Cerebrovascular and Peripheral Vascular Regulation: Role of Oxidative Stress

by

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B.H.K., The University of British Columbia, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE COLLEGE OF GRADUATE STUDIES

(Interdisciplinary Studies)

THE UNIVERSITY OF BRITISH COLUMBIA

(Okanagan)

October 2017

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Abstract

Oxidative stress is ultimately determined by the balance between pro- (e.g. free radicals) and antioxidants (e.g. vitamin C). The purpose of this thesis was to examine the role and effect of a combined mixture of oral antioxidants (vitamin C, E, and alpha-lipoic acid) on peripheral and cerebrovascular regulation at both sea-level (344m; n = 11 participants) and at high altitude (5050m; n=10) where oxidative stress is markedly elevated. In a randomized and double-blinded design, flow-mediated dilation, cardiorespiratory, cerebral blood flow, and CO_2 and hypoxic cerebrovascular reactivity tests were conducted before and 90 mins following antioxidants (vitamin C, E, and alpha-lipoic acid) or a placebo control, previous work has shown antioxidant levels increased in blood plasma after 90 mins of ingestion. Following 10-12 days at 5050m, in a similar design, flow-mediated dilation, cardiorespiratory, and resting cerebral blood flow tests were obtained. The primary findings were: 1) antioxidants did not alter flow mediated dilation, resting cerebral blood flow, cerebrovascular reactivity to CO_2 or hypoxia at sea-level; 2) similarly, at high-altitude (Nepal, 5050m), antioxidants did not alter flow mediated dilation, or resting cerebral blood flow. In conclusion, this study highlights that at both sea-level and high-altitude, acute oral administration of antioxidants does not alter FMD or cerebrovascular reactivity to CO_2 or hypoxia. Nevertheless, continued exploration of this methodology is recommended, especially in the form of more chronic dosing of antioxidants in various pathologies (e.g., chronic pulmonary obstructed disease, diabetes, coronary artery disease) who present with excessive oxidative stress and reductions in both antioxidant capacity and vascular function. This study received ethical approval from the University of British Columbia Clinical Research Ethic Board (ID: H16-00101). This research was supported by an NSERC Discovery grant and Canadian Research Chair in Cerebrovascular Physiology.

Preface

Chapter 1. I wrote Chapter 1 with extensive and critical feedback from Prof. Ainslie prior to finalization for including within this Thesis.

Chapter 2. Chapter 2 will be submitted to a relevant journal for publication. Prof. Ainslie and myself planned the experiment. Data collection was completed by myself with the assistance of Ryan Hoiland, Mike Tymko, Josh Tremblay, Dr. Nia Lewis, Dr. Howard Carter, Dr. Daniella Flück. This study was completed at the University of British Columbia – Okanagan campus, as well as, the EV-K2-CNR pyramid laboratory located in the Khumbo Valley, Nepal. Data analysis was completed by myself, with assistance by Prof. Ainslie, Ryan Hoiland, Mike Tymko, Dr. Nia Lewis, Josh Tremblay. I wrote the manuscript. Prof. Ainslie provided extensive feedback and critically reviewed the manuscript for content and data interpretation. Manuscript was edited by all co-authors. This study received ethical approval from the University of British Columbia Clinical Research Ethic Board (ID: H16-00101).

Chapter 3. I wrote Chapter 3 with the extensive and critical feedback from Prof. Ainslie. Ryan Hoiland and Mike Tymko also provided feedback prior to finalization.

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List of Abbreviations

Abbreviation	Definition
AMS	Acute mountain sickness
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CA	Cerebral autoregulation
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CaO_2	Arterial oxygen content
CBF	Cerebral blood flow
CCA	Common carotid artery
CCAv	Common carotid artery blood velocity
CDO_2	Cerebral oxygen delivery
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CoV	Coefficient of variation
CPT	Cold pressure test
CVC	Cerebral vascular conductance
CVR	Cerebral vascular resistance
ECF	Extracellular fluid
eNOS	Endothelial nitric oxide synthase
FMD	Flow mediated dilation
FBF	Forearm blood flow
GC^{+}	Guanylate cyclase
gCBF	Global cerebral blood flow
GTP	Guanosine triphosphate
H^{+}	Hydrogen
H_2O_2	Hydrogen peroxide
HR	Heart rate

AHVR	Acute hypoxic ventilatory response
ICA	Internal carotid artery
ICAv	Internal carotid artery blood velocity
L-NNMA	N^G-monomethyl-L-arginine
LOOH	Lipid hydroperoxide
MAP	Mean arterial pressure
MCA	Middle cerebral artery
MCAv	Middle cerebral artery velocity
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide
OH^-	Hyrdoxide
$OONO^-$	Peroxynitrite
OSI	Oscillatory shear index
$PaCO_2$	Partial pressure of arterial carbon dioxide
PaO_2	Partial pressure of arterial oxygen
$P_{ET}CO_2$	Partial pressure of end-tidal carbon dioxide
$P_{ET}O_2$	Partial pressure of end-tidal oxygen
pH	Potential of hydrogen
PUFAS	Polyunsaturated fatty acid chains
QCCA	Common carotid artery blood flow
QICA	Internal carotid artery blood flow
QVA	Vertebral artery blood flow
ROS	Reactive oxygen species
SaO_2	Arterial blood saturation of oxygen
SEM	Standard error of the mean
SNA	Sympathetic nervous activity

SOD	Superoxide dismutase
SR	Shear rate
SR_{AUC}	Shear rate area under the curve
TCD	Trans-cranial Doppler
VA	Vertebral artery
VAv	Vertebral artery blood velocity
VE	Ventilation

Acknowledgements

First I would like to thank my supervisor Prof. Philip Ainslie for his support throughout my fourth year of my undergraduate degree through the completion of my Master's degree. You have provided me with the opportunity to conduct research at both the Barcroft research laboratory at White Mountain in California at 3800m, as well as the EV-K2-CNR research pyramid in the Khumbo Valley in Nepal at 5050m. Your enthusiastic drive for research and knowledge has sparked my interest in research and science and has motivated me to pursue higher learning. Your teaching and encouragement has not only allowed me to develop academically, but has lit a fire for an underlying obsession with climbing.

Thank you to my committee Dr. Alison McManus and Dr. Glen Foster for their continued help and support throughout my Master's degree.

I would like to thank the Ainslie lab team who have helped me throughout my Master's degree. Spending countless hours offering edits on both my thesis and paper, assisting with analysis, and preparing my thesis defense presentation. There has been no better team to be a part of.

To my parents Brad and Lorraine Hansen who have supported me throughout my undergraduate degree and Master's degree - I am forever in their debt. I am thankful for your guidance and unconditional support. Without your support through the good times and the bad, I may not be where I am today. I am forever grateful.

Dedication

This is dedicated to my loving parents Brad and Lorraine Hansen, who have always stood by me and supported me during both the good and bad times. My sister Alicia has also been one of my biggest supporters. My Grandmother Shirley Hansen, who has been my biggest fan. I would also like to dedicate this to my late Grandfathers, Carl Hansen and Vincenzo Peroni.

1 Chapter: Introduction

This literature review will begin with an overview on the key mechanisms that regulate cerebral blood flow (CBF) and peripheral blood flow. Key mechanism(s) that influence CBF and vascular function during acute and chronic hypoxia will also be outlined, including both endothelial dependent (shear mediated dilation) and independent (smooth muscle) vasodilation. Furthermore, this literature review will assess the punitive role of reactive oxygen species in the regulation of cerebrovascular and vascular tone. Based on this overview, gaps on the influence of oxidative stress on CBF and peripheral vasculature regulation within the literature will be identified. The overall goal of this thesis is to examine how antioxidant supplementation (as a means to manipulate oxidative stress) might impact CBF and vascular function.

1.1 Overview of cerebrovascular regulation

This section of the literature review will highlight the key mechanism(s) of cerebrovascular regulation. At rest, adequate CBF is regulated through reflexive responses in the following order of regulatory importance: Fluctuating arterial blood gases (in particular, the partial pressure of carbon dioxide [$PaCO_2$]), cerebral metabolism, arterial blood pressure, and neurogenic activity. This section will provide a summary on these latter primary mechanisms. Extended reviews on each topic can be found elsewhere: (Ainslie & Duffin, 2009; Ainslie & Tzeng, 2010; Numan et al., 2014; Phillips et al., 2016).

1.2 Cerebral blood flow regulation by arterial blood gases

1.2.1 Regulation by changes in the partial pressure of arterial carbon dioxide

The regulation of CBF is highly sensitive to changes in the partial pressure of arterial carbon dioxide ($PaCO_2$) and, to a lesser extent, the partial pressure of arterial oxygen (PaO_2) (Ainslie & Duffin, 2009; Ide et al., 2003). Cerebrovascular sensitivity to these changes in $PaCO_2$ is often termed cerebrovascular reactivity. Elevations in $PaCO_2$ (hypercapnia) will cause vasodilation and result in an increase in CBF whereas reductions in $PaCO_2$ (hypocapnia) result in vasoconstriction and subsequent reductions in CBF (Verbree et al., 2014); however,

the vascular response to hypercapnia (e.g., 4%/mmHg change in CBF/$PaCO_2$) is greater than the vascular response to hypocapnia (e.g., 2%/mmHg change in CBF/$PaCO_2$). Changes in $PaCO_2$ will reciprocally influence extracellular fluid (ECF) pH levels (Harper & Bell, 1963), and it is via this mechanism that CBF is altered. For example, when artificial cerebrospinal fluid (CSF, an index of ECF) is infused with a high pH, causing acidosis, there is a dilation of the pial vessels, and vice versa without the need to alter $PaCO_2$ (Kontos et al., 1977); therefore, cerebrovascular sensitivity to changes in $PaCO_2$ is more accurately determined via changes in ECF pH [reviewed in: (Ainslie & Duffin, 2009)].

1.2.2 Regulation by changes in partial pressure of arterial oxygen

To a lesser extent compared to $PaCO_2$, reductions in PaO_2 will also induce changes in CBF. For the scope of this review, the focus will be primarily on hypoxemic hypoxia (the reduction in arterial tension). Reduction in PaO_2 (hypoxemia) and elevations in PaO_2 (hyperoxemia) will have contrasting effects on cerebral vasculature. For example, hyperoxemia and high levels of arterial O_2 (PaO_2 >300mmHg) can cause a reduction in $PaCO_2$ partly due to hyperoxic hyperventilation (an increase in respiratory ventilation) and induce cerebral vasoconstriction and hence reduce CBF (Willie et al., 2014). Separate from hyperoxemia, the effect of hypoxemia causes dilation throughout the cerebrovascular tree from the large cerebral arteries (e.g. ICA), to the inter-cranial arteries (e.g. MCA) (Imray et al., 2014; Wilson et al., 2011)). Typically this increase in CBF due to hypoxemia occurs around when PaO_2 drops <50 mmHg (Willie et al., 2014). The purpose for the increase in CBF during hypoxemia is to compensate for the reduction in arterial oxygen content (CaO_2) to maintain adequate delivery to the brain (i.e., CDO_2 = CBF * CaO_2). So, rather than PaO_2 per se, CaO_2 (via hemoglobin reduction) is likely an important regulator for cerebral vasodilation during both normobaric and hypobaric hypoxia [reviewed in: (Hoiland et al., 2016)]. One mechanism involved with CBF regulation during changes in CaO_2 is deoxyhemoglobin-mediated release of nitric oxide (NO) metabolites (through nitrite storage pools) and adenosine tri-phosphate (ATP), ATP is released from activated or stressed cells during inflammation, hypoxia, and/or apoptosis, and can be released from endothelial cells (Faas et al., 2017). The increase of deoxyhemoglobin during hypoxia causes a reduction in nitrite stores releasing NO, incurring vasodilation of the endothelium (Doyle et al., 1981). Other

key mechanisms involved go beyond the scope of this review, but are detailed elsewhere [Hoiland et al. (2016)].

1.2.2.1 Normobaric hypoxia (laboratory) versus hypobaric hypoxia (high altitude)

When the environment shifts from an acute and laboratory exposure to hypoxia (normobaric hypoxia) to a more chronic exposure (hypobaric or high altitude) the brain will display different adaptations to each of these stressors. Blood flow to the brain during chronic high altitude exposure has been reviewed (Ainslie & Subudhi, 2014). As such, this brief overview will provide a summary of the primary mechanisms involved with chronic exposure. For example, upon initial exposure (hours) to high altitude an increase in CBF of up to 24 % has been reported, with further declines towards baseline values thereafter with respect to time at altitude (Severinghaus et al., 1963).

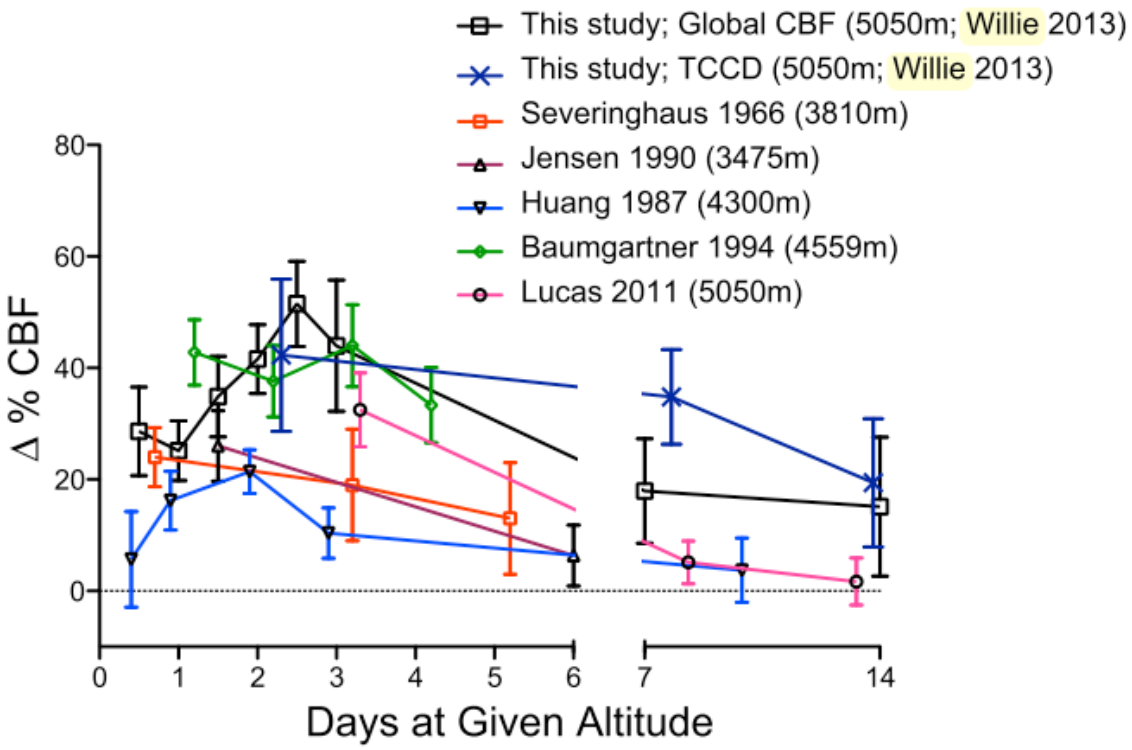

Figure 1.1 Percent change in cerebral blood flow (CBF). In response to the amount of days at altitude. CBF increases during the initial acute period of being at altitude. Chronically showing a decline in CBF over time back close to baseline levels (taken from Willie et al., 2013) (permission not required).

This elevation in CBF has been reported and confirmed in more recent publications (Subudhi et al., 2014; Willie et al., 2014). As noted above in acute hypoxia, the elevations in CBF upon ascent to high altitude seem to compensate for the reduction in CaO_2 and to maintain cerebral O_2 delivery. For further review, Figure 1.1 shows the typical elevation in CBF over the course of the initial ascent (hours – days) to high altitude followed by a return towards baseline values during the acclimatization period (days – weeks).

1.3 Cerebral blood flow and cerebral metabolism

The brain is metabolically one of the most active organs in the body. It comprises only 2% of total body weight, but receives 15-20% of cardiac output and uses 20% of total body oxygen and 25% of total body glucose (Kety & Schmidt, 1948; Holliday, 1971; Sokoloff, 1999). Within normal levels of global CBF, at rest, the brain extracts about 35% of the oxygen and 10% of the glucose and 2-4 % of lactate from arterial blood. Metabolism increases when brain energy demands increases which occurs during various situations such as sensory stimulation, cognitive and motor activity (Dienel & Cruz, 2002). Thus, it should not be surprising that to maintain this high-energy demand and adequate blood flow delivery, a myriad of regulatory mechanisms and fuel sources are involved (Kety, 1945, Lassen et al., 1978, Lassen, 1985). The three major parameters linked to brain energy metabolism – CBF, oxygen consumption, glucose and lactate metabolism - can all be measured independently in humans. Maintaining adequate CBF is essential to providing a constant supply of metabolites and removal of byproducts to maintain cerebral tissue homeostasis (Hom et al. 2001).

1.4 Cerebral blood flow and autoregulation

Cerebral autoregulation (CA) is defined as the relationship between mean arterial pressure and CBF, as is often classified into two categories; static (steady-state, occurring over mins to hrs), and dynamic (transient, occurs over secs) [(Aaslid, 1987; Numan et al., 2014)]. It is known that CA is more effective against buffering transient increases in blood pressure, compared to transient reductions (Brassard et al., 2017). The mechanisms of CA have been described in detail elsewhere (Donnelly et al., 2016). The likely role of the sympathetic nervous system is involved in the buffering of the increase in blood pressure with constriction to the cerebral arteries to prevent over-perfusion (Numan et al., 2014). The

cerebral response to changes in buffering blood pressure is opposite to that which occurs in the peripheral vasculature to changes in buffering blood pressure (Brassard et al., 2017).

