a marked EPO increase at a specific point in time following the hyperoxic exposure. The authors suggested that such hyperoxia-induced EPO production is because of the sudden and sustained decrease in renal O_2 tension ('relative hypoxia') upon the transition from a hyperoxic to a normoxic breathing condition. However, such a mechanism was not confirmed by the present results.

Indeed, the EPO secretion seems to follow a natural diurnal variation, which, in the present study, might have been disturbed by the short period of O_2 breathing. The results of studies regarding the circadian rhythm on EPO concentration are equivocal (Gunga *et al.* 2007). Some of them have detected pronounced changes during the course of the day (Cotes & Brozovic 1982, Wide *et al.* 1989, Cahan *et al.* 1992, Klausen *et al.* 1993, Kokot *et al.* 1994b), while others have not (Miller *et al.* 1981, Gunga *et al.* 1996, Roberts & Smith 1996). The observed diurnal variation is described by nadir values of EPO in the morning hours, and zenith levels during the evening and night hours. Even though the present data do not permit us to draw firm conclusions regarding circadian rhythm of EPO secretion, it is noteworthy that they are indeed consistent with such a rhythm, characterized by the lowest values at 08:00–09:00 hours and peak values at 18:00–19:00 hours. These changes are unlikely to be caused by differences in volume distribution along the body axis (Kirsch *et al.* 2005) as a consequence of body

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Table 1 Haematological variables Pre, Mid, Post, 1 and 2 weeks after the air (NOR) and 100% normobaric O_2 (HYPER) breathing intervention

	NOR					HYPER				
	Pre	Mid	Post	1 week	2 weeks	Pre	Mid	Post	1 week	2 weeks
Hb ($g\ dL^{-1}$)	15.0 ± 0.7	14.9 ± 0.7†	15.0 ± 0.8	15.4 ± 0.5†	15.5 ± 0.6*	14.8 ± 0.6	14.7 ± 0.4	14.8 ± 0.5	15.3 ± 0.6‡	15.3 ± 0.7*
Hct (%)	0.44 ± 0.03	0.43 ± 0.02	0.44 ± 0.03	0.44 ± 0.02	0.45 ± 0.02	0.43 ± 0.02	0.43 ± 0.01	0.43 ± 0.02	0.44 ± 0.02	0.44 ± 0.02
Reticulocyte count (%)	0.8 ± 0.3	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.2
RBC ($10^{12}\ L^{-1}$)	5.0 ± 0.2	5.0 ± 0.2	5.0 ± 0.2	5.2 ± 0.2*	5.2 ± 0.2*	4.9 ± 0.2	4.9 ± 0.2	5.0 ± 0.2	5.1 ± 0.2*	5.1 ± 0.2*

Values are means ± SD. Hb, haemoglobin; Hct, haematocrit; RBC, red blood cell count.
*Statistically significantly different from Pre, Mid and Post.
†Statistically significantly different from Mid.
‡Statistically significantly different from Pre and Mid.

movements (Gunga *et al.* 1996) or intense exercise tasks (Schwandt *et al.* 1991, Roberts *et al.* 2000), as the participants were instructed to remain in the laboratory throughout the testing day either in a supine or in a sitting position and to refrain from any strenuous physical activity the day before the tests. Furthermore, the total amount of blood (30 mL) that was collected up to the sample 48 h after the exposure could not, in itself, be responsible for any changes in the EPO concentration (Roberts & Smith 1996).

Inter-individual variability of EPO response

In the present study, only three of ten males exhibited higher values of EPO in the HYPER compared to the NOR condition 32 h after the breathing intervention. These responses were not related to subjects' aerobic fitness ($r = -0.10$) or basal values of Hb ($r = 0.23$). Likewise, based on unpublished observations, Balestra *et al.* (2004, 2006) reported that only two of five divers markedly increased their EPO levels after a series of breath-hold dives. In this regard, several investigations have confirmed the wide inter-individual variability of EPO response to acute (Friedmann *et al.* 2005, Mackenzie *et al.* 2008) or chronic hypoxic stimulus (Gunga *et al.* 1994, Chapman *et al.* 1998, Ge *et al.* 2002) that may be linked to specific genetically inherited traits (Ou *et al.* 1998, Jedlickova *et al.* 2003). Hence, we cannot exclude genetic determinants of individual variability of the EPO response to normobaric hyperoxia that may enlighten the inconsistencies between the present findings and those of Balestra *et al.* (2004, 2006).

Clinical perspectives

Following the observation that EPO concentration increased in one patient suffering from chemotherapy-induced anaemia after repeated exposures to O_2 (Burk 2007), and based on the theory of a 'normobaric O_2 paradox' (Balestra *et al.* 2006), O_2 treatment has been advocated as a means of increasing EPO in anaemic patients (Balestra *et al.* 2010, De Bels *et al.* 2010). It appears that longitudinal studies regarding the effect of repeated exposures of O_2 on haematological variables are required before considering O_2 as an adjuvant therapy in anaemic patients. Notably, the present single O_2 exposure did not significantly increase reticulocyte count or RBC from baseline values.

Methodological considerations

In the present study, the EPO concentration was determined in plasma, in contrast with Balestra *et al.* (2004, 2006), who measured it in the serum. However, it is unlikely that the different results could be explained

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by the different specimen, given that no concentration differences have been detected between serum and plasma samples in previous studies (Eckardt *et al.* 1988, Lindstedt & Lundberg 1998, Jedlickova *et al.* 2003). Moreover, the high sensitivity and the low coefficient of variation of the current analysis enforce the reliability and validity of the present findings.

In conclusion, the results of the present study demonstrate that a relatively brief exposure to normobaric hyperoxia does not increase the production of EPO in aerobically fit and healthy males. On the contrary, the increased O_2 tension suppresses the production of EPO 3–5 h after the hyperoxic breathing intervention after which the EPO concentration seems to recommence a natural circadian rhythm. Thus, the present results do not support the notion of a 'normobaric oxygen paradox'.

Conflict of interest

The authors state that there is no personal conflict of interest in the present study.

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Appendix C

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ORIGINAL ARTICLE

Long-term intermittent hyperoxic exposures do not enhance erythropoiesis

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ABSTRACT

Background Based on a report of a marked increase in the erythropoietin concentration ([EPO]) a few hours after the cessation of a single 2-h session of O_2 breathing, short periods of O_2 administration have been advocated as a therapy for anaemia. Accordingly, the purpose of the present study was to evaluate this theory by investigating the effect of 10 daily short-term exposures to normobaric O_2 over a 2-week period on the plasma [EPO] in healthy individuals.

Material and methods Twenty men were assigned to either an experimental (NBO_2) or to a control (AIR) group. The NBO_2 group breathed 100% normobaric O_2 for 2 h every weekday over a 2-week period. The AIR group breathed air within the same time protocol. Blood samples were collected at the pre-, mid- and post-intervention periods to determine [EPO].

Results [EPO] of the NBO_2 group was significantly lower than that of the AIR group during the mid- and post-periods ($P < 0{\cdot}001$). [EPO] of the NBO_2 group showed a slight, albeit statistically nonsignificant, decrease during the mid ($\sim$ 11%)- and post ($\sim$ 16%)-periods.

Conclusions Daily short-term exposures to normobaric hyperoxia do not increase the [EPO] in healthy individuals. The increased O_2 tension suppresses [EPO]. Hence, administration of pure O_2 to enhance erythropoiesis is not warranted.

Keywords Anaemia, erythropoietin, hyperoxaemia, 'normobaric O_2 paradox', oxygen therapy.

Eur J Clin Invest 2011

Introduction

Recent publications advocate the use of pure O_2 breathing as a treatment for anaemia [1] and as an adjuvant therapy for patients with cancer [2]. The justification for these recommendations is based on a finding that the transition from an acute hyperoxic exposure (breathing 100% O_2 for 2 h) to normoxia (breathing normal room air) induced erythropoiesis. The term 'normobaric O_2 paradox' was coined to define the serendipitous observation of a marked paradoxical increase in the endogenous erythropoietin concentration ([EPO]) 24 and 36 h after the cessation of an acute normobaric O_2 breathing [3,4]. The suggested mechanism for such a hyperoxia-induced [EPO] production is the sudden and sustained decrease in tissue O_2 level ('relative hypoxia') upon the transition from hyperoxic to normoxic breathing conditions [3].

This theory conflicts with the reported O_2-induced erythropoietic suppression in animals [5,6] and humans [7–11]. Keramidas *et al.* [7] observed that a 2-h exposure to normobaric hyperoxia did not increase the production of [EPO] in healthy men during a single-blinded counter-balanced crossover investigation. On the contrary, the increased O_2 tension suppressed plasma [EPO] 3–5 h after the breathing intervention, confirming similar previous findings [5,6,8,9]. Thereafter, the plasma [EPO] levels appeared to resume their natural circadian rhythm [12,13]. Likewise, McGuire *et al.* [10] and Momeni *et al.* [11] failed to detect any significant differences in plasma [EPO] in healthy individuals after an acute O_2 breathing intervention.