1.5 Cerebral blood flow and neurogenic activity

Returning to the metabolic complexity and versatility of the human brain, the topic of neuronal control of CBF has been one of constant controversy. Neuronal control is comprised of a network of small and large cerebral arteries that are innervated with sympathetic alpha [(alpha 1 and 2 –adrenoceptor; e.g., Bevan et al (1987)], [beta; e.g., Tsukahara et al (1986)], and parasympathetic (cholinergic) receptors Bevan et al (1987). The majority of this work has been completed within canines or felines (Nielsen & Owman, 1967), species that likely have differences in neuronal vascular receptors from the human counterparts (Ainslie & Brassard, 2014). This extensive neuronal network will innervate a vascular response, depending on the location, type, sensitivity and density of the receptor that is being stimulated at that particular time point. These complex topics have been extensively reviewed elsewhere (Ainslie & Brassard, 2014; Bevan et al., 1987; Nielsen & Owman, 1967; Tsukahara et al., 1986).

1.6 Reactive oxygen species and cerebrovascular regulation

The regulation of CBF is multifaceted and complex. There is evidence of regulation of CBF by nitric oxide (NO) and reactive oxygen species (ROS). The by-product of NO and oxygen, nitrite (NO_2^-), acts as a storage pool for NO (Cosby et al., 2003; Doyle et al., 1981); in turn nitrite contributes to steady state CBF (Hoiland et al., 2016). It is established that NO is an inflammatory mediator and free radical which serves as a homeostatic regulator for the cardiovascular, neuronal, and immune systems (Carmeli et al., 2016). NO is also involved with neurotransmission with the central nervous system among other physiological functions (Carmeli et al., 2016). Changes in NO have been linked to the generation of ROS, important molecules involved in oxidative stress. While by-products of NO are reflected as nitrite and nitrate, nitrite is likely the more meaningful measurable biomarker (Carmeli et al., 2016). Figure 1.2 presents the biochemical pathway of ROS and the interaction of ROS and NO forming peroxynitrite, which will reduce the bioavailability of NO within the vasculature.

There have been multiple animal studies showing the attenuation of vasodilation of the cerebral vasculature by inhibiting NO synthesis during hypercapnia (Buchanan & Phillis, 1993; Dirnagl et al., 1993; Niwa et al., 1993; Pelligrino et al., 1993). In contrast, the effects of NO inhibition during reductions in $PaCO_2$ (hypocapnia) does not show an attenuation in vasoconstriction of the cerebral-vasculature (Faraci & Heistad, 1992);(Iadecola & Zhang, 1994). It is important to note that these studies were completed within animal models, and there have been few studies applied to the human model. In contrast to reactivity, however, there are also reports that NO blockade does not compromise normal dynamic CA (Zhang et al., 2004). An in-depth review of the influence of NO on the cerebral-vasculature has been published elsewhere [Faraci & Brian Jr., (1994)]. In humans, at least as assessed via TCD, there has been no direct link with CBF and NO with the attenuation of NO, with use of NO inhibitor L-NMMA, on CBF (Ide et al., 2007). However, this is not a universal findings and a MRI study reported attenuation of CBF (Van Mil et al., 2002) during an hypoxic stimulus. These latter differences could be explained by the different methods to assess CBF (TCD vs MRI), control of $PaCO_2$, with either maintaining an isocapnic or poikilocapnic protocol.

As previously stated the brain is a complex organ and highly sensitive to adverse changes affecting the overall homeostasis. Due to the amount of peroxidizable polyunsaturated fatty-acid side chains (PUFAs) located within the neuronal membrane (where the highest concentration of PUFAs located directly within the nerve tissues), the brain itself is highly prone to oxidative stress (Bailey et al, 2009). Both the neuronal membrane and nerve tissues have shown a low defense system, demonstrating the brain to be vulnerable to redox mediated changes. A possible reason explaining the increased effect of antioxidants on the brain might lie in the high concentration of PUFAs exposed to an O_2 flux which, in turn, effects the regulation of neurotransmission within the brain (Bailey et al., 2009). The brain's high concentration of iron stores might account for the increased vulnerability to oxidation, potentially leading to increased perioxide.

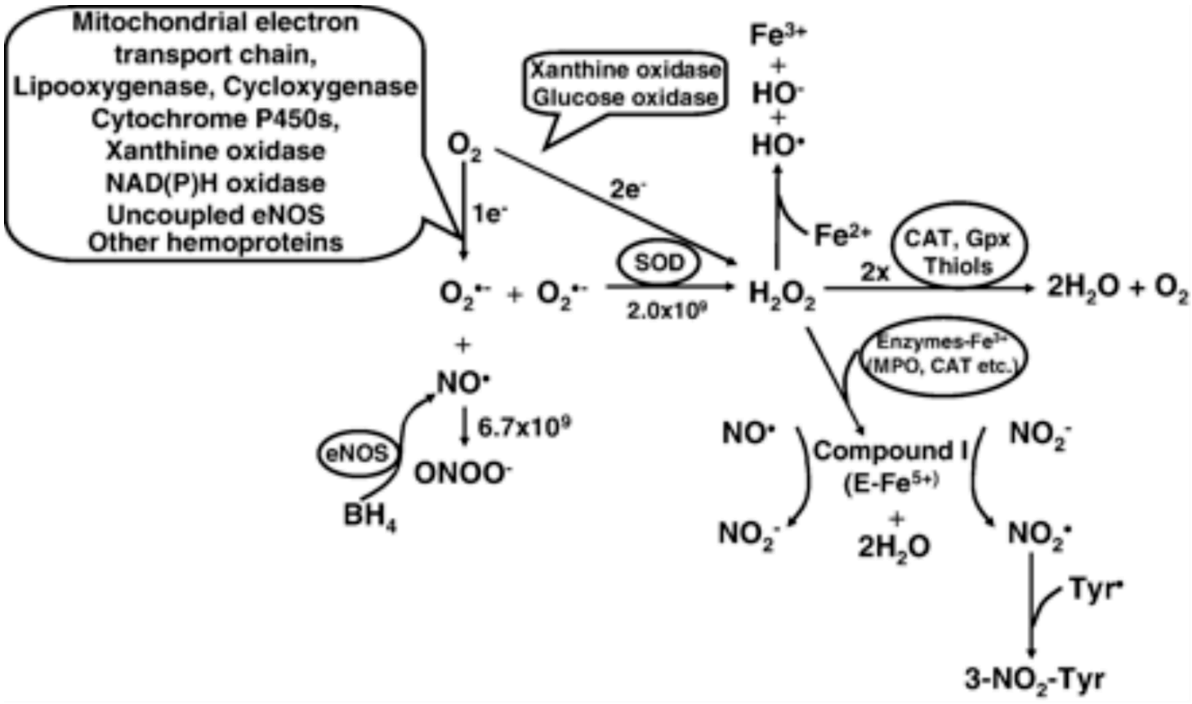

Figure 1.2 Biochemical pathway of hydrogen peroxide (H2O2) generation and metabolism. Showing the reduction from molecular oxygen (O_2) to form superoxide (O_2^-) or hydrogen peroxide (H_2O_2). Hydrogen (H) is derived from superoxide dismutase (SOD). H can interact directly as a singling intermediate, but also indirectly by exerting its biological effects via metabolites such as hydroxyl radical. Superoxide (O_2^-) also interacts with nitric oxide (NO) to form peroxynitrite ($NO + O_2^- = ONOO^-$) (taken from Cai, 2005) (permission received).

The combination of high concentrations of neuronal membrane and nerve tissue PUFAs, along with high iron stores, show the reduced capacity of the brain to regulate the potentially damaging free-radical chain reactions (Bailey et al., 2009).

1.6.1 Antioxidants

Being that the brain is sensitive to adverse changes in homeostasis and oxidative stress, there are also small but effective antioxidants defence mechanisms. Oxidative stress increases in many physiological factors such as: 1) cardiovascular and cerebrovascular diseases (Silva et al., 2012); 2) exercise (Vianna et al., 2015); 3) aging (Chrissobolis & Faraci, 2008) 4) and prolonged stays at high altitude and hypoxic exposure (Bailey et al., 2009). For the scope of this review the focus will pertain on acute and chronic hypoxia and the punitive role of antioxidants. As previously stated, the effects of exposure to hypoxia can lead to an increase

of ROS within the vasculature (Bailey et al., 2009); such increases of oxidative stress might therefore have important influences on the PUFA's within the brain. Despite the knowledge of the sensitivity of the brain and oxidative stress, however, very little work has examined the role in ROS on the cerebral vasculature during hypoxia. Hartmann et al., (2015) reported no effect of infusion of vitamin C (an effective antioxidant) on cerebrovascular and ventilator response to hypoxia. Later, however, Hartmann et al., (2015) stated that the vitamin C dosage might have become a pro-oxidant. If so, this might explain the lack of an influence of this vitamin infusion (Hartmann et al., 2015). For further review, Table 1.1 presents a summary of studies reviewing free radicals and cerebrovascular function with and without antioxidants. This summary table highlights the complexity of the effects of oxidative stress on cerebrovascular function with animal studies showing the increase of oxidative stress causing vasodilation on cerebral pial arterioles. Further studies to examine the impact of oxidative stress on human in-vivo cerebrovascular function is warranted.

Table 1.1 Summary of studies reviewing free radicals and cerebrovascular/cardiovascular function with and without antioxidants

Study	Method	Timeline	Study population	Main result
Rosenblum, 1983	Mouse pial arterioles were exposed to free radical generating reactants.	Single experimental trial	Male experimental mice	Free radicals caused dilation of pial arterioles. Whereas, high concentrations cause initial constriction then dilation. Free radical scavengers caused inhibition of this dilation.
Pokorski et al., 2003	HVR was assessed with progressive eucapnic hypoxia before and after ten days of oral ascorbic acid supplementation. Respiratory variables were recorded breath by breathy, and hypoxic sensitivity was assessed from the linear slopes of minute ventilation and mouth occlusion pressure plotted against SpO2.	Ten days of oral ascorbic acid supplementation	Eighteen healthy older females (60-80 yrs.).	Ascorbic acid increased HVR by 44%, this effect being driven by a higher occlusion pressure. The augmented HVR by ascorbic acid may have therapeutic potential in pathologies associated with hypoxia, which develop in old age.

Study	Method	Timeline	Study population	Main result
Bailey et al., 2009	Whether hypoxia causes free radical-mediated disruption of BBB. Subjects equipped with arterial-jugular catheters. Subjects exposed to nine hours of hypoxia (FiO2 = 12.9%).	Nine hours of passive exposure to normobaric hypoxia (FiO2 = 12.9%).	Ten healthy male adults	lipid-derived alkoxyl-alkyl free radicals increased and lipid hydoperoxides, cerebral oxygen metabolism remained preserved. stimulates cerebral oxidative-nitrative stress.
Bailey et al., 2011	Subjects were assessed at rest and during semi-recumbent cycling till exhaustion. Cerebral autoregulation and CBF was assessed via MCAv through TCD. EPR spectroscopy was used to assess free radical's ad nitric oxide metabolites.	Single experimental trial day.	Eight healthy male adults	Exercise caused a mild reduction in the autoregulation index. Exercise increased in ascorbate radical. Intense exercise has the potential to increase blood-brain barrier permeability without causing brain structure damage.
Pena Silva et al., 2012	Endothelial function, expressions of angiotensin system components, NADPH oxidase subunits, and pro-inflammatory cytokines were examined in cerebral arteries.	Single experimental trial for each mouse.	Wild-type mice both adult (12 month) and old (24 month) and similar aged angiotensin-converting enzyme type 2 (ACE2) knockout mice.	Vasodilation to acetycholine was impaired in adult ACE2 KO mice compared to WT mice. In older mice, dilation is impaired in WT mice, and severely impaired in KO mice.
Hartmann et al., 2015	CBF was measured via MCAv through TCD. Subjects were given intravenous vitamin C. Before and after a five-minute hypoxic challenge	Two testing protocols, either placebo or drug. 45 min washout in-between each trial.	Healthy volunteers between ages (20-79 yrs.), (younger 20-39; Older and COPD: 55-79 yrs.)	Vitamin C had no effect on hypoxic ventilator response, but selectively decrease blood flow sensitivity in younger only. Vitamin C doesn't appear to have a large influence on cerebrovascular or ventilatory response during acute hypoxia.
Irarrazaval et al., 2017	Measuring the effects of acute hypobaric hypoxia. Oxidative stress was determined through blood profile tests performed 24 hours before and after high altitude exposure. Dietary habits were assessed using Chilean Mediterranean diet index. Arterial oxygen saturation and cardiac rate was determined.	High altitude exposure occurred over 36 hours.	Ten male healthy adults (24.9 – 32.7 yrs.)	All subjects had a 17% decrease in SaO2. Plasma lipid oxidative damage increased after the expedition. Acute hypobaric hypoxia induced AMS and an increment in oxidative stress markers 24 hours after altitude exposure.

Studies are arranged in chorological order by year published.

1.7 Regulation of peripheral vasculature function

The vascular endothelium is a layer of endothelial cells that line the inner layer of blood vessels. This endothelial layer contributes to the vasodilation and constriction of the vessels, along with vasoprotection functions [e.g. protection of coronary arteries, and reductions in myocardial ischemia] (Pyke & Tschakovsky, 2005)]. Vascular diseases such as atherosclerosis and stenosis has been shown to cause endothelium dysfunction (Ross, 1993).

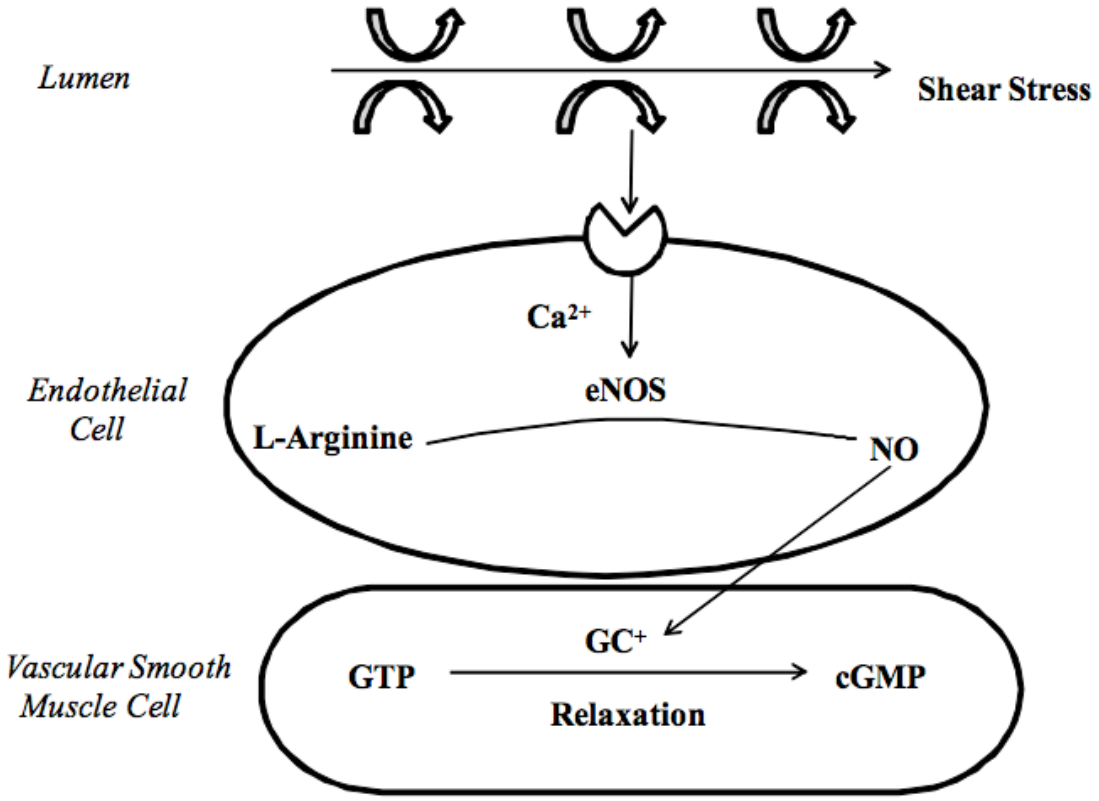

Figure 1.3 The NO pathway. In response to shear stress. Calcium – activated potassium channels, which cause calcium ions (Ca^{2+}) to enter the endothelial cell which will activate endothelial NO synthase (eNOS) and converts L-arginine to NO. NO will enter the vascular smooth muscle cells and activate guanylate cyclase (GC^{+}) which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), influencing vascular smooth muscle vasodilation (taken from Ganz, 2002) (permission received).

Blood flow within the peripheral vasculature is regulated, amongst other factors, by vasoactive substances that regulate vascular tone, driven by metabolism, blood pressure, and heart rate. The pioneering work completed by Furchgott & Zawadzki, (1980) discovered that when experimentally prepared strips of a rabbits aorta to acetylcholine (*in vitro*) vessels constricted; however, when the aorta was left intact, meaning that the endothelial layer was still present, they found that the aorta dilated (Furchgott & Zawadzki, 1980). They concluded that the endothelium layer was subsequently the primary determinant of either vasodilation or vasoconstriction of the vessel itself. This important work contributed to the initial findings of endothelial dependent vasodilation. Further work on the interaction between blood flow and the endothelium elucidated that an increase in blood flow will induce shear stress on the endothelium, releasing vasoactive relaxation factors, causing vasodilation. This shearing effect on the endothelium will release endothelial NO (eNOs), combined with L-arginine will release NO into the endothelial cells, which begins the NO cyclic cascade (Ignarro et al., 1999; Moncada et al., 1988).