Despite conflicting evidence regarding the erythropoietic potential of pure O_2, the 'normobaric O_2 paradox' continues to

be promoted as an efficient treatment for the enhancement of haemoglobin (Hb) for several clinical conditions (i.e. anaemia, cancer and sepsis) [1,2,14–16]. However, advocates of this therapy base their recommendation on the findings of a study investigating the effect of a single acute exposure to hyperoxia [3], disregarding the paucity of data on the long-term effects of O_2 breathing on erythropoiesis. It may be argued that the response of plasma [EPO] to a single 2-h period of breathing pure O_2 is of limited value. For O_2 therapy to be of any practical clinical value in this context, it would need to provide a long-term stimulus for erythropoiesis, which may require repeated hyperoxic exposures.

Accordingly, the present study evaluated whether the cumulative effect of daily hyperoxic exposures induces erythropoiesis. Specifically, the 'normobaric O_2 paradox' theory was challenged by investigating the effect of 10 daily short-term exposures to normobaric O_2 over a 2-week period on plasma [EPO] in healthy aerobically well-trained men. We hypothesized that although the evidence of erythropoiesis following a single hyperoxic exposure is equivocal, repeated stimuli of increased O_2 tension might give rise to a cumulative effect, which would potentially have clinical relevance.

Materials and methods

Subjects

Twenty healthy men participated in the study. All of them were near-sea-level residents and were not exposed to altitude > 500 m during the month preceding the experiments. They were nonsmokers [17] and had no history of any renal, haematological, heart or lung disease [18–20]. They had not used any drugs acting as prostaglandin inhibitors [21] during the month preceding the experiments. All subjects were physically active on a recreational basis; however, none of them were engaged in a formal sport-training programme nor were any of them scuba divers. The subjects were informed in detail regarding the experimental procedures and risks involved and gave their written consent. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia and conformed to the Declaration of Helsinki.

Experimental protocol

The experimental protocol consisted of ten 2-h sessions, during which either 100% normobaric O_2 or room air was inspired. Each condition comprised three testing phases (Fig. 1): a) pre-tests: 1 day before the 1st exposure session, b) mid-tests: 1 day after the 5th exposure session and c) post-tests: 1 day after the 10th exposure session. During the three testing phases, blood samples were collected. Moreover, in the pre-testing phase, the subjects performed an incremental exercise test to exhaustion ($\dot{V}O_{2max}$) to ensure that they had similar levels of aerobic fitness.

Throughout the 2-week experimental period, the subjects followed their normal daily routine of physical activity (no more than 3–5 h of exercise per week) and were asked to record their activities in individual diaries. However, they were instructed not to engage in any strenuous activity and to abstain from consuming alcohol or any caffeinated product a day before the tests.

Air and hyperoxic exposure. After completing all baseline tests, the subjects were assigned to either the control group that breathed air (O_2: 20·9%) for 2 h (AIR) or the experimental group that breathed 100% normobaric O_2 for the identical time (NBO_2), groups being balanced with respect to age and aerobic capacity (Table 1).

All subjects were exposed to normobaric O_2 or air 5 days per week for 2 weeks, each exposure being supervised by the same investigator. The exposures were conducted at the same time of the day for each subject (between 8:00 and 17:30); the environmental conditions were kept constant: the mean ambient temperature, relative humidity and barometric pressure were 24·0 ± 0·8 °C, 35·9 ± 3·9% and 979 ± 4 mb, respectively.

The subjects were naive regarding the breathing gas. During each session, they were seated while breathing the gas mixture through a low-resistance two-way respiratory valve (Model 2, 700 T-Shape; Hans Rudolph, Inc. Shawnee, KS, USA). The inspiratory side of the respiratory valve was connected via

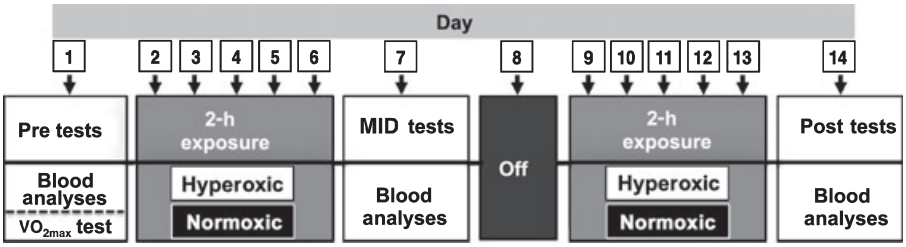

Figure 1 Schematic representation of the overall study protocol.

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Table 1 Descriptive characteristics of the control (AIR) and hyperoxic (NBO_2) groups

	AIR group (n = 10)	NBO_2 group (n = 10)
Age (years)	24·0 ± 3·2	25·1 ± 3·1
Stature (cm)	181·1 ± 5·4	181·1 ± 7·5
Body mass (kg)	78·9 ± 8·3	75·6 ± 7·3
Body fat (%)	11·8 ± 4·1	12·0 ± 8·2
$\dot{V}O_{2max}$ (mL/kg per minute)	50·6 ± 6·7	49·4 ± 6·3
PPO (Watts)	348 ± 30	331 ± 38

Values are mean ± SD.
$\dot{V}O_{2max}$, maximal O_2 uptake; PPO, peak power output.

respiratory corrugated tubing to a 200-L Douglas bag, which was continuously filled with the pre-mixed humidified breathing mixture. Throughout each exposure, heart rate (HR) was recorded continuously using a heart rate monitor (RS800CX; Polar, Kempele, Finland); the subject provided ratings of perceived exertion (RPE; 0–10) for dyspnoea-respiratory discomfort (D-RPE) at 15-min intervals. Moreover, during each breathing intervention, capillary oxyhaemoglobin saturation (SpO_2) was monitored continuously using a finger pulse oxymeter (BCI 3301, Waukesha, WI, USA) to ensure that SpO_2 values remained normal (≥ 95%) throughout the exposure; subjects were not provided any feedback from the physiological responses.

Blood analyses

During the tests (pre, mid, post), each subject reported to the laboratory at 8:00 in the morning, after an overnight fast. He rested for 5 min in a semi-reclining chair, and venous blood samples were collected in two different EDTA tubes from the antecubital vein to determine the [EPO], the complete blood count and the reticulocyte count. The blood sample for the EPO determination was immediately centrifuged and the plasma frozen to −80 °C for subsequent analyses. During the subsequent blood analyses, the investigator was blinded to each specimen.

Erythropoietin. The [EPO] was determined by sandwich enzyme-linked immunoassay (Quantikine IVD EPO ELISA; R&D Systems, Minneapolis, MN, USA) using 100 μL of plasma. Optical density was quantified on a microplate reader Quant (Bio-Tek instruments, Winooski, VT, USA) set at 450 nm and corrected at 600 nm. The method has been validated previously [22]. All techniques and materials were in accordance with the protocol provided by the company. All samples were assayed in duplicate. The estimated coefficient of variation of the analysis was 3·1%, and the sensitivity of the measurement was 0·6 mU/mL.

Complete blood count and reticulocyte count. Venous blood samples (2 mL) were collected for complete blood count and reticulocyte count. The complete blood count, including analysis of total red blood cells (RBC), Hb and haematocrit (Hct), and the reticulocyte count were obtained with an automated laser-based hematology analyzer (Advia 120; Siemens, Munich, Germany) within 6 h after the blood sampling. The apparatus was calibrated before each measurement. All samples were assayed in duplicate. The estimated coefficient of variation for the RBC, Hb, Hct and reticulocyte count was 1·4, 0·6, 2·1 and 3·1%, respectively.

Statistical analysis

Statistical analyses were performed using STATISTICA 5·0 (StatSoft, Inc., Tulsa, OK, USA). All data are reported as mean (SD), unless otherwise indicated. A two-way analysis of variance (ANOVA) for repeated measures was used for the haematological variables (group × testing period). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVA revealed significant F-ratio for main effects. Differences in median (range) D-RPE were evaluated with a Wilcoxon matched pairs nonparametric test. The alpha level of significance was set *a priori* at 0·05.

Results

Heart rate and dyspnoea-respiratory discomfort

The mean HR during all the breathing sessions was significantly lower in the NBO_2 than in the AIR group (AIR: 66 ± 8 beats/min; NBO_2: 57 ± 7 beats/min; P = 0·03). There was no difference in D-RPE between the exposures [AIR: 0·5 (0·5–1); NBO_2: 0 (0–0·5); P > 0·05].