The NO cyclic cascade is multifaceted and complex; however, at a basic level of understanding the breakdown of L-arginine (a chemically unstable intermediate) will release NO [a lipophilic vasodilator directly lying within smooth muscle (Axelsson et al., 1982; Ignarro et al., 1999; Moncada et al., 1988) known as nitric oxide synthases (NOS)]. Accompanying L-arginine breakdown, there is an accumulation of tissue guanosine triphosphate (GTP) (Axelsson et al., 1982; Axelsson et al., 1979). The accumulation of tissue GTP will release cyclic-guanosine 3',5'-monophosphate (cGMP) and, when combined with NO within the endothelium, will eventually lead to vascular relaxation (Axelsson et al., 1982). Furthermore, this cascading effect will cause smooth muscle relaxation within the endothelium of the vessel (Celermajer et al., 1992). This NO pathway is influenced (but not exclusively mediated) by reactive hyperemia (increased blood velocity) and the sheering response of blood cells with the endothelial wall, known in part as sheer stress. Furthermore, Figure 1.3 presents the NO cyclic cascade in response to a shear stress stimulus.

With the understanding of the initial work completed by Furchgott, and the discovery of the endothelium-derived relaxation factor NO, Celermajer and colleagues published a technique

known as endothelial dependent flow mediated dilation or flow mediated dilation (FMD) (Celermajer et al., 1992). This FMD technique is a commonly used index of cardiovascular health (Thijssen et al., 2011). Since the first introduction 25 years ago, the FMD technique has been further modified in order to standardize and reduce variability between measures (Thijssen et al., 2011).

1.8 Endothelial dependent flow mediated dilation

Endothelial dependent vasodilation (shear mediated dilation) can be assessed using FMD - section 2.2.4.2 "*Peripheral vascular function* in *Methods*" for further details. This FMD is an *in vitro* measurement tool used to assess peripheral cardiovascular health (e.g. atherosclerosis), which is usually assessed in the brachial, radial or femoral arteries. A 1% increase in FMD is reported to be associated with a 9% reduction in cardiovascular risk (Green et al, 2011). FMD uses an acute ischemic bout to a section of the artery distal from the elbow in order to induce reactive hyperemia creating a shearing effect in the endothelial wall, which releases NO into the smooth muscle (as previously stated) (Thijssen et al., 2011). Brachial FMD can be influenced by multiple physiological factors such as 1) changes in shear stress and related hemodynamics; 2) oxidative stress; 3) and sympathetic nervous activity (SNA). The next sections will briefly detail the key mechanisms for each of these physiological factors.

1.8.1 Changes in shear stress and hemodynamics

Shear stress or rate is the frictional force of the interaction between blood cells and the endothelium of the conduit artery, usually caused by reactive hyperemia in antegrade flow (forward moving blood flow), as well as caused by retrograde flow (backward moving blood flow). Antegrade shear can induce shear-mediated vasodilation, which releases endothelial NO (eNOS). The rapid diffusion of NO into the endothelium causes vascular relaxation of the peripheral arteries (Green et al., 2011; Joannides et al., 1995). This rapid NO diffusion has been shown during an acute shear response, but when the vessel is introduced to a chronic shear response, vascular relaxation is attenuated (Mullen et al., 2001). In contrast, retrograde shear may attenuate brachial vasodilation – this has been reported immediately following exercise (Johnson et al., 2012) and during prolonged exposure to high altitude

(Lewis et al., 2014). Along with NO, other subsequent vasodilators are released into the endothelium due to the shear stress stimulus these include: prostaglandins and endothelial hyperpolarizing factor (Pyke & Tschakovsky, 2005). However, for the scope of this review, it is reported that NO contributes to around 50% of endothelium vasodilation (Green et al., 2013).

1.8.2 Hypoxia and FMD

Impairment of the brachial artery vasodilation, as assessed by FMD, has been reported to be impaired at high altitude (5050m) (Lewis et al., 2014), although this is not a universal finding (Bruno et al., 2015; Bruno et al., 2016). The attenuation of FMD at altitude likely are related to: 1) A reduction in FMD SR_{AUC}; 2) decline in baseline blood flow and increase in retrograde and oscillatory shear; 3) impaired endothelial function and smooth muscle vasodilation; 4) and an increase in SNA. SR_{AUC}, which indicates the shear stress stimulus upon the release of the cuff. During hypoxia SR_{AUC} can be attenuated by 21% for the first hour of hypoxia, and recovers back to baseline after six hours (Lewis et al., 2014). Although shear rate remains constant during prolonged stays at high altitude, there is a reduction in antegrade shear in contrast to a marked increase in retrograde and oscillatory shear; these changes indicate arterial dysfunction (Tremblay et al., 2017). Acute and prolonged stays at altitude will increase sympathetic nervous activity (SNA); (Hansen & Sander, 2003). An increase in SNA can contribute to an attenuation of the shear stress response on the endothelium (Atkinson et al., 2015; Hijmering et al., 2002; Lewis et al., 2014). The decrease in FMD at high altitude cannot be solely explained by NO bioavailability since NO is likely liberated, at least as indexed by NO_2^-. Along with the increase in NO bioavailability, there are elevations in hydroperoxide (LOOH) (Lewis et al., 2014) and potential upregulation of superoxide – both these factors could hinder the signalling process of NOS and the NO cyclic cascade (Dweik, 2005). For further review of FMD and hypoxia Table 1.2 presents the summary of studies about systemic vascular function and structure after exposure to hypoxia. Table 1.2 summarizes the change in peripheral vascular function with exposure to high-altitude, assessing the differences in measurement tools, stimulus given and acute versus chronic exposure.

Table 1.2 Summary of studies about systemic vascular function and structure after exposure to hypoxia.

Study	Technique	Exposure	Timeline	Study population	Main results
Otsuka et al., 2005	PWV heart-ankle pulse wave velocity, cardio-ankle vascular index.	Elderly community at 3524m compared to Japanese town	Prolonged residence at high altitude	40 elderly high altitude natives (19 men, 21 women)	Elderly living at high altitude have a higher risk of cardiovascular disease then sea-level, with an increased cardo-ankle vascular index.
Frick et al., 2006	FMD, nitroglycerin mediated dilation	Location A (576m), location B (1700m)	Day one at moderate altitude, and three weeks at moderate altitude	18 patients with coronary risk factors	FMD remained unchanged after initial day at altitude. After prolonged stay FMD reduced by 3.6%
Thomson et al., 2006	Systemic vascular resistance index (SVRI), and arterial stiffness (AI)	Normobaric hypoxia SO2 of 82.6%.	One hour of hypoxia	Eight healthy men	Hypoxia reduced SVRI (-15.2%), and AI (-10.7%).
Frobert et al., 2008	FMD, nitroglycerin-mediated dilation	Normobaric hypoxia 12.5% O2, 100% O2 supplementation	One day, and 3 months	10 males with increased risk of cardiovascular disease, and 10 age-matched controls	Oxygen supplementation evoked vasoconstriction and hypoxia reduced FMD/NMD
Vedam et al., 2009	Arterial stiffness augmentation index (AIx)	Normobaric hypoxia (SO2 80%), room air control	20 min	Hypoxia 12 healthy subjects, 5 healthy controls breathed room air	Hypoxia caused a 6% increase in AIx, before decreasing back to baseline values. After hypoxia AIx decreased a further 6%.
Rhodes et al., 2011	Arterial stiffness and tone	3450m and 4770m	11 days	17 lowlander subjects (three with mild hypertension)	At 3450m arterial stiffness unchanged, and tone decreased 4% from baseline. AT 4770m arterial tone decreased 4.5% from baseline.
Rimoldi et al., 2012	FMD, arterial stiffness, carotid intima-media thickness (IMT)	Bolivia 3600m	Born and permanently living at high altitude	23 CMS patients without additional cardiovascular risks. 27 age-matched healthy highlanders	CMS patients had a reduced FMD 4.6%, and greater pulse wave velocity and IMT, compared to control group FMD 7.6%.
Bailey et al., 2013	FMD, arterial stiffness, and carotid intima-media thickness (IMT)	Bolivia 3600m	Highlanders are permeant residence	25 male highlanders both with and without CMS, 12 age-matched male lowlanders.	Vascular function remained unchanged in highlanders without CMS. Vascular dysfunction in highlanders with CMS.

Study	Technique	Exposure	Timeline	Study population	Main results
Parati et al., 2013	PWV	4559m by passive cable car, one day of trekking	24-72h	42 healthy lowlanders	PWV unchanged
Johansson et al., 2014	Hyperemic responses to brachial artery occlusion by peripheral arterial tonometry	Normobaric hypoxia equivalent to 4500m	2-4 hrs	12 healthy subjects	Post-occlusion/pre-occlusion ratio (reactive hyperemia index) reduced from normoxia (1.80) to hypoxia (1.62), and increased during normoxia recovery (2.43)
Lewis et al., 2014	FMD, PWV, carotid stiffness	Ev-K2-CNR pyramid laboratory (5050m), 9-11 days of high altitude trekking	3-4 days and 12-14 days	12 healthy lowlanders	FMD reduced from sea-level (7.9 ± 0.4 to 6.8 ± 0.4%) at initial arrival.
Bruno et al., 2015	FMD, PWV, carotid stiffness	3842m via passive ascent (cable car)	4h exposure, and 24h exposure	34 healthy lowlander volunteers	No change in FMD
Iglesias et al, 2015	FMD	Normobaric hypoxia (simulated equivalent to 4000m)	4 hrs.	Ten healthy subjects	FMD was unchanged in hypoxia compared to normoxia.
Tremblay et al., 2017	FMD, 30 min distal cuff occlusion	Sea-level (344m). Passive ascent to 3800m at Barcroft Laboratory	Prolonged stay at altitude, before and after 30 min of distal cuff occlusion	12 young healthy lowlander participants	Brachial artery was constricted at baseline at HA. FMD was unchanged at SL. FMD was reduced by 26% at HA.
Tymko et al., 2017	FMD	Sea-level (344m). Passive ascent to 3800m Barcroft Laboratory	Prolonged stay at altitude, before and after 30min of moderate exercise.	Nine young healthy lowlander participants.	FMD unchanged from sea-level to high altitude. FMD reduced by 2.9% following exercise, but was abolished with prazosin. Unchanged after exercise at altitude with and without prazosin.

Studies are arranged in chronological order by year published.

1.8.3 Sympathetic nervous activity

As previously mentioned within section 1.8.2, an increase in SNA or sympathoexcitation can attenuate conduit artery FMD. Increased SNA has been linked to vascular impairment and cardiovascular disease, altering hemodynamics (heart rate, mean arterial pressure [MAP]) (Dyson et al., 2005) and reducing FMD. Changes in SNA can affect FMD both acutely and chronically, acutely depending on the type and duration of the stress (e.g., mental versus cold pressor) (Lind et al., 2002; Spieker et al., 2002). For example, Lind et al., (2002) reported that with a cold pressor test (CPT)– which markedly increases SNA – there is acute endothelium dysfunction (Lind et al., 2002). Dyson et al., (2005) confirmed this finding showing that CPT caused a 27% drop in FMD when compared to the control baseline group (Dyson et al., 2005). Likewise, using 10-min of lower body negative pressure (to elevate SNA), there is an reduction in antegrade shear and increases in retrograde shear; together, these changes mediate a reduction in FMD% (Thijssen et al., 2014). Acute increases in SNA also occur with an hypoxic stimulus, and has also been reported to reduce FMD (Duplain et al., 1999; Hansen & Sander, 2003). The attenuation of FMD is reversed in acute hypoxia upon administration of alpha1-adrenorecptor blockade (prazosin), thus further highlight the importance of SNA (Lewis et al., 2014).

1.8.4 Reactive oxygen species and FMD

Along with increasing SNA, acute hypoxia has also shown to increase ROS and oxidative stress (Bailey et al., 2009; Dweik, 2005). There has been a degree in variation within the amount of influence that ROS has on peripheral vascular function (Caruana & Marshall, 2015; Crecelius et al., 2010; Donato et al., 2010; Eskurza et al., 2004; Jablonski et al., 2007b; Kirby et al., 2009a; Ranadive, Joyner et al., 2014b; Richards et al., 2015; Richardson et al., 2007; Trinity et al., 2016; Wray et al., 2012; Wray et al., 2009). The mechanistic influence of ROS primarily occurs within the endothelial layer of the vessel, resulting in vasoconstriction (Wray et al., 2012). Vasodilation is seen in both the brachial (Green et al., 2011) as well as the radial artery (Joannides et al., 1995), post cuff occlusion during the FMD testing stimulus. There are excessive elevations in ROS during times of stress such as cardiovascular diseases (atherosclerosis) (Joannides et al., 1995), chronic obstructed

pulmonary disease (COPD) (Hartmann et al., 2016), and congestive heart failure (Belch et al., 1991), type 2 diabetes (Butkowski & Jelinek, 2016), normal aging (Wray et al., 2017) and high altitude trekking (Lewis et al., 2014). These increases in ROS and oxidative stress, when excessive, can lead to vascular dysfunction and arterial stiffening (Wray et al., 2013). Mechanistically, as previously mentioned, vascular dilation is established through the release of relaxation mediators or NO into the smooth muscle of the endothelium, during hyperemia (Green et al., 2011). Vascular vasodilation from the release of endothelium NO can become impaired due to the interaction with oxidative stress. Impairment of peripheral vascular function seems to be a consequence of a redox imbalance i.e., ROS has overwhelmed the antioxidant capacity results in reductions in NO and hence arterial stiffening (Wray et al., 2017).

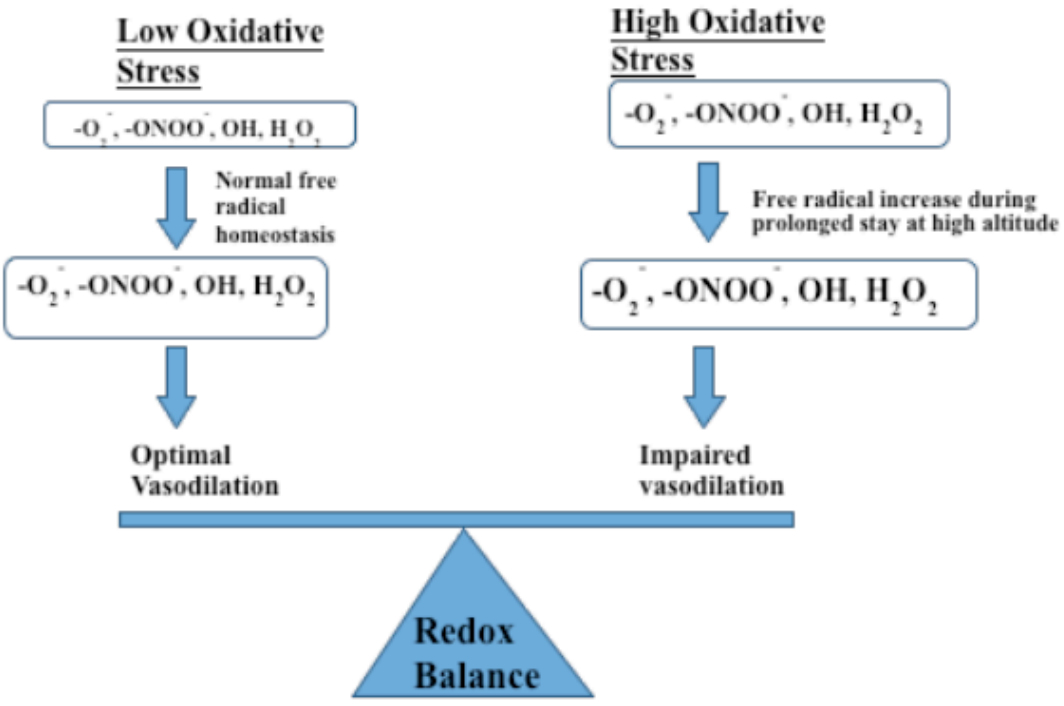

Figure 1.4 Redox balance in response to changes in oxidative stress. High oxidative stress in chronic hypoxia might be reflected with an upregulation of superoxide anions, leading to reductions in NO bioavailability and attenuating vasodilation. Low oxidative stress results in optimal peripheral vascular endothelium vasodilation and hence little or no impairment of NO bioavailability (adapted from Trinity et al., 2016) (permission received).

Although complex, increases of ROS have been reported at altitude (Bailey, 2003; Bailey et al., 2009). In brief, hypoxia exposure upregulates NADPH oxidase and forms superoxide, which in turn will impair the upregulation of endothelium NO and attenuate peripheral artery vasodilation (Dweik, 2005). Lewis et al (2014) reported increases in NO (measured through blood nitrite [NO_2^-] and in lipid hydroperoxide (LOOH) during a prolonged stay at high altitude (5050m). Both NO_2^- and LOOH remained elevated over prolonged high altitude exposure, compared to sea-level (Lewis et al., 2014). So, in agreement with previous work (Dweik, 2005; Lewis et al., 2014), NO bioavailability was reduced as relation to peripheral vascular impairment at altitude; however, it remains possibly that impairment in FMD is reflective from increases in LOOH within this study, or increased upregulation of superoxide, which will ultimately scavenge the increased NO and reflect in vascular impairment (Dweik, 2005; Lewis et al., 2014). Furthermore, Figure 1.4 presents the redox balance in response to changes in oxidative stress.