Erythropoietin

Figures 2 and 3 present the mean absolute values and the relative changes of [EPO] of both groups, respectively. Pre values of [EPO] were similar in the AIR and NBO_2 groups, whereas both mid and post [EPO] values were lower (P < 0·001) in the NBO_2 than in the AIR group. [EPO] of the AIR group was increased during the mid-period compared to pre values (∼ 41%; P = 0·02). The increase seemed to be maintained in the post-period (∼ 31%), but the difference was not statistically significant compared to the pre-levels (P > 0·05). Conversely, [EPO] of the NBO_2 group showed a slight decrease during the mid (∼ 11%)- and post (∼ 16%)-tests that was not statistically different from the pre values (mid: P = 0·88; post: P = 0·67).

Complete blood count and reticulocytes

Mean values of Hb, Hct, reticulocyte count and RBC are summarized in Table 2. In both groups, Hb, Hct and RBC decreased

in the mid-test ($P \leq 0{\cdot}05$); there were no differences in any of these variables between the groups. The reticulocyte count did not alter throughout the experimental period in either of the groups ($P > 0{\cdot}05$).

Discussion

The present study clearly demonstrates that a daily 2-h exposure to normobaric O_2 over a 2-week period does not increase plasma [EPO] in aerobically fit men, confirming the established O_2-induced erythropoietic suppression and disapproving the notion of a 'normobaric O_2 paradox' in healthy individuals. Advocating the use of pure O_2 breathing as a method to enhance erythropoiesis is thus not warranted.

The term 'normobaric O_2 paradox' was coined to define the supposedly paradoxical increase in endogenous [EPO] 24 and 36 h after the cessation of a short-term normobaric O_2 breathing in response to a sharp decrease in tissue O_2 level ('relative hypoxia') upon the transition from a hyperoxic to a normoxic condition [3]. Notwithstanding, the present findings disprove the aforementioned notion and, in fact, confirm the already known suppressive role of pure O_2 to erythropoiesis [7–9]. Namely, in a previous study [7], we observed that a short-term exposure to normobaric O_2 causes a marked decrease in [EPO] 3 and 5 h after the cessation of breathing. Eventually, that drop was followed by a slow return of [EPO] to its natural circadian rhythm and the reversal to its basal values 8 h later. However, a small decline of [EPO] ($\sim$ 11%) was still evident 24 h after the breathing intervention, despite the lack of any statistical difference from the control values. Likewise, in the current study, we detected an equivalent drop, albeit statistically nonsignificant decrease in [EPO] of the NBO_2 group during the mid ($\sim$ 11%)- and post ($\sim$ 16%)-testing period.

Contrary to the NBO_2 group, [EPO] increased significantly in the AIR group during the mid- and post-periods. This increment was unexpected, because the AIR group breathed normoxic air during all the sessions; none of the participants performed any strenuous activity a day before the blood tests nor did they change their normal daily routines throughout the 2-week period [23], as evident from their individual activity diaries, which they maintained during this period. Present results do not allow us to discern which mechanisms might underlie this enhancement of [EPO]. Conceivably, it is attributable to the slightly diminished levels of Hb and/or Hct [24–28], which, in turn, might reflect a seasonal variation of Hct [29] (the study was performed during June and July) and/or the level of hydration of the subjects. That the drop in Hb correlated with the increase in [EPO] in the AIR group ($r = -0{\cdot}63$; $P = 0{\cdot}03$) and that no such correlation was observed in the NBO_2 group ($r = -0{\cdot}39$; $P > 0{\cdot}05$) support the notion that the [EPO] increased in the AIR group was secondary to the Hb drop and that the O_2 intervention prevented such [EPO] increase in the NBO_2 group. Regardless of the underlying mechanisms, both groups presented similar decrements in Hb and Hct during the course of the breathing regimen. Therefore, and because [EPO] was considerably lower in the NBO_2 than in the AIR group during and immediately after the breathing regimen, present results support the notion that normobaric hyperoxia suppresses erythropoiesis.

By contrast, Balestra *et al.* [3] did not detect any significant decline of [EPO] at least during the initial period following the O_2 breathing. This disparity might be due to the wide inter-individual variability of the [EPO] response to the O_2 stimulus [30–32] and as regards the natural circadian rhythm of [EPO] [12,13].

The mechanism for the O_2-induced suppression of [EPO] remains unresolved. Considering the shape of the oxyhaemoglobin binding curve, the O_2 content of arterial blood will increase very little ($\sim$ 5%) during O_2 breathing, and hence, it is unlikely that the hyperoxia-induced suppression of [EPO] release is attributable to increased O_2 availability in the renal tissue [25]. Recent studies regarding the presence of humoral factor(s) stemming from the hypothalamic-hypophyseal system that, at least in part, have a regulating effect on renal EPO

Table 2 Haematological variables pre-, mid- and post-breathing intervention period of the control (AIR) and hyperoxic (NBO_2) groups

	AIR group			NBO_2 group		
	Pre	Mid	Post	Pre	Mid	Post
Hb (g/dL)	15·5 ± 0·7	14·9 ± 0·6*	14·9 ± 0·7	15·6 ± 1·1	14·9 ± 0·9*	15·4 ± 0·9
Hct (%)	0·48 ± 0·02	0·46 ± 0·02*	0·47 ± 0·02	0·48 ± 0·04	0·47 ± 0·03*	0·48 ± 0·03
Reticulocyte count (%)	1·5 ± 0·5	1·6 ± 0·4	1·5 ± 0·4	1·3 ± 0·2	1·3 ± 0·2	1·2 ± 0·2
RBC (10^{12} per litre)	5·3 ± 0·3	5·2 ± 0·3*	5·2 ± 0·3	5·3 ± 0·5	5·1 ± 0·4*	5·3 ± 0·4

Values are mean ± SD.
Hb, haemoglobin; Hct, haematocrit; RBC, red blood cell count.
*Statistically significant difference from the Pre testing period.

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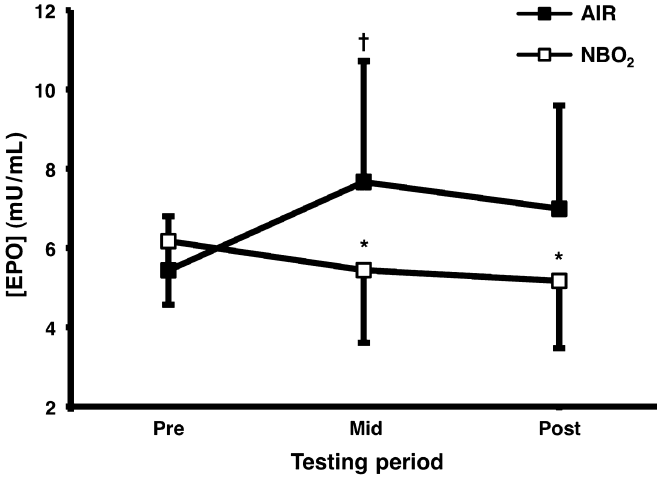

Figure 2 Absolute values of erythropoietin concentration ([EPO]) during the pre-, mid- and post-breathing intervention periods for the control (AIR) and hyperoxic (NBO_2) groups. Values are mean ± SD. (†) Significant difference from the pre-testing period. (*) Significant difference between the AIR and NBO_2 groups ($P \leq 0{\cdot}05$).

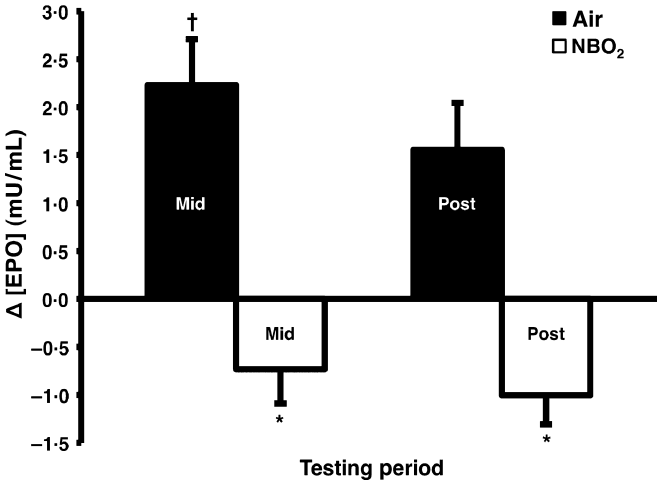

Figure 3 Changes from pre values of erythropoietin concentration (Δ[EPO]) during the mid- and post-breathing intervention periods for the control (AIR) and hyperoxic (NBO_2) groups. Values are mean ± SE. (†) Significant difference from the pre-testing period. (*) Significant difference between the AIR and NBO_2 groups ($P \leq 0{\cdot}001$).

secretion [33,34], and given the existence of sensors in the brain capable of detecting changes in arterial PO_2 [35], the role of the central nervous system on the suppression of [EPO] should be taken into account.