1.9 Antioxidant supplementation on peripheral vascular increases in ROS

Excessive elevations in ROS lead to the so-called redox imbalance. One acute approach to alleviate this imbalance is to administer antioxidants. For example, oral or intravenous vitamin C has been used to improve peripheral endothelium vasodilation. Taddei et al., (2001) reported an increase in forearm blood flow (FBF), measured via strain-gauge venous plethysmography with volume assessed per the water displacement method, by +598% in hypertensive subjects (aged; 31-60 years) upon intravenous administration of vitamin C. However, intravenous vitamin C improved FBF by a lesser extent (+395%) after the same subjects were given L-NMMA. These findings highlight that the vitamin C is acting, in part, by an NO liberating related mechanisms (Taddei et al., 2001). Although intravenous vitamin C infusions can provide a redox balance (Eskurza et al., 2004), oral supplementation of vitamin C has not shown the same effects. For example, Eskurza et al., (2004) reported no change in FMD after a chronic oral dose of 500mg/day of vitamin C for 30 days, in young and old sedentary and trained men (Eskurza et al., 2004). A single dose of oral vitamin C supplementation also resulted in no change in FMD; however, using an acute and combined oral dose of 1000mg of vitamin C, 600IU of vitamin E, and 900mg of alpha-lipoic acid, Wray et al., (2012) reported an attenuation of brachial FMD within a young healthy

population. In contrast, using the same approach, FMD was improved within an aging population (Wray et al., 2012). Within the older population FMD increased from placebo (5.2 ± 0.4%) compared to antioxidant (8.2 ± 0.6%) (Wray et al., 2012). This important study highlights the sensitivity of the redox balance, as well as showing a decrease in NO bioavailability within the aging population.

As shown, there has been extensive work completed with the use of antioxidant supplementation and endothelium function in both healthy young and aging populations at sea-level (Caruana & Marshall, 2015; Crecelius et al., 2010; Donato et al., 2010; Jablonski et al., 2007; Kirby et al., 2009; Moreau, DePaulis et al., 2007; Ranadive et al., 2014; Richardson et al., 2007; Rossman et al., 2015; Trinity et al., 2016). In contrast, there is very little work done that has examined how antioxidant supplementation may influence the integrated cerebrovascular and peripheral vascular function during acute and chronic hypoxia. Since both conditions likely increase superoxide anions and hence reduce bioavailability of NO within the endothelium (Dweik, 2005), the utility of antioxidant supplementation may offer some vascular benefits. For further review Table 1.3 presents a summary of studies about the use of antioxidants on peripheral vascular regulation. Table 1.3 summarizes the effect of antioxidants on peripheral vascular function and overall it displays that antioxidants allow for an improvement in vascular function and blood flow.

Table 1.3 Summary of studies about the use antioxidants on peripheral vascular regulation.

Study	Antioxidants used	Method	Timeline	Study population	Main result
Levine et al., 1996	Orally two grams of ascorbic acid	Brachial artery FMD was assessed before and two hours after either oral antioxidants or placebo.	One day, before and after two hours of oral administration.	46 patients with coronary artery disease	Placebo show no effect on dilation. Whereas, antioxidants showed an increase in dilation.
Ting et al., 1996	Intravenous infusion of vitamin C	Endothelium-independent dilation was assessed with nitroprusside. Blood flow was determined by venous occlusion plethysmography.	Each test was separated by 60min to re-establish resting blood flow.	Ten diabetic patients (47 ± (35-55yr) and ten age-matched controls	Diabetic patients, FMD was augmented by vitamin-C. Whereas, independent dilation had no effect. In controls, vitamin-C had no effect on FMD nor independent dilation.

Study	Antioxidants used	Method	Timeline	Study population	Main result
Eskurza et al., 2004	Intravenous infusion of vitamin C. Chronic ingestion of 500mg of vitamin C	Three groups were asked to supplement with 500mg of vitamin C for 30 days, and were given intravenous infusion of vitamin C before and after 30 days. FMD was measured before and after infusion.	30 days	Young sedentary (n = 11; 25 ± 1 yrs.), old sedentary (n=9; 64 ± 2 yrs.), older endurance trained (n=9; 64 ± 2 yrs.).	FMD was 45% lower in older compared to young, but preserved in exercise-trained. Infusion restored FMD in older group, with no effect in other two. Oral supplementation did not affect FMD in any group.
Peluso et al., 2004	Chemically generated peroxyl radicals and injected antioxidant protection	Adult male rats were acclimatized to a 12-hour dark and light cycle. Blood pressure was measured, and rats were injected with the radicals to increase BP. Antioxidants injected	One week	Adult male wistar rats.	Blood pressure baseline increased due to the radicals. With an exponential decrease with the injected antioxidants.
Jablonski et al., 2007	Intravenous infusion of ascorbic acid.	Resting leg blood flow was measured during either intravenous infusion of ascorbic acid and placebo. Plasma oxidized LDL, absolute resting femoral artery blood flow.	Two separate experimental testing days.	10 young healthy adults (25 ± 1 yrs.), and 11 older healthy adults (63 ± 2 yrs).	Resting femoral blood flow 25% lower in the older men when compared to the healthy young. Femoral artery blood flow increased by 37% in the older adults after the infusion of ascorbic acid.
Richardson et al., 2007	Vitamin C, E, and alpha lipoic acid	Subjects performed a submaximal forearm hand grip exercise, with either placebo or antioxidant. Brachial artery diameter and flow was measured. 12 subjects underwent an cycling exercise.	Two separate days, separated by 4 days, to take in account for proper washout.	25 young healthy subjects (25 ± 2 yrs.), 12 subjects underwent a cycling exercise, whereas, 13 subjects underwent the handgrip exercise.	EPR spectroscopy showed a 98% reduction in circulating free radicals. Brachial dilation was greater in placebo then antioxidant during handgrip exercises (7.4 ± 1.8% vs 2.3 ± 0.7%)
Kirby et al., 2009	Infusion of ascorbic acid (AA)	FBF was measured using ultrasound during single muscle contractions of 10, 20, and 40% MVC, before and after infusion. Also, muscle blood flow during continuous handgrip at 10%.	Acute one day experimental trial.	14 young healthy adults (22 ± 1 yrs.), and 14 healthy older men and women (65 ± 2 yrs.)	Infusion of AA had no effect on response in either age group. AA infusion had no effect on the young adults, but older adults had an increase in FBF after infusion.

Study	Antioxidants used	Method	Timeline	Study population	Main result
Wray et al., 2009	Vitamin C, E, and alpha-lipoic acid.	Six mildly hypertensive subjects underwent 6-week training program visiting the lab 3 times a week. BP and FMD was measured. Antioxidants was given to subjects on days 3-6	6-week aerobic exercise program, days 3-6 of training program, then re-evaluated after 6 weeks.	Six older (71 ± 2 yrs.) mildly hypertensive men	Antioxidant did not change resting FMD or BP. Exercise program improved FMD and BP, but antioxidants attenuated improvements.
Donato et al., 2010	Vitamin C, E, and alpha – lipoic acid	Subjects performed submaximal forearm handgrip exercise with either control or AOC. Older completed knee-extensor exercise training. Brachial artery diameter and flow was measured.	Two separate study days, 4 days apart. To take in account for washout.	Eight healthy young (26 ± 2 yrs.) and eight healthy old (71 ± 6 yrs.)	The old group had an attenuated brachial dilation during hand grip compared to young with placebo. This was reversed with antioxidants and hand grip in the old.
Crecelius et al., 2010	Acute infusion of ascorbic acid	Forearm blood flow was measured via Doppler ultrasound, during rhythmic handgrip exercise at 10% MVC. Baseline 5-min steady-state exercise was performed, with AA infused for 10min during exercise. Two groups of eight subjects were then either infused with PG inhibitor (ketorolac) or NOS inhibitor (L-NMMA)	Acute one day experimental trial.	14 healthy older adults (64 ± 3 yrs.).	AA infusion during exercise increased FBF by 25%. When L-NMMA as infused, this reversed the results in eight subjects, and reduced FBF by 20%. Proving that the increase in FBF during exercise in older adults infused with AA is mediated by an increase in NO bioavailability.
Wray et al., 2012	Oral vitamin C, E, and alpha-lipoic acid	FMD was measured before and after either antioxidants or placebo. Blood velocity, BA diameter was assessed before and after 5-minute forearm circulatory test	Two separate days, each day was separated by 4 days to account for washout.	87 volunteers (42 young: 25 ± 1 yrs.; 45 older; 71 ± 1 yrs.).	FMD was reduced in older group compared to young (5.2 ± 0.4%; vs 7.4 ± 0.6%). Antioxidant supplementation improved FMD in elderly, and reduced FMD in young. Antioxidants improve endothelial function in old, and reduces in old.

Study	Antioxidants used	Method	Timeline	Study population	Main result
Ives et al., 2014	Oral antioxidant combination (vitamin C, E, alpha-lipoic acid)	Brachial artery flow mediated dilation and carotid-radial pulse wave velocity were assessed using ultrasound Doppler.	Acute single day experimental trial	30 COPD patients, and 30 age and sex matched controls	Antioxidants significantly improved (3.1 ± 0.5 vs 4.7 ± 0.6%; placebo versus antioxidants). Control subjects showed no change. COPD patients exhibit free-radically mediated vascular dysfunction.
Ranadive et al., 2014	Intra-arterial infusion of vitamin C	Twelves healthy young adults performed rhythmic handgrip exercise during both normoxia and hyperoxia, with both either saline or vitamin C. Forearm blood flow was measured, along with forearm vascular conductance.	Each participant came in for two separate days.	Twelve heathy young adults	Vitamin C increased forearm blood flow, and forearm vascular conductance with hyperoxia. Vitamin C improved the subjects that were most affected by hyperoxia. While it had a reduced effect on those that were not.
Caruana et al., 2015	2000mg of vitamin C	FBF was measured using venous occlusion plethysmography, at rest and following static handgrip at 60% MVC for 2min. Also, following 2min of arterial occlusion, and breathing air or 40% O2. With either antioxidant or placebo	Two separate days in a cross-over study.	10 male subjects (21.1 ± 0.84 yrs.)	During air breathing, vitamin C increased the peak increase in FVC following static contraction, or release of arterial occlusion by 50-60%. Breathing 40% O2, vitamin C reduced the peak increase in FVC following static contraction, or release of arterial occlusion. Placebo at 40% O2 had no effect.
Richards et al., 2015	Intra-arterial infusion of ascorbic acid	Protocol 1) older healthy male subjects participated in handgrip exercise while forearm blood flow was measured using Doppler ultrasound. With and without ascorbic acid. Protocol 2) AA would not enhance sympatholysis in older adults during handgrip.	Acute dose of antioxidants. Participants came on two separate days.	Eight older heathy males (65 ± 3 yrs.) participated in protocol one, whereas, 10 older heathy adults (63 ± 2 yrs.) participated in protocol 2	Protocol 1) AA did not influence blood flow at both 15% and 25% handgrip MVC, blood flow increased due to conductance. Protocol 2) with AA, blood flow was elevated at 15% MVC vasoconstriction to reflex increases in sympathetic activity. AA improves muscle blood flow and VO2 in older adult via vasodilation.

Study	Antioxidants used	Method	Timeline	Study population	Main result
Rossman et al., 2015	Vitamin C, E, and alpha-lipoic acid	16 COPD patients and 16 healthy controls performed submaximal single leg knee extensor exercise. Leg blood flow was measured, with MAP, leg vascular conductance, and SpO2, leg O2 consumption with direct Fick was assessed before and after placebo or antioxidants.	Two separate days.	16 subjects with COPD, and 16 healthy controls	Antioxidants improved leg blood flow, leg vascular conductance, leg O2 consumption during exercise in COPD. Whereas, no effect was observed in heathy subjects.
Trinity et al., 2016	Ascorbic acid	Infusion of ascorbic acid, along with infusion of L-NMMA during handgrip increasing.	Acute single day infusion of ascorbic acid, L-NMMA	older healthy subjects (men n=3, women n=4; 69±2yr).	improved brachial diameter at the greater handgrip forces (9kg, 12kg). L-NMMA blunted brachial.

Studies are arranged chronologically by year published.

1.10 Purpose and hypothesis

The aim of this study is to investigate the effects of oral antioxidant supplementation (1000mg vitamin C, 600IU vitamin E, and 900mg of alpha-lipoic acid) on peripheral and cerebral endothelial function at both sea-level and high altitude. To date, the use of oral antioxidant supplementation on cerebral vascular endothelium function at sea-level and peripheral *and* cerebral at high-altitude has not been investigated. The goal is to elucidate the possible mechanism(s) that regulate endothelial function in both peripheral and cerebral vasculature, and better the understanding of the possible role of oxidative stress. The hypotheses that were examined were: 1) at sea-level (when oxidative stress is low), peripheral and cerebral vascular function will be reduced after the administration of oral antioxidants; 2) at high-altitude (when oxidative stress is high), peripheral and cerebral vascular function will be improved after administration of oral antioxidants.

2 Chapter: Cerebrovascular and Peripheral Vascular Function: Role of Oxidative Stress

2.1 Background

Oxidative stress is ultimately determined by the balance between pro- (e.g. free radicals) and antioxidants (e.g. vitamin C). Free radicals act as important mediators and modulators of cell signaling, and contribute to other biological functions, which regulate the activity of transcription factors and gene expression (Donato et al., 2010; Richardson et al., 2007; Ristow et al., 2009; Shoemaker et al., 1997; Wray et al., 2012; Wray et al., 2009). The biological importance of free-radicals in physiological doses are reflected in the marked abolishment in vascular endothelial function (as assessed via flow-mediated dilation [FMD]) upon administration of a dosage of oral antioxidants (vitamin C, vitamin E, and lipoic acid) in otherwise healthy young humans (Wray et al., 2012). In contrast, in the aging and populations with pre-existing vascular disease (diabetes, heart failure, COPD, etc.) the same reduction in vascular endothelial function is not observed upon administration of oral antioxidants (Donato et al., 2010). Surprisingly, although a number of studies have examined the effects of antioxidants within the peripheral vascular system (Donato et al., 2010; Richardson et al., 2007; Ristow et al., 2009; Shoemaker et al., 1997; Wray et al., 2012, 2009), only one study in humans has assessed the basic response on the cerebral vasculature (Hartmann et al., 2015). This study assessed intra-cranial responses to antioxidants using the approach of trans-cranial Doppler (TCD) ultrasound at rest and during acute hypoxia and showed no augmentation in cerebrovascular response as well as ventilation during acute hypoxia, within the healthy young population. The assumption of TCD, however, is that it assumes that the diameter of cerebral blood vessels remains constant (Hartmann et al., 2015). Recent evidence suggests that this assumption does not always hold true, and diameter of the cerebral blood vessels changes with alterations in oxygen and carbon dioxide (Ainslie & Hoiland, 2014; Coverdale et al., 2014; Coverdale et al., 2015; Imray et al., 2014; Verbree et al., 2014; Willie et al., 2012; Wilson et al., 2011). To avoid this potentially major confound, different ultrasound approaches to measure volumetric cerebral blood flow (CBF) is required.

Although the regulation of cerebral blood flow (CBF) is multifaceted and complex, there is some evidence of regulation by nitric oxide (NO) and reactive oxygen species (ROS). The by-product of NO and oxygen, nitrite (NO_2^-) acts as a storage pool for NO (Hoiland et al., 2016), in turn nitrite contributes to steady state CBF (Hoiland et al., 2016). NO is an inflammatory mediator and free radical, which serves as a homeostatic regulator for the cardiovascular, neuronal, and immune systems, as well as involved with neurotransmission with the central nervous system among other physiological functions. Changes in NO have been linked to the generation of ROS, which are important molecules involved in oxidative stress. The by-products of NO are reflected as nitrite and nitrate, for which nitrite is likely the more meaningful measurable biomarker (Carmeli et al., 2016; Du et al., 2015; Ide et al., 2007; Shoemaker et al., 1997).

The brain is highly prone to oxidative stress, such as hydrogen peroxide (H_2O_2) which binds with NO and forms peryoxynitrite ($ONOO^-$) and reduces NO bioavailability, due to the amount of peroxidizable polyunsaturated fatty-acid (PUFAs) side chains, for which are located within the neuronal membrane, with the highest concentration of PUFAs located directly within the nerve tissues (Bailey et al., 2009). Both the neuronal membrane and nerve tissues have shown a low defense system, in turn shows the brain is vulnerable to redox mediated changes. A reason behind the increased effect of antioxidants on the brain could be that the high concentration of polyunsaturated fatty acids that are exposed to an O_2 flux, which affect neurotransmission within the brain (Bailey et al., 2009). The brain's high concentration of iron stores, could also account for the increased vulnerability to oxidation, which can potentially increase hydroxide (OH^-). The combination of high concentrations of neuronal membrane and nerve tissue polyunsaturated fatty acids, along with high iron stores, show the reduced capacity of the brain to regulate the potentially damaging free-radical chain reactions (Bailey et al., 2009). Despite the potentially important role of oxidative stress on cerebrovascular function, no studies have examined the impact of antioxidants on integrative cerebrovascular function (e.g. reactivity) in humans both at sea-level and high altitude (e.g., COPD in which patients have excessive levels of oxidative stress; Ives et al., (2014).