Clinical perspectives

Despite the implausible premise for the existence of a 'normobaric O_2 paradox' in healthy individuals and the lack of evidence concerning its haematopoietic effects in patients, O_2 breathing is being advocated both as an effective treatment for primary anaemia and as an adjuvant therapy for patients with cancer suffering from chemotherapy-induced anaemia [1,2,14–16]. It is noteworthy that the erythropoietic suppression resulting from a long-term (≤ 5 days) continuous normobaric O_2 breathing has been confirmed in anaemic subjects [36,37]. Therefore, and because long-term treatment with normobaric O_2 might have side effects [cf. 38, 39], to emphasize the use of O_2 breathing as a treatment to increase EPO in anaemic patients might be unwarranted. In addition, considering that potential confounders (e.g. drugs, sleep apnoea) are common in patients, anecdotal findings regarding erythropoiesis in single anaemic individuals should be interpret circumspectly. Thus, specific randomized controlled long-term studies are required before considering O_2 therapy as an adjuvant treatment for anaemia.

In conclusion, the results of the present study demonstrate that daily short-term exposures to normobaric hyperoxia do not increase the [EPO] in healthy male subjects. Hence, the present findings do not support the theory of a 'normobaric O_2 paradox' in healthy population.

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Conflict of interest

The authors state that there is no financial or personal conflict of interest in the present study.

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Appendix D

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Muscle and cerebral oxygenation during exercise performance after short-term respiratory work

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ABSTRACT

The purpose of the study was to investigate the effect of 30-min voluntary hyperpnoea on cerebral, respiratory and leg muscle balance between O_2 delivery and utilization during a subsequent constant-power test. Eight males performed a $\dot{V}O_{2max}$ test, and two exercise tests at 85% of peak power output: (a) a control constant-power test (CPT), and (b) a constant-power test after a respiratory maneuver (CPT_{RM}). Oxygenated ($\Delta[O_2Hb]$), deoxygenated ($\Delta[HHb]$) and total ($\Delta[tHb]$) hemoglobin in cerebral, intercostal and vastus lateralis were monitored with near-infrared spectroscopy. The performance time dropped ~15% in CPT_{RM} (6:55 ± 2:52 min) compared to CPT (8:03 ± 2:33 min), but the difference was not statistically significant. The vastus lateralis and intercostal $\Delta[tHb]$ and $\Delta[HHb]$ were lower in CPT_{RM} than in CPT ($P \leq 0.05$). There were no differences in cerebral oxygenation between the trials. Thus, respiratory work prior to an exercise test influences the oxygenation during exercise in the leg and respiratory muscles, but not in the frontal cortex.

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1. Introduction

Whether exercise-induced respiratory demands for O_2 can influence exercise tolerance; and if so, what could be the potential mechanisms for this limitation, remains unresolved. Several experimental approaches have been used to determine the contribution of respiratory muscle fatigue to endurance exercise performance (Romer and Polkey, 2008). One of these approaches is the pre-fatigue of respiratory muscles through either voluntary hyperpnoea (global fatigue of respiratory muscles) or resistive external loads (fatigue of inspiratory or expiratory muscles) at rest, followed by a whole body exercise performance. Indeed, the findings from studies using such a protocol remain equivocal: some of them have observed significant reductions of exercise performance after pre-fatigue of the respiratory muscles (Mador and Acevedo, 1991; Martin et al., 1982; Verges et al., 2007), while others have not (Dodd et al., 1989; Sliwinski et al., 1996; Spengler et al., 2000).

It has been speculated that the mechanism by which pre-fatigue of respiratory muscles limits exercise tolerance is through activation of the respiratory muscle metaboreflex, resulting in reduced blood flow to the exercising limb muscles (Dempsey et al., 2002, 2006). Likewise, it has been proposed that a competition for blood flow between the locomotor and the respiratory muscles exists, in such a way that respiratory muscle blood flow may increase at the expense of blood flow to working limb muscles (Harms et al., 1997, 1998). However, none of these studies, which used the experimental approach of the pre-fatigue of respiratory muscles followed by endurance exercise test, monitored blood flow or the levels of oxygenation of respiratory and locomotor muscles.

Aside from peripheral fatigue, previous findings have revealed that cortical deoxygenation in prefrontal regions has a potentially pivotal role in determining maximal exercise performance in healthy individuals exposed to hypoxia (Subudhi et al., 2007) and in patients with terminal lung disease (Jensen et al., 2002); but it does not limit normoxic exercise performance (Amann et al., 2007; Billaut et al., 2009). Nevertheless, it is still unknown whether the frontal cortex area becomes involved in the proposed competition of respiratory and locomotor muscles for blood flow in healthy individuals under normoxic exercise conditions preceded by respiratory muscle fatigue.

During exercise in humans it is not feasible to directly measure blood flow simultaneously in several muscle groups (Nielsen et al., 2001). However, near-infrared spectroscopy (NIRS) offers noninvasive, real-time assessment of local differences in the balance between O_2 consumption ($\dot{V}O_2$) and delivery (Van Beekvelt et al., 2001); and is popularly used to monitor cerebral (Amann

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et al., 2007; Billaut et al., 2009), intercostal (Nielsen et al., 2001; Vogiatzis et al., 2009) and leg muscle (Subudhi et al., 2007) oxygenation during exercise.

Accordingly, the purpose of the present study was to investigate the effect of a 30-min voluntary isocapnic hyperpnoea task on cerebral, respiratory and leg muscle oxygenation during a subsequent constant-power cycle ergometry test. We hypothesized that during the exercise performance test following the respiratory maneuver: (a) the leg muscle oxygenation will be reduced due to exaggerated respiratory muscle metaboreflexes in the pre-fatigued muscles, and (b) the cerebral oxygenation will be unimpaired confirming the notion that it does not constitute a limiting factor during normoxic exercise.

2. Material and methods

2.1. Subjects

Eight healthy, well-trained males (age 23.9 ± 4.6 years, body mass 72.56 ± 6.65 kg, stature 181.33 ± 5.30 cm, body fat 12.99 ± 3.67%) took part in the study. All participants were non-smokers and free of heart and lung diseases and had normal resting pulmonary function, as assessed by a standard pulmonary function test (PFT). The subjects were informed in detail about the experimental procedures and risks involved with the experimental methodology, and gave their informed consent. They were instructed not to engage in any physical activity and not to drink or eat any caffeinated product on testing days. The experimental protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of Republic of Slovenia and conformed to the Declaration of Helsinki.

2.2. Experimental protocol

On a preliminary visit to the laboratory, participants were thoroughly familiarized with the equipment and experimental procedures (cycle ergometer and respiratory maneuver). Thereafter, they participated in three separate trials. During the first session, they performed an incremental exercise test to exhaustion to determine their maximal oxygen uptake ($\dot{V}O_{2max}$) and peak power output (PPO). On later occasions, each subject performed two constant-power tests in a counter-balanced order, at the same time of the day separated by at least 48-h of resting. Namely, they carried out: (a) a control constant-power test (CPT), and (b) a constant-power test after a 30-min respiratory maneuver (CPT_{RM}) (described below). All the exercise tests were performed on an electrically braked cycle-ergometer (Daun Electronic, Furth, Germany). During the entire experimental period, the mean ambient temperature, relative humidity and barometric pressure were 20.3 ± 1.0 °C, 46.1 ± 3.2% and 906.2 ± 1.9 mm Hg, respectively.

2.2.1. $\dot{V}O_{2max}$ testing

The $\dot{V}O_{2max}$ test commenced with a 5-min rest period, followed by a 2-min warm up on a cycle-ergometer at a work rate of 60 W. Thereafter, the load was increased by 25 W min^{-1} until exhaustion. Attainment of $\dot{V}O_{2max}$, defined as the highest $\dot{V}O_2$ averaged over 60 s, was confirmed according to the following classical criteria, listed in priority order: (a) severe fatigue or exhaustion resulting in an inability to maintain exercise at a given work rate (cycling cadence lower than 60 rpm), (b) a plateau in oxygen uptake, (c) a subjective rating of perception of effort at or near maximal, and/or (d) a respiratory exchange ratio >1.10.

2.2.2. Constant-power tests

During both constant-power tests, the subjects were required to complete a 2-min warm-up on a cycle ergometer at an individualized work rate of 1.5 W kg^{-1} body weight. Subsequently, they cycled at 85% of PPO (mean power output = 304 ± 46 W). The participants selected their preferred pedal cadence (between 60 and 90 rpm) and they maintained it via visual and verbal feedback throughout the trial. The investigators terminated the test when the pedal cadence dropped below 70% of the self-selected cadence for ≥5-s (task failure). During all tests, subjects remained seated on the cycle ergometer to minimize changes in muscle recruitment; and they received verbal encouragement always by the same investigators.

2.2.3. Respiratory maneuver (RM)

All participants used a respiratory endurance-training device (Spirotiger®, Idiag, Fehraltorf, Switzerland), which consisted of a hand-held unit with a pouch and a base station (Keramidas et al., 2010). A two-way piston valve connected to a re-breathing bag permitted the addition of fresh inspired air into the bag in order to maintain a constant isocapnic end-tidal CO_2 fraction (Renggli et al., 2008). Personal target values were entered into the base unit that monitored the breathing frequency (f_R), set threshold limits for breathing patterns, and displayed visual and acoustic feedback to allow the subject to breathe within the threshold values for isocapnia.

The RM was performed in an upright standing posture. The duration of the RM was 30 min, and the participants allowed brief respites (30–60 s), when they were unable to maintain the requested f_R. The volume of the bag (V_{BAG}) was set at a value representing approximately 60% of the subject's slow vital capacity (SVC). The f_R was then determined by dividing 80% of maximum voluntary ventilation (MVV) by the bag volume such that f_R = MVV (0.80)/V_{BAG}. Finally, there was a 12–17-min interval between the RM and the subsequent exercise test.

2.2.4. Pulmonary function

Prior to the start of RM, the participants performed the PFT. Pulmonary function was assessed using a Cardiovit AT-2 plus (Schiller, Baar, Switzerland) spirometer, according to the criteria by Miller et al. (2005). The spirometer was calibrated before every test with a 2-L syringe (Schiller, Baar, Switzerland). Each subject performed each test three times and the highest of the three values was used for subsequent analysis. The PFT was used to obtain measures of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), peak expiratory flow (PEF), slow vital capacity (SVC) and maximum voluntary ventilation (MVV). The PFT was also repeated after the end of RM in order to assess the RM effect on MVV.

2.3. Instrumentation

Respiratory measurements. During the exercise tests, oxygen uptake ($\dot{V}O_2$), ventilation ($\dot{V}E$), carbon dioxide production ($\dot{V}CO_2$), tidal volume (VT) and f_R were measured on-line with a metabolic cart (Quark CPET, Cosmed, Rome, Italy). The gas analyzers and pneumotachograph were calibrated before each test with two different gas mixtures and a 3-L syringe (Cosmed, Rome, Italy), respectively. Data were averaged each minute.

Heart rate (HR) and PPO. HR was measured and recorded using a heart rate monitor (Polar S810i, Kempele, Finland). PPO was calculated by the equation: PPO = PO_{FINAL} + (t/60 × 25 W), where PO_{FINAL} refers to the last workload completed, and t is the number of seconds for which the final, uncompleted workload was sustained.

Arterial oxygen saturation (Sp_{O2}). Sp_{O2} was monitored with a finger pulse oxymeter (BCI 3301, Wisconsin, USA), with an accuracy of ±2 units across the range of 70–100% and an acceptable resilience to motion artifact (Langton and Hanning, 1990).

Ratings of perceived exertion (RPE). During the respiratory maneuver and the exercise tests, subjects were requested to pro-

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vide ratings of perceived exertion for dyspnoea – respiratory discomfort (D-RPE; Wilson and Jones, 1991), and leg effort (L-RPE). The modified scale (0–10) was explained to the participants by the investigators during the familiarization session and prior to the RM. RPE readings were obtained at rest, every 5-min during the RM, and every minute during the exercise tests.

Blood lactate concentration. At the 3rd-min of recovery, two blood samples were taken from the tip of the left index finger to measure the blood lactate concentration. Before every collection, the finger was cleaned and dried in order to avoid contamination from sweat and dirt. The skin was punctured with a lancet (Accu-Chek, Scoftclix Pro, Basel, Switzerland); the second drop of blood was placed on a strip (BM-lactate, Roche, Basel, Switzerland) and immediately analyzed with a portable analyzer (Accutrend Lactate, Roche, Basel, Switzerland).

Near-infrared spectroscopy (NIRS). During the RM and the constant-power tests, the cerebral, intercostal and leg muscle oxygenation was monitored by three pairs of continuous-wave infrared spectroscopy (NIRS) probes (Artinins Medical System, Oxymon MKIII, Zetten, the Netherlands). In particular, the cerebral probe was positioned over the left prefrontal cortex between Fp1 and F3, according to the modified international EEG 10–20 system; the respiratory muscle probe was positioned over the right 7th intercostal space of the serratus anterior muscle (Guenette et al., 2008; Vogiatzis et al., 2009) and the leg muscle probe above the vastus lateralis, ~15 cm above the proximal line of the patella and ~5 cm lateral to the midline of the thigh. The probes consisted of one emitter and one detector housed in a black, plastic holder that was stabilized on the shaved and cleaned skin with double-sided adhesive tape. A bandage covered and stabilized each probe holder in order to reduce the intrusion of external light and the loss of transmitted NIR light from the measuring area. The inter-optode distance was kept at 4.5 cm to minimize the influence of skin blood flow. All technical considerations (probe position and stabilization) were taken into account according to the previously published reports using the same NIRS device (Billaut et al., 2009; Subudhi et al., 2007).

The NIR light consisted of two wavelengths (780 and 850 nm), and the micromolar changes in tissue oxygenation – oxygenated ($\Delta[O_2Hb]$) and deoxygenated (Δ[HHb]) hemoglobin – were calculated from the age-dependent differential path-length factors (DPF; range: 4.95–6.12) (Duncan et al., 1995). In addition, total hemoglobin (Δ[tHb]), which is the sum of $\Delta[O_2Hb]$ and Δ[HHb], used as an index of change in regional blood volume (Van Beekvelt et al., 2001).

The theory, limitations and reliability of the cerebral and muscle measurements obtained with the NIRS device during exercise have been detailed previously (Perrey, 2008; Subudhi et al., 2007). The NIR light is absorbed by heme groups both within hemoglobin (Hb) and myoglobin (Mb). However, the relative contribution of Hb seems to be substantially greater than that of Mb, since the Hb is tetrameric and exists in appreciably greater concentrations than the monomeric Mb pigment (Wilson et al., 1989). Moreover, it has been shown that Mb desaturation occurs at low work intensities (<50% of PPO), suggesting that changes in the NIRS signal occurring at higher intensities originate mainly from changes in Hb absorption (Richardson et al., 1995). Furthermore, it is known that the thickness of adipose tissue may influence the NIRS signal (McCully and Hamaoka, 2000), potentially biasing comparison of signal magnitudes between subjects.

NIRS data were recorded at 50 Hz and stored in a PC for further analysis. Because the exact DPF was unknown, cerebral and muscle measurements were normalized to reflect the magnitude of changes from the resting period of each test (arbitrarily defined as 0 μM) (Subudhi et al., 2007). Data were averaged every minute.

Table 1
Mean (±SE) changes from resting values in cerebral, intercostal muscles and vastus lateralis total hemoglobin (Δ[tHb]), oxyhemoglobin ($\Delta[O_2Hb]$) and deoxyhemoglobin (Δ[HHb]) during the respiratory maneuver (RM).

	RM	Main effect (*P*)
Cerebral		
Δ[tHb] (μM)	5.24 ± 051	0.00
$\Delta[O_2Hb]$ (μM)	3.74 ± 0.65	0.01
Δ[HHb] (μM)	1.50 ± 0.64	n.s.
Intercostal muscles		
Δ[tHb] (μM)	9.66 ± 0.93	0.00
$\Delta[O_2Hb]$ (μM)	4.45 ± 0.80	0.03
Δ[HHb] (μM)	5.20 ± 0.53	0.00
Vastus lateralis		
Δ[tHb] (μM)	4.36 ± 0.92	n.s.
$\Delta[O_2Hb]$ (μM)	1.03 ± 0.62	n.s.
Δ[HHb] (μM)	3.32 ± 0.82	n.s.

2.4. Statistical analysis

Statistical analyses were performed using Statistica 5.0 (StatSoft, Inc., Tulsa, USA). All data are reported as mean (SD), unless otherwise indicated. Statistical significance of maximal values of exercise tests and mean values of the RM were assessed with a *t*-test analysis. A two-way analysis of variance (ANOVA) for repeated measures was used for absolute or relative submaximal values of constant-power tests (condition × overtime). The Tukey *post hoc* test was employed to identify specific differences between means when ANOVAs revealed significant *F*-ratio for main effects. Differences in D-RPE and L-RPE were evaluated with a Wilcoxon matched pairs non-parametric test. The alpha level of significance was set a priori at 0.05.