Using a double blinded, randomized, placebo controlled design, the primary purpose of this study was to examine the role and effect of a combined mixture of antioxidants on cerebrovascular regulation using hypercapnia, normobaric isocapnic hypoxia, and hypobaric hypoxia. The effects of antioxidants on peripheral vascular regulation at both sea level and during hypobaric hypoxia were also determined. We hypothesized that: 1) at sea-level, peripheral and cerebral vascular function will be reduced after the administration of oral antioxidants; and 2) at high-altitude, peripheral and cerebral vascular function will be improved after administration of oral antioxidants after a prolonged (days – weeks) stay at high-altitude (5050m).

2.2 Methods

2.2.1 Ethics

Study 1 and 2 were approved by Human Ethics Committee of British Columbia and the Nepal Health Medical Research Council, and confirmed with the standards set by the *Declaration of Helsinki*. Verbal and written consent was obtained by all participants, both in Kelowna [British Columbia (344m)] for Study 1, and at the Ev-K2-CNR Research Pyramid in the Khumbo Valley, Nepal (5050m) for Study 2.

2.2.2 Study 1 – Sea-level laboratory testing, in Kelowna, British Columbia (344m)

2.2.2.1 Participants

Twelve healthy young individuals (age: 24.3 ± 3.0yrs; body mass index: 24.1 ± 1.9kg/m^2) volunteered to participate in Study 1. Of the twelve participants, two were female. Participants were screened to ensure reliable ultrasound measurements of brachial, internal carotid, and common carotid arteries. All participants were free of cardiovascular, respiratory & cerebrovascular diseases, were non-diabetic, and were not taking any prescription drugs (other than oral contraceptives n=2) at the time of participation.

2.2.2.2 Protocol design

This study involved both a placebo trial and a drug trial, on two separate days with 48 hrs in-between each day to account for adequate drug washout. Each trial was completed within the laboratory of the University of British Columbia Okanagan campus, in Kelowna British Columbia (344m). The intervention was randomized and blinded to the ultrasound scanners, to minimize measurement subjectivity. Participants arrived at the laboratory having abstained from caffeine for 12hrs, and exercise and alcohol for 24hrs, as well as 2hrs fasted. Participants were requested to lie supine for 15 mins upon arrival, while being instrumented with experimental equipment, following the supine rest, a brachial flow mediated dilation test (FMD) would take place.

Following FMD, the participant began breathing on the pneumotachograph and dynamic end-tidal forcing was used to control $P_{ET}O_2$ and $P_{ET}CO_2$ at resting baseline. During this time point (5 mins), cardiorespiratory measurements were also recorded (VE, HR, MAP, MCAv, $P_{ET}O_2$, $P_{ET}CO_2$, Q_{ICA}, Q_{CCA}). Following a two-minute baseline period, the participant underwent a total of five minutes of increased $P_{ET}CO_2$ (hypercapnia; +9mmHg from resting $P_{ET}CO_2$). Ultrasound images of ICA and CCA were recorded for the total two mins of baseline, and five mins of hypercapnia (the final minute of steady state hypercapnia was used for analysis). Rest in-between hypercapnia and isocapnic hypoxia was five mins, which was enough time for CO_2 washout to occur, as well as a decrease in SNA.

Following hypercapnia and related recovery, the participants underwent 10 mins of isocapnic hypoxia ($P_{ET}O_2$ = 45mmHg). Ultrasound images were recorded throughout the two-minute baseline, and ten-minute trial, with the final minute of steady state used for analysis. After the breathing trial was completed, the participant ingested either a placebo (five sugar pills) or antioxidant dose of water – soluble vitamin C (500mg), and both fat – soluble vitamin E (400IU), and alpha lipoic acid (300mg). A second antioxidant dose (or placebo) of vitamin C (500mg), vitamin E (200IU), and alpha lipoic acid (300mg) ingested 30 mins later. These antioxidants were chosen based on previous work completed by Wray et al., (2012). Post testing resumed one hour after the second dose of either placebo or antioxidants as described by (Wray et al., 2012). The described protocol (Figure 2.1) was then repeated.

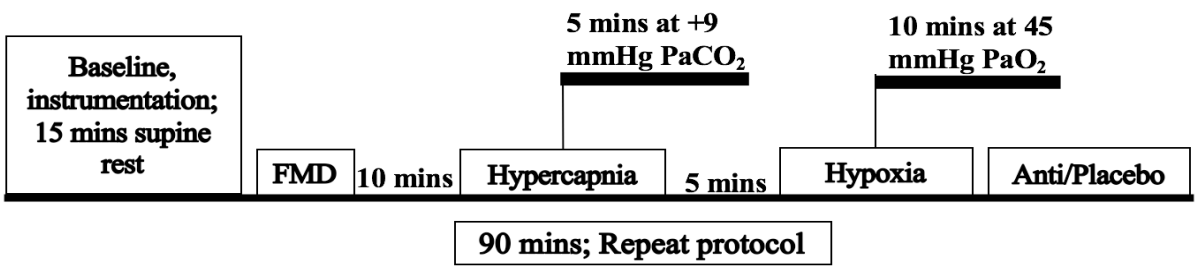

Figure 2.1 Schematic of the experimental protocol for study 1 representing sea-level (Kelowna, 344m) measurements. FMD = time of flow-mediated dilation measurement. Q_{ICA} and Q_{CCA} = time of blood flow through the internal carotid artery and blood flow through the common carotid artery. For hypercapnia end-tidal CO_2 was increased +9 mmHg above baseline resting values. For hypoxia end-tidal O_2 was decreased and aimed at 45mmHg.

2.2.3 Study 2 - High altitude testing at the EVR-K2-CNR Research Pyramid Khumbu Valley, Nepal (5050m):

2.2.3.1 Participants

Nine healthy young individuals (age: 24.2 ± 4.1yrs; body mass index: 23.1 ± 2.3kg/m^2) volunteered for study 2 (all subjects were different from study 1). Out of the nine participants, two were female. Participants were screened to ensure reliable ultrasound measurements of brachial, internal carotid, and vertebral arteries. All participants were free of cardiovascular, respiratory & cerebrovascular diseases, were non-diabetic, and were not taking any prescription drugs (other than oral contraceptives n=1, one female was on an IUD) at the time of participation.

2.2.3.2 Protocol design

This study involved both a placebo trial and a drug trial, on two separate days with 48 hrs in-between each day to account for adequate drug washout, it was made sure there was no cross-over between the other studies taking place. Each trial was completed within the Ev-K2-CNR pyramid laboratory located in Khumbu valley, Nepal (5050m) after 12 days of

high-altitude acclimatization. The intervention was randomized and blinded to the ultrasound scanners, to minimize measurement subjectivity. Participants arrived at the laboratory having abstained from caffeine for 12hrs, and exercise and alcohol for 24hrs, as well as 2hrs fasted. Participants were request to lie supine in a sleeping bag for 15 mins upon arrival.

Cardiorespiratory measures as previously described was taken at the start of both the pre-and post-testing trials (e.g., MAP, HR, $P_{ET}CO_2$). Following the collection of the cardiorespiratory variables, two-minute resting cerebral blood flow measures of both the ICA and VA were taken. Following CBF measures, a FMD test would take place. Following the FMD, participants were asked to ingest two doses of either placebo or antioxidant combination pills (vitamin C, E, alpha-lipoic acid) separated by 30 minutes, and starting 90 minutes before post-trial testing. Antioxidant dosage was the same as previously describe in the "*sea-level*" study design. Post testing resumed one hour after the second dose of either placebo or antioxidants (Wray et al., 2012). The described protocol (Figure 2.2) was then repeated.

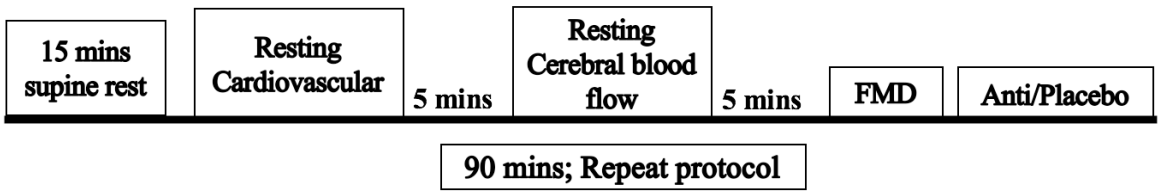

Figure 2.2 Schematic of the experimental protocol for Study 2, during chronic exposure to high-altitude. Performed at the Everest-K2-CNR pyramid laboratory (Khumbu valley, Nepal, 5050m). FMD = time of flow mediated dilation measurement. Q_{ICA} and Q_{VA} = time of measurement of blood flow through the internal carotid artery and vertebral artery. During the resting, cardiovascular measures SPO_2 and end-tidal CO_2 was measured.

2.2.4 Experimental measures

2.2.4.1 Cardiorespiratory measures

All cardiorespiratory variables were sampled continuously throughout the protocol at 1KHz via an analogue-to-digital converter (Powerlab, 16/30; ADInstruments, Colorado Spring, CO). Heart rate (HR) was measured by a 3-electrode electrocardiogram (ADI bioamp ML132), and beat-to-beat blood pressure by finger phtotoplethysmography (Finometer PRO, Finapres Medical Systems, Amsterdam, Netherlands). The Finometer reconstructed brachial waveform was used for the calculation of mean arterial pressure (MAP) after values were back calibrated to the average of three automated brachial blood pressure measurements made over 5-minutes at rest (Tanog+; SunTech, Morrisville, NC). Partial pressure of end-tidal CO_2 ($P_{ET}CO_2$) and O_2 ($P_{ET}O_2$) was sampled at the mouth, and recorded by a calibrated gas analyzer (model ML206, ADInstruments), ventilation (VE) and respiratory flow was measured by a pneumotachograph (model HR 800L, HansRudolph, Shawnee, KS) connected to a bacteriological filter. Arterial oxyhemoglobin saturation (SaO_2), was measured using a pulse-oximeter. All data measurements were displayed on LabChart (version 7), and analyzed offline post measurements. Measurements of brachial vascular function were conducted as described below (see section 2.3.4.2 "*Peripheral Vascular Measures*"). All cerebral blood flow (CBF) measurements were conducted as described below (see section 2.3.4.3 "*Cerebral Vascular Measures*"). Average values of final minute of each stage were recorded and analyzed (see section 2.3.4 "*Experimental Protocol*"). Analysis and collection of all data were performed blinded to the protocol (i.e., placebo vs antioxidants).

Cardiorespiratory measurements taken at the pyramid include recordings of both systolic and diastolic blood pressure, for which MAP was calculated along with HR (HEM-775CAN, Omron Healthcare, Bannockburn, IL, USA), $P_{ET}CO_2$ (EMMA, Mainstream Capnometer, Danderyd, Sweden), along with respiratory rate, with arterial oxyhemoglobin saturation (SaO_2) using a portable device (Vacu-med, Ventura, California). All measurements were taken after ten minutes of supine rest.

2.2.4.2 Peripheral vascular measures

Following 15 mins of supine rest, blood velocity and diameter was recorded in the peripheral brachial artery, using 10MHz multi-frequency duplex ultrasound (Terason T3200, Teratech, Burlington, MA). Artery diameter was assessed using B-mode imaging while pulse-wave mode was used to simultaneously measure peak blood velocity. Participants remained supine, with their right arm extended at a 90-degree angle from their body, all noise and lighting was reduced during scans. A brachial blood pressure cuff was placed on the arm, three centimeters distal from the elbow, respectively. Brachial artery diameter and blood velocity was measured proximal to the artery bifurcation. One minute diameter and blood velocity baseline was recorded, recording was then paused while the brachial cuff is inflated to 250 mmHg to occlude blood flow through the artery. Following five minutes of cuff inflation, the final 30 secs of inflation is recorded before cuff release, which extended three minutes of recording post release. This technique was conducted under the guidelines put forth by (Thijssen et al., 2011).

All video files are stored for further offline analysis, using edge detection and wall tracking software. Flow mediated dilation percent (FMD%) is calculated via the software using baseline diameter and peak diameter post cuff release. Shear rate, antegrade shear, and retrograde shear are all measured through the software. The software reduces objector error during analysis.

2.2.4.3 Cerebral vascular measures

Blood velocity through the right middle cerebral artery (MCAv) was measured using a 2MHz transcranial Doppler ultrasound (TCD; Spencer Technologies, Seattle, WA). The TCD probes were secured in place using a specialized headband (model M600 bilateral head frame, Spencer standardized techniques (Willie et al., 2011).

Blood velocity and vessel diameter of the internal carotid artery (ICA) and common carotid artery (CCA) were measured using a 10MHz multi-frequency duplex ultrasound (Terason T3200, Teratech, Burlington, MA). Arterial diameter was assessed with B-mode imaging

while pulse-wave mode was used to simultaneously measure peak blood velocity. Measures of ICA (Q_{ICA}) and CCA (Q_{CCA}) blood flow were made ipsilateral to MCA, respectively. Diameter and velocity of ICA was measured at least 1.5 cm distal to the common carotid bifurcation to eliminate measures of turbulent and retrograde flow, while diameter and velocity of the CCA was made 1.5 cm prior to the bifurcation to avoid as previously mentioned, respectively. Diameter and velocity of the VA were measured between C4-C5, C5-C6, or proximal to entry into the vertebral column. These locations were determined on an individual basis, with the same location repeated in each participant to acquire reproducible measures.

Screen capture software is used for ultrasound recordings, and video files are stored for offline analysis. Measures of diameter and peak blood velocity are acquired at 30Hz using customized edge detection and wall tracking software. At least twelve consecutive cardiac cycles were used to determine Q_{ICA}, Q_{CCA}, and Q_{VA}. Volumetric blood flow was calculated using the following formula:

$$Q_{ICA},\ Q_{CCA},\ \text{or}\ Q_{VA} = \frac{\text{Peak Envelop Velocity}}{2} * [\pi(0.5*\text{Diameter})^2]$$

Cerebrovascular conductance (CVC) was determined to account for MAP, during analysis of CBF response. This was calculated for both ICA, CCA and VA (e.g., Q_{ICA}/MAP). Videos of both ICA, CCA and VA were completed and analyzed while blinded to the protocol (placebo vs antioxidant).

Global cerebral blood flow was calculated using the following formula:

$$gCBF = (Q_{ICA} + Q_{VA}) \cdot 2$$

2.2.4.4 End-tidal forcing

Both $P_{ET}CO_2$ and $P_{ET}O_2$ were controlled using dynamic end-tidal forcing system. The system uses independent gas solenoid valves for O_2, CO_2, and N_2 and controls the volume of each

gas delivered into a reservoir through a mixing and humidification chamber. $P_{ET}O_2$, $P_{ET}CO_2$, expiratory and inspiratory tidal volume, frequency of breathing, and minute ventilation were determined for each participant on a breath by breath basis in real time using a custom designed software (Labview 13.0, National Instruments, Austin, TX, USA). Feedback information allows for adjustments of inspirate during real time, regarding $P_{ET}CO_2$, $P_{ET}O_2$, and inspiratory and expiratory tidal volume to control the desired target end-tidal gases. Controls are established using an integral error reduction system. Feed-Forward control is based on basal O_2 metabolism and CO_2 production on each breath by breath expiration. Steady state as described in section 2.3.4.1 "*Cardiorespiratory measures*" is achieved when three consecutive breaths are within ±1mmHg. The operation and use of the dynamic end-tidal forcing system has been further assessed in detail elsewhere (Tymko et al., 2016; Tymko et al, 2015) and proven to be an accurate experimental tool.

2.2.5 Data Analysis

All statistical analysis for both sea-level and high-altitude was performed using IBM SPSS Statistics (Version 24, IBM statistics), and are reported as mean ± SEM. Statistical significance was assumed at $P < 0.05$. For this study, all cardiovascular, cerebrovascular variables, and peripheral vascular variables were analyzed between day and within trial (i.e., placebo & antioxidant; pre & post) using a two-way repeated measures ANOVAs, with a Bonferroni correction. Two-way repeated measures ANOVA was used to compared FMD, CBF and cardiovascular variables placebo vs antioxidants, pre-versus post intervention at sea-level. Post-hoc calculations were made post testing.

2.3 Results

2.3.1 Sea-level experiments

Cardiovascular and cerebrovascular baseline variables for placebo and antioxidant with isooxic hypercapnia (+9mmHg CO_2) and isocapnic hypoxia (45mmHg) are presented in Table 2.1 and 2.2, respectively. There were elevations in MAP, HR, $P_{ET}CO_2$, and V_E with hypercapnia compared to baseline ($P < 0.05$). Similarly, MAP, HR, and V_E increased during isocapnic hypoxia ($P < 0.05$ vs baseline). There were no significant differences in these

responses between placebo and antioxidant. As such, although QICA, ICAv, ICA diameter, ICA CVC, QCCA, CCAv, CCA diameter, MCAv, and CCA CVC all increased with hypercapnia ($P < 0.05$), there were no between-trial differences. Likewise, although QICA, ICAv, ICA diameter, ICA CVC, QCCA, CCA diameter, and CCA CVC increased with hypoxia ($P < 0.05$), there was no influence of antioxidants. Therefore, both cerebrovascular reactivity to hypercapnia and hypoxia were not significantly different between placebo and antioxidant conditions (see Table 2.3 and Figures 2.1 and 2.2).