3. Results

3.1. $\dot{V}O_{2max}$ testing

The average value of $\dot{V}O_{2max}$ was 63.5 ± 7.9 mL kg^{-1} min^{-1} and PPO was 371 ± 55 W. Moreover, the maximal HR, $\dot{V}E$ and Sp_{O2} were 187 ± 8 beats min^{-1}, 165.2 ± 24.6 L min^{-1} and 86.4 ± 6.1%, respectively; and the median (range) L-RPE and D-RPE were 10 (7–10) and 9 (8–10), respectively.

3.2. Respiratory maneuver

During the 30-min RM, the mean HR was 122 ± 12 beats min^{-1}; and the median (range) L-RPE and D-RPE were 1 (0–4) and 4.5 (2–7), respectively. The mean changes in the cerebral and muscle Δ[tHb], $\Delta[O_2Hb]$ and Δ[HHb] measured during the RM are displayed in Table 1. In particular, the Δ[tHb], $\Delta[O_2Hb]$ and Δ[HHb] of serratus anterior and the Δ[tHb], $\Delta[O_2Hb]$ of cerebral (Fig. 1) increased significantly compared to resting values. However, the Δ[tHb], $\Delta[O_2Hb]$ and Δ[HHb] of vastus lateralis remained unchanged throughout the RM.

3.3. Pulmonary function test

The mean values (% of predicted value) of FVC, FEV_1, PEF and SVC were 5.81 ± 0.71 L (107.6%), 4.65 ± 0.64 L (102.6%), 11.20 ± 1.34 L s^{-1} (110.4%) and 5.56 ± 0.55 L (98.3%), respectively. The MVV did not change after the RM (Pre RM = 205.93 ± 25.04 L min^{-1}; Post RM = 203.15 ± 22.68 L min^{-1}).

3.4. Constant-power tests

Mean and individual results of constant-power tests to exhaustion are shown in Fig. 2. The performance time was 8:03 ± 2:33 min

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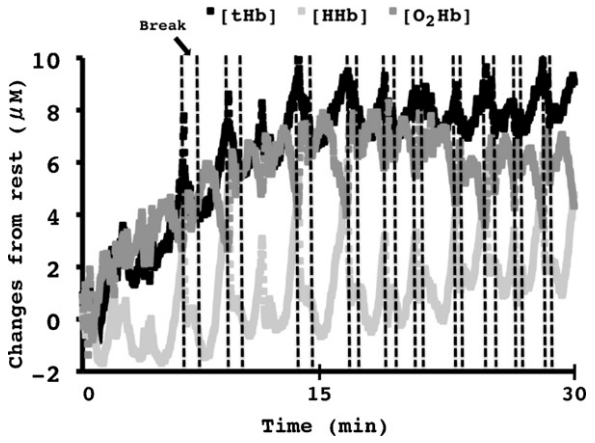

Fig. 1. Representative changes in cerebral oxyhemoglobin ([O_2Hb]), deoxyhemoglobin ([HHb]) and total hemoglobin ([tHb]) from a single participant performing the voluntary isocapnic hyperpnoea task.

in CPT and 6:55 ± 2:52 min in CPT_{RM} (~15% decrement), but the difference was not statistically significant between the two trials ($P = 0.42$). Specifically, seven participants reduced their time to exhaustion after the RM (~18%), and only one increased it approximately 6%.

Cardiorespiratory responses. Mean values of the cardiorespiratory variables during the CPT and CPT_{RM} are summarized in Table 2. $\dot{V}O_2$, $\dot{V}E$, HR, L-RPE and D-RPE were similar in the two performance tests ($P > 0.05$). However, $\dot{V}E/\dot{V}_{CO_2}$ was significantly lower during CPT_{RM} than during CPT ($P \leq 0.05$). Moreover, PET_{CO_2} was lower and f_R was higher during the CPT_{RM} than in CPT ($P \leq 0.05$), whereas VT did not differ between the two trials ($P > 0.05$) (Fig. 3). Furthermore, there was no difference in Sp_{O2} (CPT: 86.1 ± 3.4%; CPT_{RM}: 85.3 ± 5.1%) or in blood lactate concentration (CPT: 15.1 ± 4.6 mmol l^{-1}; CPT_{RM}: 13.6 ± 1.9 mmol l^{-1}) at the maximal intensity between the two conditions ($P > 0.05$).

Vastus lateralis oxygenation. Δ[O_2Hb] of the vastus lateralis decreased progressively during exercise up to 20% of performance time, and thereafter plateaued until the end of test without any difference between the two trials (Fig. 4). However, Δ[HHb] and Δ[tHb] showed significantly different responses in the two tests (Fig. 4). Especially, at any given time, exercise-induced changes in Δ[HHb] and Δ[tHb] were lower in CPT_{RM} than in CPT by approximately 37% and 67%, respectively.

Serratus anterior oxygenation. In both trials, Δ[O_2Hb] of the serratus anterior gradually declined until the end of exercise, but the drop was more pronounced during the CPT_{RM} at 40, 60 and 80% of performance time (Fig. 4). Likewise, Δ[HHb] increased in both tests, but at 60, 80 and 100% the increment was less in the CPT_{RM} (Fig. 4). Δ[tHb] did not alter during the CPT, whereas it was significantly reduced throughout the CPT_{RM} (Fig. 4).

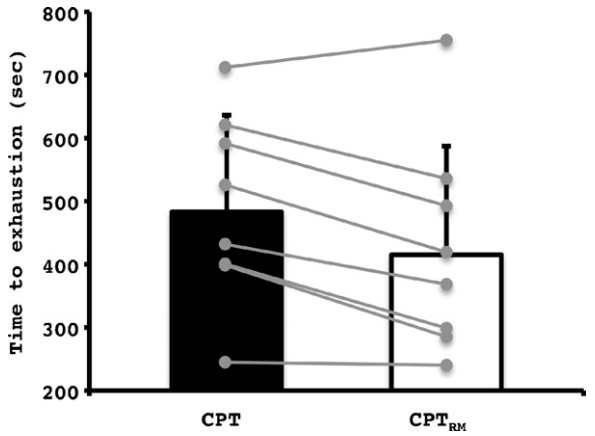

Fig. 2. Mean (±SD) and individual time to exhaustion for the control constant-power test (CPT) and the constant power test after the respiratory maneuver (CPT_{RM}); ($P > 0.05$).

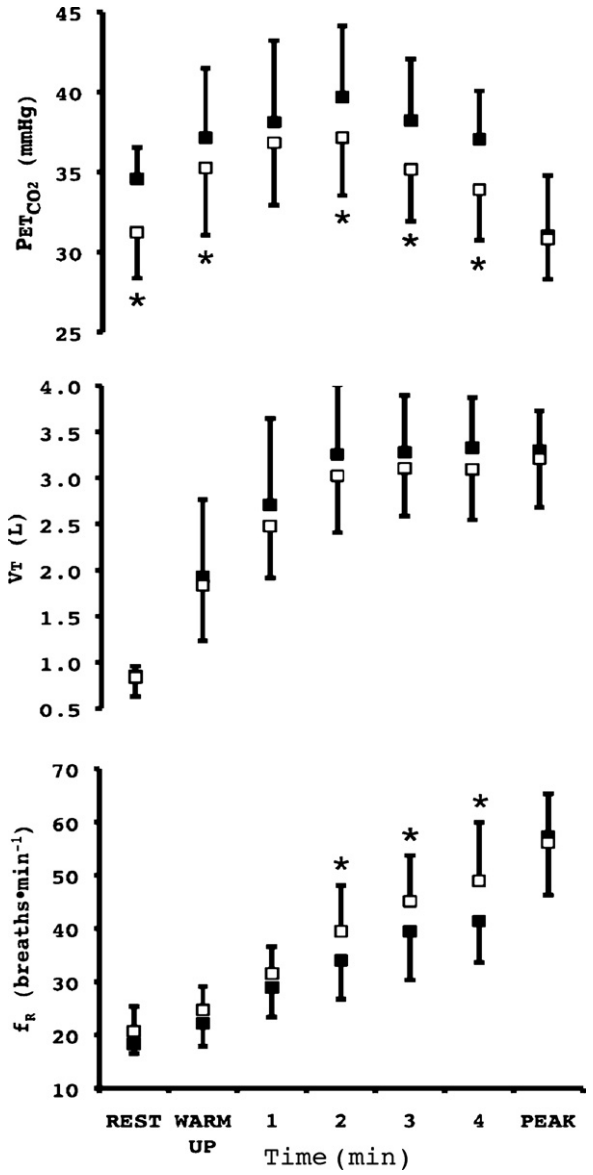

Fig. 3. Partial pressure of end-tidal carbon dioxide (PET_{CO_2}), tidal volume (VT) and respiratory frequency (f_R) during the control constant-power test (CPT; filled square) and the constant-power test after the respiratory maneuver (CPT_{RM}; open square) at the same absolute performance time; (means ± SD). Data in both conditions were significantly different over time; *significant differences between CPT and CPT_{RM}; ($P \leq 0.05$).

Cerebral oxygenation. The changes in cerebral hemodynamics are shown in Table 3. In both tests, Δ[O_2Hb] remained constant throughout the test, whereas Δ[HHb] significantly increased after 60% of performance time compared to resting values; and Δ[tHb] increased at the end of exercise. There were no differences in any of the measured parameters between the CPT and CPT_{RM}.

4. Discussion

The principal finding of the present study is that a 30-min voluntary isocapnic hyperpnoea task at rest decreases the Δ[tHb] and Δ[HHb] in the vastus lateralis and the serratus anterior during a

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Table 2
Cardiorespiratory values during the control constant-power test (CPT) and the constant-power test after the respiratory maneuver (CPT_{RM}) at the same relative performance time.

	CPT					CPT_{RM}				
	REST	20%	60%	80%	100%	REST	20%	60%	80%	100%
$\dot{V}O_2$ (L min^{-1})	0.53 ± 0.07	3.23 ± 0.69	3.94 ± 0.60	4.07 ± 0.60	4.05 ± 0.59	0.54 ± 0.10	3.07 ± 0.59	3.93 ± 0.58	4.03 ± 0.55	4.06 ± 0.57
$\dot{V}E/\dot{V}CO_2$	33.4 ± 2.9	28.2 ± 3.7	31.6 ± 4.8	34.1 ± 4.1	37.6 ± 5.0	38.4 ± 3.6*	30.7 ± 4.1*	33.9 ± 4.0	35.7 ± 3.8	37.1 ± 3.7
$\dot{V}E$ (L min^{-1})	14.7 ± 2.1	95.8 ± 31.3	135.8 ± 21.5	149.7 ± 19.6	161.7 ± 19.8	15.7 ± 2.7	94.3 ± 30.8	145.5 ± 24.9	155.4 ± 19.8	157.6 ± 13.1
HR (beats min^{-1})	80.4 ± 12.6	161.5 ± 8.3	178.5 ± 6.6	183.4 ± 7.8	185.0 ± 9.4	87.2 ± 12.1	162.1 ± 9.4	175.9 ± 7.3	180.5 ± 7.6	181.2 ± 7.8
L–RPE (1–10)	0.5 (0–2)	3.25 (1–7)	7.5 (3–9)	9 (4–10)	10 (7–10)	0.5 (0–1)	3 (2–6)	7 (5–9)	8 (4–9)	10 (7–10)
D–RPE (1–10)	0 (0–0.5)	3 (2–5)	5.5 (4–9)	6 (5–8)	8 (7–10)	0.5 (0–2)	2.5 (2–6)	6 (4–9)	6.25 (4–8)	9 (5–10)

Values are means ± SD for $\dot{V}E/\dot{V}CO_2$ ventilatory equivalent of carbon dioxide, $\dot{V}E$ expired ventilation, HR heart rate.
Values are median (range) for L-RPE leg effort and D-RPE dyspnoea-respiratory discomfort.
* Statistically significant difference between CPT and CPT_{RM}; ($P \leq 0.05$).

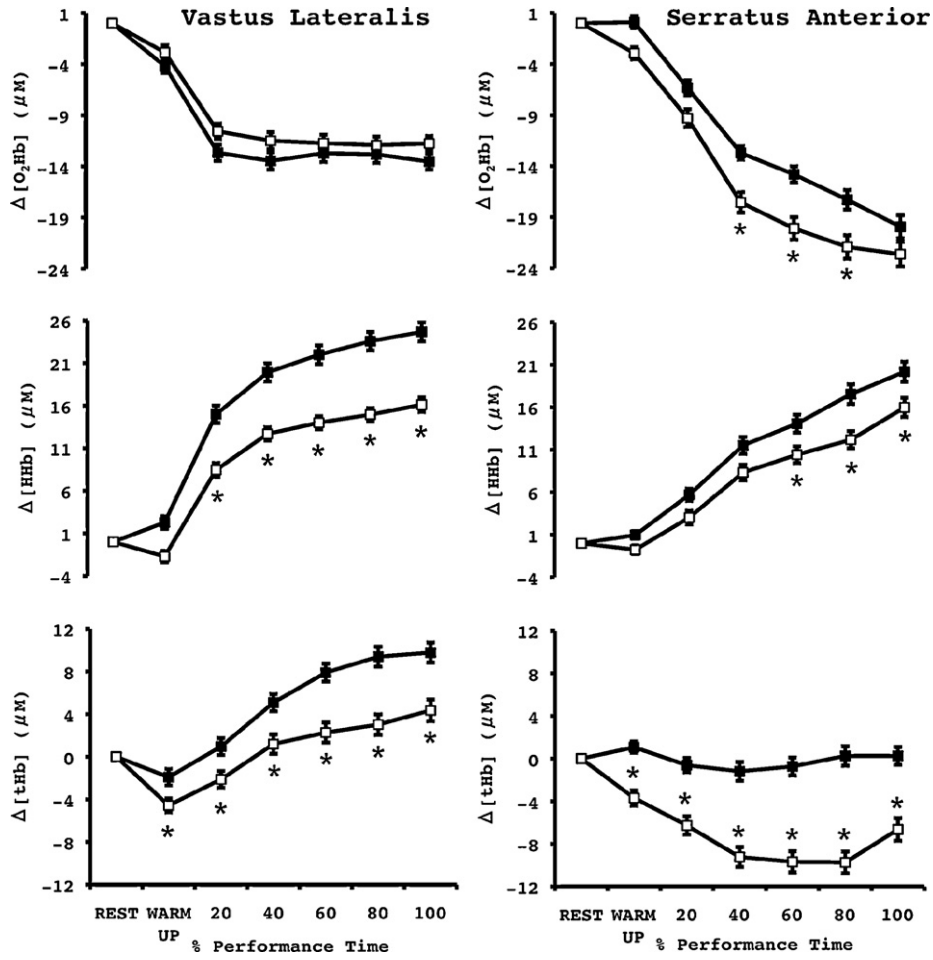

Fig. 4. Changes from resting values in serratus anterior and vastus lateralis total hemoglobin (Δ[tHb]), oxyhemoglobin (Δ[O_2Hb]) and deoxyhemoglobin (Δ[HHb]) during the control constant-power test (CPT; filled square) and the constant-power test after the respiratory maneuver (CPT_{RM}; open square) at the same relative performance time; (means ± SE). Data in both conditions were significantly different over time; *significant differences between CPT and CPT_{RM}; ($P \leq 0.05$).

Table 3
Changes from resting values in cerebral total hemoglobin (Δ[tHb]), oxyhemoglobin (Δ[O_2Hb]) and deoxyhemoglobin (Δ[HHb]) during the control constant-power test (CPT) and the constant-power test after the respiratory maneuver (CPT_{RM}) at the same relative performance time.

	CPT					CPT_{RM}				
	REST	20%	60%	80%	100%	REST	20%	60%	80%	100%
Δ[tHb] (μM)	0	−0.9 ± 0.7	1.6 ± 0.8	2.5 ± 0.9	5.6 ± 0.9†	0	−0.4 ± 0.7	0.3 ± 0.9	3.3 ± 0.8	4.8 ± 0.8†
Δ[O_2Hb] (μM)	0	−1.5 ± 0.7	−1.1 ± 0.7	−1.1 ± 0.7	0.5 ± 0.8	0	−2.4 ± 0.7	−2.4 ± 0.8	−1.2 ± 0.7	−1.9 ± 0.8
Δ[HHb] (μM)	0	0.5 ± 0.4	2.7 ± 0.5†	3.6 ± 0.5†	5.1 ± 0.5†	0	2.0 ± 0.6	2.7 ± 0.5†	4.6 ± 0.5†	6.7 ± 0.7†

Values are means ± SE.
† Statistically significant changes from rest and 20%; ($P \leq 0.05$).

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subsequent constant-power test to exhaustion. The performance time seems to be affected by the RM (decrement ~15%), but this difference was not statistically significant. Moreover, the prior loading of respiratory muscles does not affect the cerebral oxygenation during exercise.