Table 2.1 Cerebral vascular, hemodynamics, and respiratory variables at baseline and during hypercapnia following placebo or antioxidants

		Placebo		Antioxidant	
		Baseline	**CO_2 (+9mmHg)**	**Baseline**	**CO_2 (+9mmHg)**
$P_{ET}O_2$	**Pre**	91.1 ± 13.0	92.5 ± 5.1	92.4 ± 4.9	91.4 ± 3.9
(mmHg)	**Post**	94.7 ± 4.8	93.2 ± 4.9	89.1 ± 11.9	91.8 ± 4.4
P-value		Pre v Post P=0.29; CO_2 P=0.92; interaction P=0.51		Pre vs Post P=0.33; CO_2 P=0.76; interaction P=0.16	
$P_{ET}CO_2$	**Pre**	42.4 ± 2.3	52.3 ± 2.4*	43.4 ± 2.1	52.8 ± 1.8*
(mmHg)	**Post**	42.3 ± 2.0	51.8 ± 1.8*	42.9 ± 2.2	52.7 ± 2.2*
P-value		Pre vs Post P=0.89; CO_2 P<0.05; interaction P=0.05		Pre v Post P=0.89; CO_2 P<0.05; interaction P=0.579	
VE	**Pre**	13.1 ± 3.5	40.0 ± 15.1*	13.8 ± 4.8	38.2 ± 13.0*
($L \cdot min^{-1}$)	**Post**	14.5 ± 5.3	37.6 ± 16.1*	14.1 ± 4.5	39.9 ± 13.2*
P-value		Pre vs Post P=0.84; CO_2 P<0.05; interaction P=0.40		Pre vs Post P=0.36; CO_2 P<0.05; interaction P=0.44	
MAP	**Pre**	89.2 ± 11.8	97.8 ± 14.0*	91.5 ± 8.1	96.6 ± 6.3*
(mmHg)	**Post**	88.6 ± 9.8	96.7 ± 9.4*	91.7 ± 13.4	97.4 ± 8.7*
P-value		Pre vs Post P=0.05; CO_2 P<0.05; interaction P=0.05		Pre vs Post P=0.64; CO_2 P<0.05; interaction P=0.86	
HR	**Pre**	63.7 ± 10.9	74.0 ± 13.2	59.9 ± 9.4	68.0 ± 9.0*
($beats \cdot min^{-1}$)	**Post**	59.5 ± 10.4	68.6 ± 16.0	61.2 ± 11.9	68.1 ± 12.7*
P-value		Pre vs Post P=0.09; CO_2 P=0.09; interaction P=0.79		Pre vs Post P=0.70; CO_2 P<0.05; interaction P=0.63	
S_PO2	**Pre**	96.4 ± 2.8	96.3 ± 2.8	97.8 ± 0.7	97.8 ± 0.7
(%)	**Post**	97.7 ± 1.2	97.3 ± 0.9	96.4 ± 3.7	96.9 ± 0.9
P-value		Pre vs Post P=0.05; CO_2 P=0.98; interaction P=0.85		Pre vs Post P=0.19; CO_2 P=0.67; interaction P=0.48	
Q_{ICA}	**Pre**	266.8 ± 61.2	415.3 ± 86.3*	274.5 ± 57.2	416.8 ± 88.7*
($mL \cdot min^{-1}$)	**Post**	257.6 ± 71.1	418.4 ± 93.1*	269.1 ± 65.4	413.9 ± 87.8*
P-value		Pre vs Post P=0.17; CO_2 P<0.05; interaction P=0.32		Pre vs Post P=0.37; CO_2 P<0.05; interaction P=0.22	

		Placebo		Antioxidant	
		Baseline	**CO_2 (+9mmHg)**	**Baseline**	**CO_2 (+9mmHg)**
ICAv	**Pre**	45.4 ± 14.8	62.6 ± 10.7*	42.8 ± 8.2	60.0 ± 11.8*
(cm · s^{-1})	**Post**	40.7 ± 9.5	60.0 ± 13.7*	42.2 ± 9.6	60.0 ± 10.7*
P-value		Pre vs Post P=0.07; CO_2 P<0.05; interaction P=0.64		Pre vs Post P=0.88; CO_2 P<0.05; interaction P=0.81	
ICA diameter	**Pre**	5.3 ± 0.53	5.3 ± 0.56*	5.1 ± 0.44	5.3 ± 0.53*
(mm)	**Post**	5.2 ± 0.50	5.4 ± 0.49*	5.0 ± 0.43	5.4 ± 0.51*
P-value		Pre vs Post P=0.99; CO_2 P<0.05; interaction P=0.08		Pre vs Post P=0.50; CO_2 P<0.05; interaction P=0.13	
ICA CVC	**Pre**	3.5 ± 1.6	4.3 ± 0.9*	2.9 ± 0.8	4.1 ± 1.1*
(mL · min^{-1} · $mmHg^{-1}$)	**Post**	3.0 ± 0.9	4.3 ± 1.2*	2.9 ± 1.1	4.3 ± 1.2*
P-value		Pre vs Post P=0.21; CO_2 P<0.05; interaction P=0.19		Pre vs Post P=0.24; CO_2 P<0.05; interaction P=0.47	
Q_{CCA}	**Pre**	394.9 ± 113.5	611.5 ± 103.0*	407.8 ± 109.0	566.5 ± 162.1*
(mL · min^{-1})	**Post**	406.7 ± 60.3	587.5 ± 123.9*	391.5 ± 122.6	577 ± 152.9*
P-value		Pre vs Post P=0.72; CO_2 P<0.05; interaction P=0.35		Pre vs Post P=0.90; CO_2 P<0.05; interaction P=0.16	
CCAv	**Pre**	37.6 ± 5.5	53.9 ± 7.7*	38.2 ± 8.9	50.1 ± 11.3*
(cm · s^{-1})	**Post**	37.8 ± 5.7	52.5 ± 9.8*	36.9 ± 11.2	51.2 ± 11.7*
P-value		Pre vs Post P=0.83; CO_2 P=0.13; interaction P=0.46		Pre vs Post P=0.09; CO_2 P<0.05; interaction P<0.05	
CCA diameter	**Pre**	6.6 ± 0.9	6.8 ± 0.5*	6.6 ± 0.3	6.8 ± 0.4*
(mm)	**Post**	6.7 ± 0.3	6.8 ± 0.5*	6.6 ± 0.3	6.9 ± 0.5*
P-value		Pre vs Post P=0.60; CO_2 P<0.05; interaction P=0.46		Pre vs Post P=0.61; CO_2 P<0.05; interaction P=0.43	
CCA CVC	**Pre**	4.5 ± 1.4	6.3 ± 1.3*	4.5 ± 1.4	5.9 ± 1.9*
(mL · min^{-1} · $mmHg^{-1}$)	**Post**	4.7 ± 0.9	6.1 ± 1.5*	4.4 ± 1.5	6.1 ± 2.1*
P-value		Pre vs Post P=0.96; CO_2 P<0.05; interaction P=0.37		Pre vs Post P=0.93; CO_2 P<0.05; interaction P=0.23	
MCAv	**Pre**	62.8 ± 10.2	86.8 ± 19.0*	60.9 ± 7.7	84.7 ± 14.3*
(cm · s^{-1})	**Post**	61.5 ± 9.9	84.0 ± 14.3*	59.8 ± 8.6	83.3 ± 14.1*
P-value		Pre vs Post P=0.29; CO_2 P<0.05; interaction P=0.67		Pre vs Post P=0.35; CO_2 P<0.05; interaction P=0.82	

For both between-trial placebo and antioxidant. (explained in section 2.3 "*Methods*"). Significance for CO_2 is indicated by *, significant difference for pre vs post is indicated by ∞.

Table 2.2 Cerebral vascular, hemodynamic, and respiratory variables at baseline and during hypoxia following placebo or antioxidants

		Placebo		Antioxidant	
		Baseline	**Hypoxia (45 mmHg)**	**Baseline**	**Hypoxia (45 mmHg)**
$P_{ET}O_2$	**Pre**	93.5 ± 5.5	50.1 ± 4.3*	93.3 ± 6.5	50.6 ± 4.0*
(mmHg)	**Post**	94.9 ± 4.0	50.4 ± 4.2*	93.7 ± 5.1	50.4 ± 4.1*
P-value		Pre vs Post P=0.22; O_2 P<0.05; interaction P=0.42		Pre vs Post P=0.36; O_2 P<0.05; interaction P=0.30	
$P_{ET}CO_2$	**Pre**	43.0 ± 3.0	43.0 ± 2.8	43.8 ± 3.6	43.2 ± 2.5
(mmHg)	**Post**	42.4 ± 2.0	42.7 ± 2.0	42.9 ± 2.5	43.3 ± 2.3
P-value		Pre vs Post P=0.19; O_2 P=0.44; interaction P=0.40		Pre vs Post P=0.48; O_2 P=0.70; interaction P=0.33	
VE	**Pre**	13.7 ± 3.6	28.1 ± 7.4*	12.6 ± 3.8	24.4 ± 10.4*
($L \cdot min^{-1}$)	**Post**	13.6 ± 3.8	26.9 ± 8.7*	15.7 ± 3.3∞	28.8 ± 11.4*∞
P-value		Pre vs Post P=0.51; O_2 P<0.05; interaction P=0.54		Pre vs Post P<0.05; O_2 P<0.05; interaction P=0.55	
MAP	**Pre**	93.8 ± 11.4	98.1 ± 13.4*	91.8 ± 9.6	95.6 ± 10.4*
(mmHg)	**Post**	93.8 ± 10.7	99.3 ± 13.7*	92.3 ± 11.0	97.3 ± 9.1*
P-value		Pre vs Post P=0.86; O_2 P<0.05; interaction P=0.51		Pre vs Post P=0.83; O_2 P<0.05; interaction P<0.05	
HR	**Pre**	60.2 ± 10.5	73.2 ± 16.8*	59.5 ± 8.8	72.2 ± 14.7*
($beats \cdot min^{-1}$)	**Post**	60.9 ± 11.7	70.6 ± 17.6*	60.0 ± 8.9	75.1 ±14.2*
P-value		Pre vs Post P=0.60; O_2 P<0.05; interaction P=0.13		Pre vs Post P=0.16; O_2 P<0.05; interaction P=0.33	
S_PO_2	**Pre**	97.5 ± 1.2	82.7 ± 4.9*	97.7 ± 0.9	82.7 ± 4.8*
(%)	**Post**	97.8 ± 0.9	82.4 ± 4.8*	97.9 ± 1.1	82.5 ± 4.9*
P-value		Pre vs Post P=0.94; O_2 P<0.05; interaction P=0.72		Pre vs Post P=0.86; O_2 P<0.05; interaction P=0.19	
Q_{ICA}	**Pre**	224.8 ± 81.4	281.8 ± 96.1*	244.9 ± 77.5	298.1 ± 83.8*
($mL \cdot min^{-1}$)	**Post**	247.5 ± 78.8	310.2 ± 103.0*	249.4 ± 78.6∞	294.7 ± 84.0 *∞
P-value		Pre vs Post P=0.05; O_2 P<0.05; interaction P=0.34		Pre vs Post P<0.05; O_2 P<0.05; interaction P=0.91	
ICAv	**Pre**	33.6 ± 9.7	40.8 ± 13.4	34.7 ± 12.0	43.1 ± 17.7*
($cm \cdot s^{-1}$)	**Post**	39.4 ± 13.7	43.8 ± 14.3	37.3 ± 10.7	45.4 ± 15.2*
P-value		Pre vs Post P=0.06; O_2 P=0.05; interaction P=0.26		Pre vs Post P=0.06; O_2 P<0.05; interaction P=0.82	
ICA diameter	**Pre**	5.2 ± 0.6	5.4 ± 0.6*	5.2 ± 0.4	5.4 ± 0.5*
(mm)	**Post**	5.2 ± 0.5	5.4 ± 0.5*	5.1 ± 0.5	5.3 ± 0.5*
P-value		Pre vs Post P=0.75; O_2 P<0.05; interaction P=0.96		Pre vs Post P=0.17; O_2 P<0.05; interaction P=0.84	
ICA CVC	**Pre**	2.4 ± 0.9	2.9 ± 0.9	2.5 ± 1.1	3.2 ± 1.4*
($mL \cdot min^{-1} \cdot mmHg^{-1}$)	**Post**	2.7 ± 1.0	3.0 ± 0.9	2.6 ± 1.2	3.2 ± 1.2*
P-value		Pre vs Post P=0.33; O_2 P=0.08; interaction P=0.06		Pre vs Post P=0.62; O_2 P<0.05; interaction P=0.35	

		Placebo		Antioxidant	
		Baseline	**Hypoxia (45 mmHg)**	**Baseline**	**Hypoxia (45 mmHg)**
Q_{CCA}	**Pre**	421.8 ± 226.4	572.7 ± 227.0*	375.0 ± 89.0	474.1 ± 150.5*
(mL · min^{-1})	**Post**	390.7 ± 105.4	508.3 ± 157.5*	399.8 ± 121.1	472.0 ± 146.4*
P-value		Pre vs Post P=0.52; O_2 P<0.05; interaction P=0.21		Pre vs Post P=0.69; O_2 P<0.05; interaction P=0.28	
CCAv	**Pre**	36.7 ± 7.9	43.4 ± 11.8*	36.3 ± 6.7	40.9 ± 10.4*
(cm · s^{-1})	**Post**	35.8 ± 9.4	42.2 ± 11.2*	38.2 ± 10.8	42.2 ± 11.2*
P-value		Pre vs Post P=0.45; O_2 P<0.05; interaction P=0.84		Pre vs Post P=0.50; O_2 P<0.05; interaction P=0.77	
CCA diameter	**Pre**	6.7 ± 1.7	7.3 ± 1.2*	6.6 ± 0.5	6.9 ± 0.5*
(mm)	**Post**	6.7 ± 0.4	7.0 ± 0.5*	6.6 ± 0.3	6.9 ± 0.4*
P-value		Pre vs Post P=0.61; O_2 P<0.05; interaction P=0.36		Pre vs Post P=0.68; O_2 P<0.05; interaction P=0.30	
CCA CVC	**Pre**	4.6 ± 2.7	5.9 ± 2.6*	4.1 ± 1.0	5.0 ± 1.7*
(mL · min^{-1} · mmHg^{-1})	**Post**	4.2 ± 1.3	5.2 ± 1.7*	4.3 ± 1.2	4.8 ± 1.4*
P-value		Pre vs Post P=0.41; O_2 P<0.05; interaction P=0.33		Pre vs Post P=0.97; O_2 P<0.05; interaction =0.23	
MCAv	**Pre**	60.2 ± 9.7	67.8 ± 12.7*	60.9 ± 6.7	66.8 ± 10.2*
(cm · s^{-1})	**Post**	58.3 ± 9.2	65.0 ± 14.1*	57.9 ± 7.1	69.0 ± 9.8*
P-value		Pre vs Post P=0.23; O_2 P<0.05; interaction P=0.25		Pre vs Post P=0.83; O_2 P<0.05; interaction P<0.05	

For both between-trial placebo and antioxidant. (explained in section 2.3 "*Methods*"). Significant difference with O_2 is indicated by *. Significant difference between pre versus post is indicated by ∞.

Table 2.3 Hypercapnic and hypoxic reactivity for ICA and CCA following placebo or antioxidants

		Placebo	Antioxidant	P-value
ICA relative reactivity ($\Delta CBF/\Delta CO_2$) (mL/100g/min/mmHg)	**Pre**	5.8 ± 2.0	5.7 ± 2.0	Pre vs Post P=0.34; interaction P=0.33
	Post	6.8 ± 2.1	5.8 ± 1.9	
ICA absolute reactivity ($\%\Delta CBF/\Delta CO_2$)	**Pre**	15.1 ± 4.5	15.1 ± 5.3	Pre vs Post P=0.52; interaction P=0.45
	Post	16.9 ± 4.6	15.0 ± 4.3	
ICA hypoxic reactivity ($\%\Delta/-\%SaO_2$)	**Pre**	1.8 ± 0.6	1.5 ± 0.7	Pre vs Post P=0.12; interaction P=0.96
	Post	1.6 ± 0.5	1.2 ± 0.8	
CCA relative reactivity ($\Delta CBF/\Delta CO_2$) (mL/100g/min/mmHg)	**Pre**	7.6 ± 8.1	6.2 ± 4.3	Pre vs Post P=0.61; interaction P=0.36
	Post	5.3 ± 2.4	6.9 ± 5.0	
CCA absolute reactivity ($\%\Delta CBF/\Delta CO_2$)	**Pre**	22.7 ± 8.0	23.2 ± 15.5	Pre vs Post P=0.71; interaction P=0.97
	Post	21.6 ± 10.7	21.8 ± 6.6	
CCA hypoxic reactivity ($\%\Delta/-\%SaO_2$)	**Pre**	8.2 ± 4.0	6.1 ± 5.2	Pre vs Post P=0.17; interaction P=0.70
	Post	7.3 ± 4.1	3.7 ± 5.9	

Variables are presented using calculations described with section 2.3 "*Methods*"

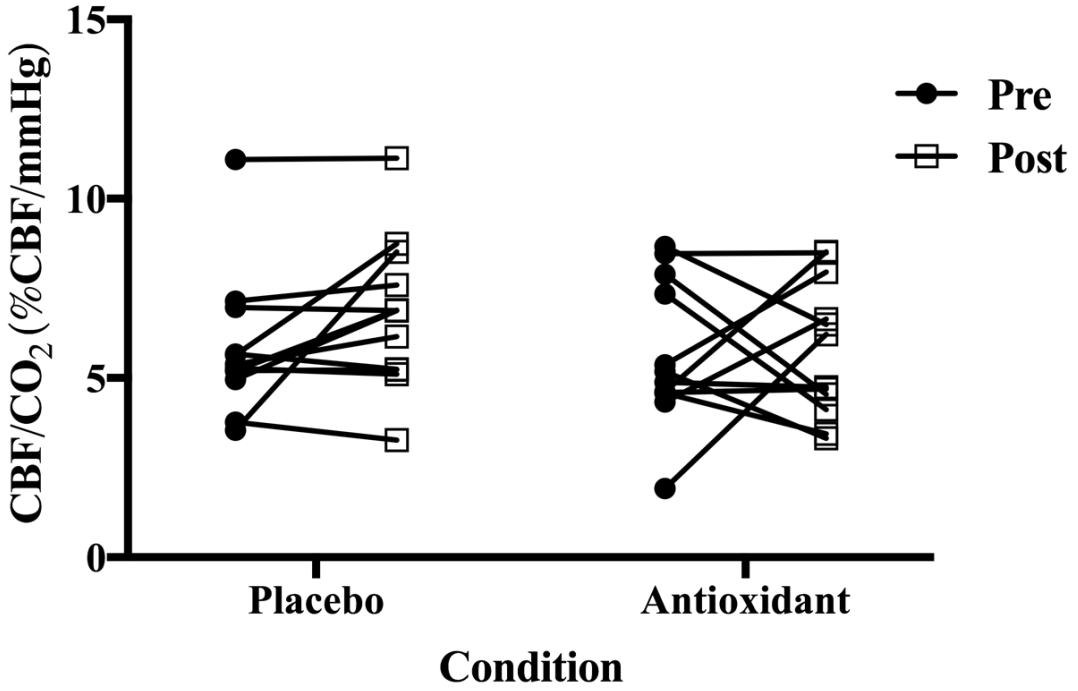

Figure 2.3 Relative CBF reactivity to CO2 in the ICA in all experimental trials at sea-level. There was no significant difference in all experimental trials at sea-level.

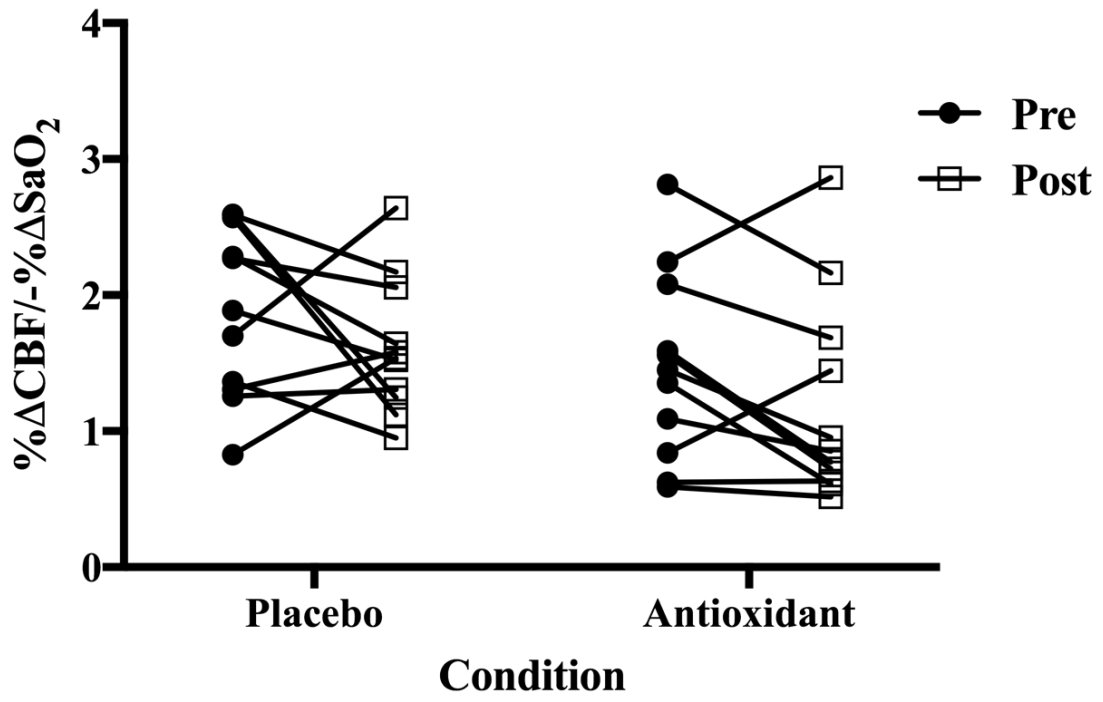

Figure 2.4 Relative CBF reactivity to hypoxia in the ICA at all experimental trials at sea-level. There was no significant difference in all experimental trials at sea-level.

Peripheral vascular variables for placebo and antioxidants are presented in Table 2.4 and Figure 2.5, respectively. There were no between-trial differences in the percent change in FMD with placebo and antioxidants (5.5 ± 2.1 vs. 6.2 ± 1.5; 5.5 ± 2.5 vs. 6.4 ± 2.5 %, respectively; P = 0.806), when considering SRAUC and baseline diameter, FMD still showed no significant change (P=0.368). Likewise, there were no significant changes in both antegrade SR and retrograde SR following the antioxidant trial, compared with the placebo trial. For example, antegrade SR variables did not change following the antioxidant intervention when compared to placebo (450.9 ± 65.2 vs 548.8 ± 154.9; 474.4 ± 212.6 vs 538.8 ± 117.6 s^{-1}; P = 0.673). Likewise, retrograde SR following the antioxidant intervention when compared to placebo was not significant (-0.33 ± 0.96 vs -14.2 ± 44.8; -1.3 ± 2.8 vs -0.25 ± 0.3 s^{-1}; P = 0.291). Nonetheless, antegrade SR and retrograde SR variables are presented in Figure 2-6. There were no other between-trial differences in peripheral vascular variables.

Table 2.4 Peripheral vascular variables before and following placebo and antioxidants at sea-level

		Placebo	Antioxidant	P-value
FMD	**Pre**	5.5 ± 2.1	5.5 ± 2.5	Pre vs Post P=0.15; interaction P=0.81
(%)	**Post**	6.2 ± 1.5	6.4 ± 2.5	
Baseline Diameter	**Pre**	0.41 ± 0.04	0.41 ± 0.1	Pre vs Post P=0.60; Interaction P=0.12
(mm)	**Post**	0.42 ± 0.1	0.41 ± 0.1	
Peak Diameter	**Pre**	0.44 ± 0.04	0.43 ± 0.1	Pre vs Post P=0.25; interaction P=0.10
(mm)	**Post**	0.45 ± 0.1	0.43 ± 0.1	
SR_{AUC}	**Pre**	25148.6 ± 7707.3	22771.1 ± 6148.3	Pre vs Post P=0.47; interaction P=0.27
(AUC)	**Post**	24381.1 ± 6273.8	26102.3 ± 7908.6	
Time to Peak	**Pre**	56.2 ± 5.7	51.9 ± 10.5	Pre vs Post P=0.09; interaction P=0.38
(s)	**Post**	47.6 ± 12.9	48.5 ± 10.4	
Total SR	**Pre**	450.6 ± 147.5	473.1 ± 212.6	Pre vs Post P=0.15; interaction P=0.81
(s^{-1})	**Post**	534.6 ± 136.7	538.51 ± 117.6	
Antegrade SR	**Pre**	450.9 ± 147.5	474.4 ± 212.6	Pre vs Post P=0.15; interaction P=0.67
(s^{-1})	**Post**	548.8 ± 154.9	538.8 ± 117.6	
Retrograde SR	**Pre**	-0.33 ± 0.96	-1.3 ± 2.8	Pre vs Post P=0.37; interaction P=0.29
(s^{-1})	**Post**	-0.22 ± 0.24	-0.25 ± 0.3	
OSI	**Pre**	0.004 ± 0.01	0.003 ± 0.01	Pre vs Post P=0.59; interaction P=0.34
	Post	0.0003 ± 0.0004	0.0004 ± 0.001	
Response flow (reactive hyperemia)	**Pre**	237.7 ± 77.3	204.5 ± 63.3	Pre vs Post P=0.60; interaction P=0.30
($mL \cdot min^{-1}$)	**Post**	215.8 ± 86.6	210.4 ± 82.0	

For both between-trial placebo and antioxidant. (explained in methods section 2.3.4.2 "*Peripheral vascular function*").

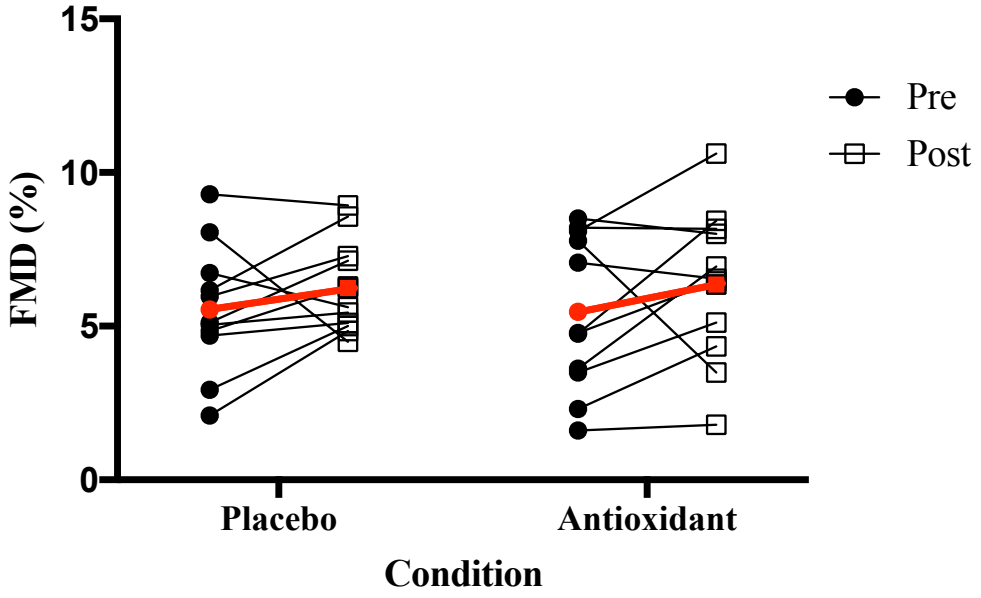

Figure 2.5 FMD percent change in all experimental trials at sea-level. The FMD percent change for between-trial for placebo and antioxidant is presented with individual data. There was no significant difference in all experimental trials at sea-level. Data mean is represented in red.

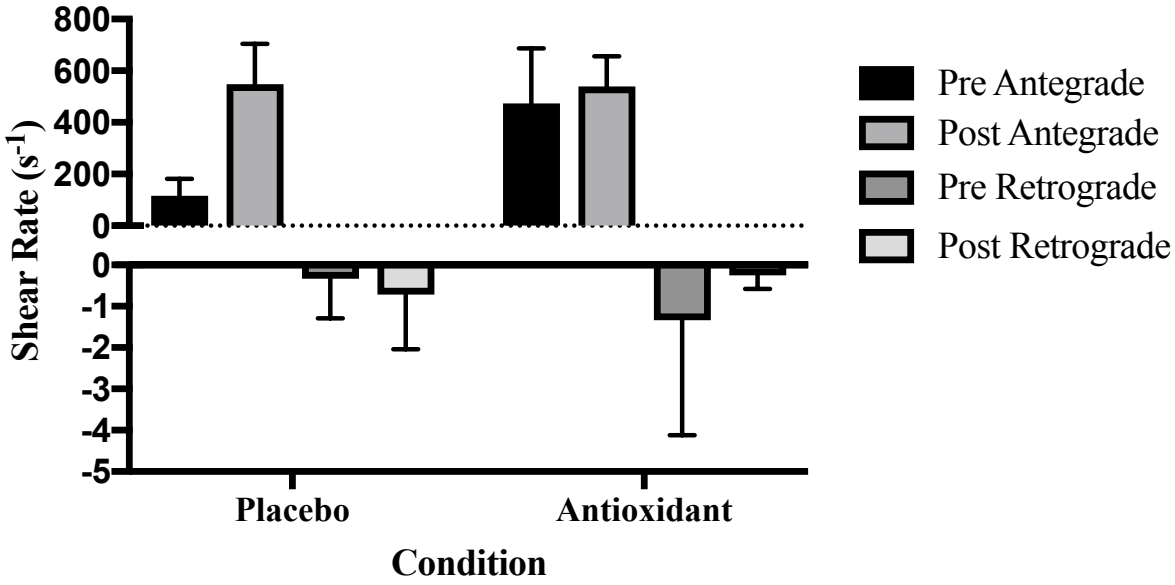

Figure 2.6 Peripheral vascular antegrade SR and retrograde SR in all experimental trials at sea-level. Peripheral vascular antegrade SR and retrograde SR established through FMD. There was no significant difference in all experimental trials at sea-level.

2.3.2 High altitude experiments

Cardiovascular (e.g., HR, MAP, and $P_{ET}CO_2$) and cerebral vascular variables (e.g., ICA, VA flow, resting global cerebral blood flow (gCBF), and related conductance) are presented in Table 2.5. There were no significant differences in cardiovascular variables between-trial with placebo and antioxidants. Figure 2.7 presents resting gCBF at high-altitude, there was no significant differences between-trial with placebo and antioxidants.

Table 2.5 Cerebral vascular, hemodynamics, and respiratory variables upon chronic exposure to high altitude following placebo or antioxidants

		Placebo	Antioxidant	P-value
$P_{ET}CO_2$	**Pre**	29.7 ± 3.1	29.2 ± 2.6	Pre-vs Post P=0.58; interaction P=0.41
(mmHg)	**Post**	29.2 ± 3.0	29.3 ± 2.3	
MAP	**Pre**	103.5 ± 10.8	103.2 ± 13.1	Pre-vs Post P=0.77; interaction P=0.29
(mmHg)	**Post**	102.0 ± 10.3	105.8 ± 10.3	
S_PO_2	**Pre**	84.5 ± 3.1	83.7 ± 1.8	Pre-vs Post P=1.00; interaction P=0.11
(%)	**Post**	83.6 ± 3.3	84.6 ± 3.0	
QICA	**Pre**	364.1 ± 64.6	393.3 ± 93.4	Pre-vs Post P=0.74; interaction P=0.22
(mL · min^{-1})	**Post**	377.4 ± 73.7	361.9 ± 84.7	
ICAv	**Pre**	39.6 ± 7.1	39.7 ± 7.8	Pre-vs Post P=0.14; interaction P=0.83
(cm · s^{-1})	**Post**	37.5 ± 8.1	38.0 ± 8.1	
ICA diameter	**Pre**	4.9 ± 0.6	5.0 ± 0.5	Pre-vs Post P=0.63; interaction P=0.18
(mm)	**Post**	5.1 ± 0.4	4.9 ± 0.6	
ICA CVC	**Pre**	2.1 ± 0.3	2.3 ± 0.6	Pre vs post P=0.76; interaction P=0.07
(mL · min^{-1} · mmHg^{-1})	**Post**	2.2 ± 0.5	2.1 ± 0.5	
QVA	**Pre**	228.6 ± 199.1	181.0 ± 155.1	Pre-vs Post P = 0.562; Interaction P = 0.264
(mL · min^{-1})	**Post**	210.7 ± 181.9	253.9 ± 149.0	
VAv	**Pre**	19.3 ± 4.0	18.1 ± 3.9	Pre-vs Post P=0.42; interaction P=0.20
(cm · s^{-1})	**Post**	19.1 ± 3.3	20.7 ± 4.9	
VA diameter	**Pre**	6.7 ± 2.7	6.0 ± 2.5	Pre-vs Post P=0.54; interaction P=0.11
(mm)	**Post**	6.3 ± 2.5	6.9 ± 2.2	

		Placebo	Antioxidant	P-value
VA CVC	**Pre**	2.4 ± 2.4	1.9 ± 1.8	Pre vs post P=0.78; interaction P=0.32
(mL · min^{-1} · mmHg^{-1})	**Post**	2.2 ± 2.0	2.4 ± 1.4	
gCBF	**Pre**	735.9 ± 126.0	835.9 ± 332.7	Pre-vs Post P=0.64; interaction P=0.42
(mL · min^{-1})	**Post**	761.8 ± 147.9	944.1 ± 323.1	
eCDO$_2$	**Pre**	132.3 ± 21.4	149.0 ± 59.2	Pre-vs Post P=0.39; interaction P=0.42
(mL · min^{-1})	**Post**	135.3 ± 22.9	170.9 ± 60.3	

For both between-trial placebo and antioxidant. (explained in methods section 2.3.4 "*Experimental measures*").