4.1. Exercise performance

The contribution of respiratory muscle fatigue on exercise tolerance is a perplexing problem. The findings of previous studies using a similar experimental approach as in our study are inconsistent. Some of them have observed a significant decrement in performance following the RM (Mador and Acevedo, 1991; Martin et al., 1982; Verges et al., 2007), while others have not detected any change (Dodd et al., 1989; Sliwinski et al., 1996; Spengler et al., 2000). The differences among the studies might be explained by the different techniques used to fatigue the respiratory muscles, and by the level of fatigue of respiratory muscles. It is conspicuous that from the studies using a voluntary isocapnic hyperpnoea method, two have not noticed any effect (Dodd et al., 1989; Spengler et al., 2000), and only one has found a significant decrement in short-term maximal running performance (Martin et al., 1982). In the present study, we did not find any statistically significant influence of a prior RM on the subsequent exercise performance. Notably, since the sample size was small in the current study, it cannot be excluded that RM does in fact reduce exercise performance. Thus, the nominal impairment of performance time was ~15%, and seven out of eight participants shortened their time to exhaustion in CPT_{RM}.

Regardless of the duration of the performance, the RM altered the breathing pattern during the exercise test, increasing the f_R but not changing the VT, as has also been shown by Sliwinski et al. (1996). Nevertheless, there was no difference in D-RPE between the two trials, indicating that the sense of dyspnoea is not responsible for the altered breathing pattern (Spengler et al., 2000) nor the discontinuance of work. Interestingly, as reported by Taylor and Romer (2008) as well, the participants terminated the trials in both conditions due to discomfort in their leg muscles, even when the RM preceded the constant-power test.

4.2. Muscle and cerebral oxygenation

Previous studies have speculated that the fatigue of respiratory muscles influences exercise performance via activation of the respiratory muscle metaboreflex, causing a redistribution of blood flow from the locomotor to the respiratory muscles (Dempsey et al., 2002, 2006). Specifically, it has been proposed that the increased blood flow is attributable to increased discharge of the respiratory muscle metaboreceptors (increased neural activity in type IV afferent nerve fibers) that increases sympathetic vasoconstrictor outflow to the exercising legs (Dempsey et al., 2006). However, none of these studies assessed blood flow or levels of oxygenation of respiratory muscles during exercise. Conversely, Vogiatzis et al. (2009), who performed simultaneous measurements of quadriceps and respiratory muscle blood flow and muscle vascular conductance during maximal incremental exercise, confirmed an enhanced sympathetic vasoconstrictor activity, but they also observed a reduction in intercostal muscle blood flow at maximal exercise. The latter observation seems to be in agreement with our findings during the CPT_{RM}, when we detected a significantly decreased regional blood volume (i.e. reduced Δ[tHb]), in both leg and respiratory muscles. Yet in the case that a redistribution of blood flow from the legs to the respiratory muscles had taken place, we could expect that the Δ[tHb] in intercostal muscles would not be different between the CPT and CPT_{RM}. *Ergo*, in accordance with Vogiatzis et al. (2009), we believe it is likely that impairment of oxygenation to the intercostal muscles during CPT_{RM} reflects the inability of the circulatory system to meet the increasing energy demands of both locomotor and respiratory muscles.

The cerebral oxygenation does not seem to be affected by the previously performed RM, since the pattern of altered Δ[tHb] was not different between the two experimental trials. In both cases, we observed, as Amann et al. (2007), a fall at the beginning of the exercise, and then a progressive increment of cerebral oxygenation. However, our findings are in contrast with Nielsen et al. (2001), who reported that moderate and intense resistive breathing during submaximal exercise increased Δ[Hb], $\Delta[O_2Hb]$ and Δ[tHb], due to elevated production of carbon dioxide (CO_2), and thus enhanced arterial CO_2 partial pressure (PA_{CO_2}). By contrast, in our protocol, the RM, which was isocapnic, did in fact induce hypocapnia after the end of hyperpnoea task as indicated by the lower resting and exercise values of PET_{CO_2}; which, however, did not seem to influence the cerebral oxygenation during exercise.

4.3. Respiratory maneuver

In the present study, the RM was performed with a commercially available respiratory endurance-training device (Spirotiger) that allows partial re-breathing of CO_2, and consequently assures isocapnic hyperpnoea (Keramidas et al., 2010; Renggli et al., 2008). A main limitation of the current protocol is the lack of actual measurements of the work of breathing, and the undefined degree of respiratory muscle fatigue induced by the specific RM. However, we presumed that the respiratory muscles would fatigue during the RM, when participants breathed at 80% of MVV, given that Mador et al. (1996) and Renggli et al. (2008) measured a significant reduction in transdiaphragmatic twitch pressure ($P_{di,tw}$) indicating diaphragmatic fatigue after about 9-min of voluntary hyperpnoea at 60% of MVV and 8-min at 70% of MVV, respectively. Furthermore, it can be assumed that the diaphragm and abdominal fatigue appears early – almost 2-min after the beginning of RM –, and lasts for more than 1-h after the cessation of the RM (Mador et al., 1996; Renggli et al., 2008). Consequently, the 12–17-min break between the end of hyperpnoea and the beginning of cycling seems to be too short for the full recovery of the respiratory muscles, but sufficient to minimize the intensity of dyspnoea (McConnell and Romer, 2004).

Furthermore, the oxygenation and the regional blood volume in the intercostal muscles are increased with the work of breathing during the hyperpnoea. However, the enhanced Δ[HHb], which is an index of tissue O_2 extraction (Grassi et al., 1996), reveals that the increasing O_2 demand of the intercostal muscles was not met by proportionally increased blood flow and oxygen delivery (Vogiatzis et al., 2009). Nevertheless, the leg oxygenation did not alter during the RM, in contrast with previously reported findings (Sheel et al., 2001, 2002; St Croix et al., 2000).

It is also notable that the cerebral Δ[tHb] and $\Delta[O_2Hb]$ were significantly increased during the RM. In view of the fact that the participants performed isocapnic hyperpnoea, we assume that this phenomenon could be explained by specific cardiovascular responses occurring during the RM. In particular, it is known that isocapnic hyperventilation via baroreflex inhibition increases arterial pressure, heart rate and sympathetic activity (Van De Borne et al., 2000), and raises cardiac output (Cummin et al., 1986). Therefore, the enhanced arterial pressure (Ide et al., 1999; Magyar et al., 2005) and/or cardiac output (Ogoh et al., 2005) could possibly have contributed to the increased cerebral oxygenation during the hyperventilation. An alternative explanation could be that the afferent feedback from the muscle mechanoreceptors increased the cerebral blood flow and oxygenation (Jorgensen et al., 1992). Along with the latter assumption, Renggli et al. (2008) have shown that early fatigue of diaphragm and abdominal muscles during the isocapnic hyperpnoea is accompanied by an increased recruitment of

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rib cage muscles in order to maintain the requested ventilatory output.

Moreover, it is remarkable that the failure of the participants to continue the maneuver, and consequently having short breaks, appears with a considerable decline of cerebral $\Delta[O_2Hb]$ (Fig. 1). Bellemare and Bigland-Ritchie (1987) have suggested that the task failure is caused mainly by the "catastrophe" of central motor drive to completely activate the fatigued muscles, rather than solely from peripherally contractile failure. However, based on our experimental protocol, it is not possible to distinguish between the central and local mechanisms; and further studies are needed to investigate the relationship of isocapnic hyperpnoea and cerebral oxygenation.

4.4. Limitations

A limitation of the current study is that blood-flow measurements of leg and respiratory muscles were not performed with an invasive method. However, the changes in regional blood volume were assessed with the NIRS technique, the reliability of which has been verified in previous studies comparing the changes in Δ[tHb] with those obtained with venous occlusion mercury strain gauge plethysmography, revealing high correlation between the two methods (De Blasi et al., 1994; Edwards et al., 1993; Van Beekvelt et al., 2001). Furthermore, measuring blood flow in the respiratory muscles is difficult owing to their complex anatomical arrangement, their extensive vascular network and the large variation in muscular recruitment with varying degrees of ventilation (Guenette et al., 2008; Vogiatzis et al., 2009). Hence, in order to interpret the results of the present study, we should consider carefully the limitations of measuring the respiratory muscle oxygenation with NIRS technology (see Section 2). That NIRS data represent regional and not global changes in oxygenation, and it has not been ascertained that the measurements in intercostal muscles could represent the overall response of recruited respiratory muscles. In particular, it remains to be elucidated whether the diaphragm, which is the main respiratory muscle during exercise, gobbles the "stolen" blood by the locomotor muscles and maintains its blood flow at the same levels in both conditions.

5. Conclusion

In conclusion, the results of the present study demonstrate that a short-term voluntary respiratory work before a subsequent exercise endurance test influences the oxygenation during exercise in the vastus lateralis and serratus anterior muscles, but not in the frontal cerebral cortex. The exercise performance seems also to be affected by the previously performed respiratory maneuver.

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