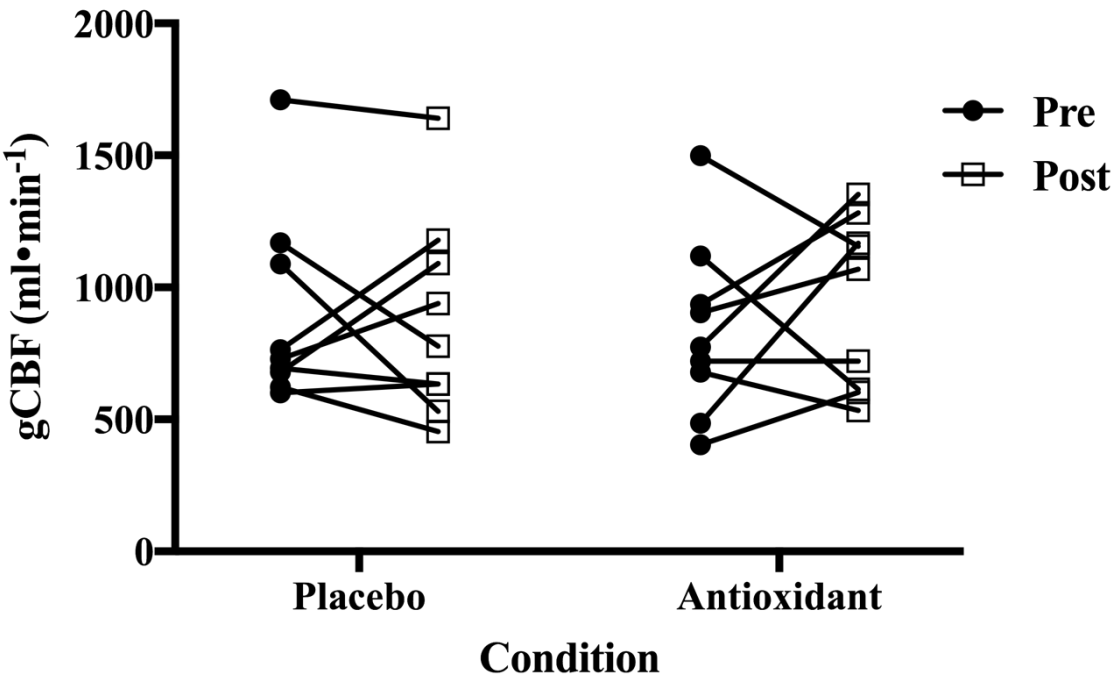

Figure 2.7 Global cerebral blood flow (gCBF) in all experimental trials after chronic exposure to high-altitude. The combination of both resting ICA and VA blood flow after chronic exposure to high-altitude. There was no significant difference in all experimental trials at high-altitude.

Changes in vascular function upon initial ascent to altitude are presented in Table 2.6. Variables for total SR are presented in Figure 2.8. There was no significant difference in total SR in all experimental trials (68.7 ± 39.7 vs 70.2 ± 45.9; 64.7 ± 37.0 vs 63.9 ± 31.3 s^{-1}; P = 0.931 Figure 2-8). Likewise, all other FMD variables showed no significant differences

between trials with placebo and antioxidants (see Figure 2-9 for selected variables). Compared to sea-level FMD results, SRAUC and baseline diameter was taken into consideration for change in FMD. When taking SRAUC and baseline diameter into account, there was still no significant change in FMD (P=0.112), showing no effect of antioxidants on peripheral vascular function at high-altitude.

Table 2.6 Peripheral vascular variables between-trial placebo and antioxidant at high-altitude

		Placebo	Antioxidant	P-value
FMD	**Pre**	7.8 ± 4.4	6.41 ± 4.0	Pre vs Post P= 0.89; interaction P=0.08
(%)	**Post**	6.5 ± 3.9	8.0 ± 2.7	
Baseline Diameter	**Pre**	0.38 ± 0.1	0.38 ± 0.1	Pre vs Post P=0.99; interaction P=0.43
(mm)	**Post**	0.38 ± 0.1	0.37 ± 0.1	
Peak Diameter	**Pre**	0.40 ± 0.1	0.40 ± 0.1	Pre vs post P<0.05; interaction P=0.069
(mm)	**Post**	0.41 ± 0.0	0.40 ± 0.1	
SR_{AUC}	**Pre**	4075.9 ± 2412.0	3807.6 ± 2175.6	Pre vs Post P=0.98; interaction P=0.97
(AUC)	**Post**	4091.5 ± 2747.0	3813.7 ± 1841.4	
Time to Peak	**Pre**	56.4 ± 9.4	58.3 ± 4.8	Pre vs Post P=0.81; interaction P=0.07
(s)	**Post**	55.5 ± 10.5	59.7 ± 0.7	
Total SR	**Pre**	68.7 ± 39.7	64.7 ± 37.0	Pre vs Post P=0.93; interaction P=0.36
(s^{-1})	**Post**	70.2 ± 45.9∞	63.9 ± 31.1*∞	
Antegrade SR	**Pre**	95.9 ± 27.4	93. 0 ± 29.8	Pre vs Post P=0.55; interaction P=0.67
(s^{-1})	**Post**	88.9 ± 38.7	89.2 ± 22.6	
Retrograde SR	**Pre**	-26.7 ± 17.9	-22.8 ± 14.2	Pre vs Post P=0.20; interaction P=0.09
(s^{-1})	**Post**	-18.0 ± 16.2	-25.3 ± 16.8	
OSI	**Pre**	0.22 ± 0.1	0.19 ± 0.1	Pre vs Post P=0.53; interaction P<0.05
	Post	0.17 ± 0.1	0.22 ± 0.1	

For both the placebo and antioxidant trial, FMD percent dilation, baseline diameter, peak diameter, SRAUC, time to peak, mean SR, antegrade SR, retrograde SR, and OSI are presented. (explained in methods section 2.3.4.2 "*Peripheral vascular function*"). Significant difference between pre versus post is indicated by ∞. Significant difference between post-condition is indicated by *.

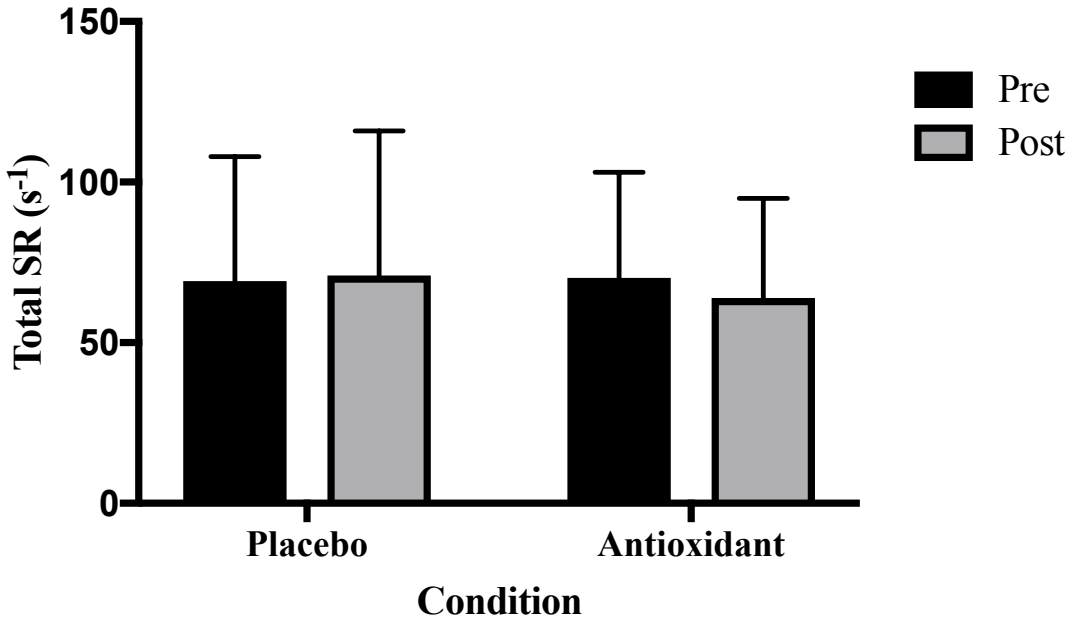

Figure 2.8 Peripheral vascular total SR in all experimental trials after chronic exposure to high altitude. There was no significant difference in total SR in all experimental trials at high altitude.

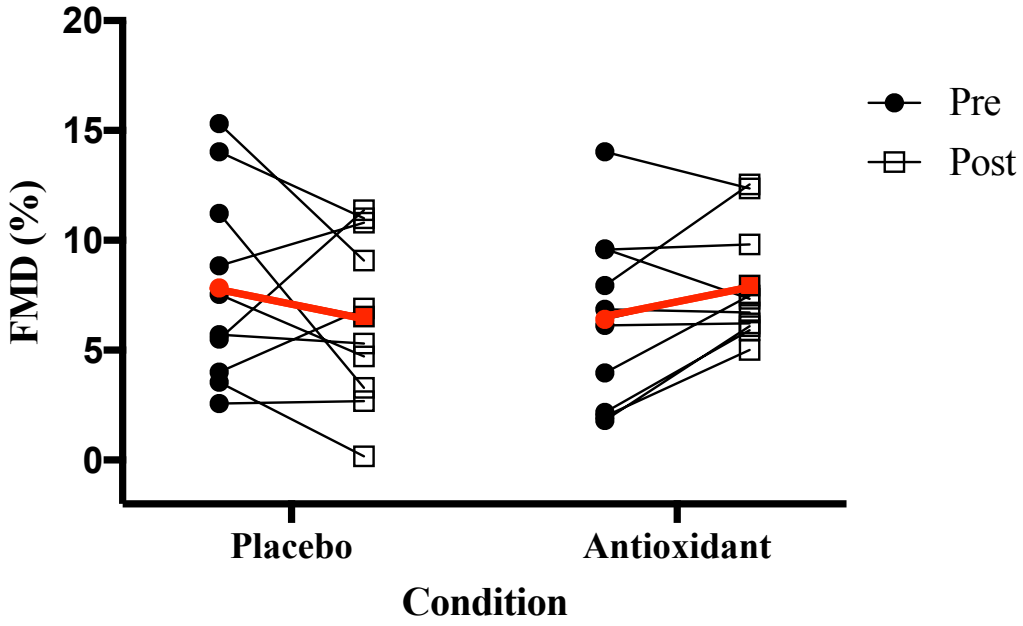

Figure 2.9 FMD percent change in all experimental trials after chronic exposure to high altitude. The FMD percent change for between-trial for placebo and antioxidant is presented with individual data. There was no significant difference in all experimental trials at high attitude. Data mean is represented in red.

2.3.3 Measurement Variability

Tables 2.7 presents both the between-day and within-day coefficient of variation at sea-level for all cerebrovascular and peripheral vascular variables. Likewise, Table 2.8 presents both the between-day and within-day coefficient of variation at high-altitude for all cerebrovascular and peripheral vascular variables.

Table 2.7 Sea-level between- and within-day coefficient of variability measures

Measurement	Between Day CoV	Within Day control CoV	Within day antioxidant CoV
FMD %	26.1	15.3	17.6
FMD baseline diameter	3.3	4.8	3.5
ICA flow	6.3	12.1	11.9
ICA baseline diameter	4.9	3.4	3.5
ICA CO_2 reactivity	15.3	14.0	6.9
ICA hypoxic reactivity	12.0	9.3	4.8
CCA flow	14.5	15.4	14.0
CCA baseline diameter	9.2	8.5	1.9
CCA CO_2 reactivity	11.9	10.1	8.9
CCA hypoxic reactivity	10.0	16.5	12.4
ICA CVC	12.5	11.3	14.9
CCA CVC	12.4	15.3	12.5

Table 2.8 High-altitude between- and within-day coefficient of variability

Measure	Between-day CoV	Within-day control CoV	Within-day antioxidant CoV
FMD %	28.4	27.7	25.3
FMD baseline diameter	7.9	5.3	5.7
ICA flow	19.3	13.0	16.0
ICA diameter	6.6	8.4	4.3
ICA CVC	20.7	15.5	17.6
VA flow	22.6	14.8	12.5
VA diameter	24.3	12.8	15.5
VA CVC	18.9	27.8	25.5
gCBF	20.1	26.5	28.7

2.4 Discussion

2.4.1 Primary findings

The primary findings of the current study are: 1) antioxidants did not alter FMD, cerebrovascular reactivity (CVR) to CO_2 hypercapnia or hypoxia at sea-level; 2) similarly, at high-altitude, antioxidants did not alter FMD, resting CBF. This study highlights that at sea-level, acute antioxidant dosing does not alter brachial artery function or cerebrovascular reactivity to CO_2 or hypoxia. Similarly, acute antioxidant dosing does not alter resting FMD or CBF at high-altitude. The following discussion outlines these findings in the context of previous literature and the strengths and limitations of the experimental design.

Our findings imply that oral antioxidant supplementation has no effect on physiological consequences on vascular function at sea-level or high-altitude. Consistent with this view, previous studies that have employed a more chronic use of antioxidants [e.g., dose (=500mg ascorbic acid), days (= 21 days), duration (= 13 days at 4300m), etc.; Subudhi et al., 2004)] during high-altitudes treks have shown no change in pro-inflammatory cytokines (e.g., TNF-alpha) (Bailey et al., 2004), or in oxygen saturation (Bailey et al., 2004). Likewise, previous work has shown that oxidative stress (e.g., lipid H_2O_2), which increases during exposure to high-altitude (Lewis et al., 2014; Pichler Hefti et al., 2016), remains unchanged when chronically supplementing with antioxidants during chronic exposure to both moderate (2743m) (Pfeiffer et al., 1999; Schmidt et al., 2002) and extreme (6210m) high-altitude (Pichler Hefti et al., 2016; Subudhi et al., 2004). These previous findings demonstrate that although there is an increase in oxidative stress at high altitude, chronic antioxidant dosing (both water and lipid soluble) has no clear effect in the reduction of oxidative stress. Likewise, the current study shows that an acute dosage (both water and lipid soluble) of antioxidants has no effect on improving vascular functionality, even though at sea-level the efficacy of the antioxidants have been proven (Richardson et al., 2007). Perhaps, then, such increases in oxidative stress are somewhat biologically important and adaptive in nature.

2.4.2 Vascular function and oxidative stress

Marked elevations in oxidative stress are present in normal aging (Wray et al., 2017) and in a multitude of cardiovascular diseases, including; atherosclerosis (Joannides et al., 1995), chronic obstructive pulmonary disease (Hartmann et al., 2016), congestive heart failure (Belch et al., 1991), and type 2 diabetes (Butkowski & Jelinek, 2016). Excessive increases in oxidative stress contribute, at least in part, to the development of vascular dysfunction and arterial stiffening (Wray et al., 2013); such decrements are likely related to alteration in NO-mediated vascular function. The underpinning mechanism driving NO-mediated dilatation involves the physical interaction of red blood cells against the blood vessel endothelium commonly termed as "shear stress". Increases in endothelial shear stress promotes the release of intracellular calcium which incurs a cascading effect that will cause an upregulation of endothelial nitric oxide (eNOS) and synthesize NO, ending with the opening of potassium channels and causing smooth muscle dilatation (Behringer & Segal, 2012). This cascading effect is interrupted due to an excessive interaction with oxidative stress (Heitzer et al., 2001; Richardson et al., 2007). Reactive oxygen species such as lipid hydrogen peroxide (H_2O_2) - a spontaneously forming weak oxide derived from superoxide anions (O_2^-) with a long half-life can diffuse within cells. Likewise, it can also diffuse across cell membranes (Powers et al., 2010), which makes H_2O_2 important in cell signalling for either cell proliferation (Geiszt & Leto, 2004) or apoptosis (Cai, 2005), depending on the subsequent levels (Cai, 2005; Veal et al, 2007). Increases in O_2^- reacts with NO forming peryoxynitrite ($ONOO^-$), a strong oxidizing agent, which alters redox and cell signalling (Moylan & Reid, 2007). The formation of $OONO^-$ reduces the bioavailability of O_2^- and NO (Powers & Jackson, 2008). Further details have been elegantly highlighted in a recent review (Trinity et al, 2016).

The impact of acute administration of antioxidants (e.g., water – soluble vitamin C, lipid – soluble vitamin E, and alpha-lipoic acid) on vascular function has been assessed in young populations (Caruana & Marshall, 2015; Donato et al., 2010; Ranadive et al., 2014; Richardson et al., 2007; Wray et al., 2012), healthy aging populations (Eskurza et al., 2004; Jablonski et al., 2007; Richards et al., 2015; Trinity et al., 2016; Wray et al., 2012), and various diseased populations (Levine et al., 1996; Rossman et al., 2015; Ting et al., 1996; Wray et al., 2009). The methodology between studies has varied in terms of the mode of

administration of antioxidants and the varying antioxidant dosages. For example, administering antioxidants have been giving orally (Caruana & Marshall, 2015; Donato et al., 2010; Levine et al., 1996; Richardson et al., 2007; Rossman et al., 2015; Wray et al., 2012; Wray et al., 2009) or intravenously (Crecelius et al., 2010; Eskurza et al., 2004; Jablonski et al., 2007; Kirby et al., 2009; Ranadive et al., 2014; Richards et al., 2015; Ting et al., 1996; Trinity et al., 2016). Despite the differing administration methodology and likely pharmacokinetics (e.g. oral, intravenous infusion, dosing combinations), antioxidants have generally demonstrated an improvement in vascular function within older participants (Wray et al., 2012) and disease populations (Ives et al., 2014); however, this is not the case in the young and healthy participants where FMD is reduced following antioxidants (Crecelius et al., 2010; Donato et al., 2010; Jablonski et al., 2007; Kirby et al., 2009; Richards et al, 2015; Rossman et al., 2015; Trinity et al., 2016; Wray et al., 2012). A potential explanation for the observed differences between the young and old populations is due to increased oxidative stress within the aging population compared with the young (Wray et al., 2017). Similar to the healthy aging population, patients (e.g. those with COPD or hypertension) show an improvement in vascular function following antioxidant supplementation (Ives et al., 2014; Levine et al., 1996; Rossman et al., 2015; Ting et al., 1996).

Despite the various methods and drugs utilized, the majority of work assessing the role of antioxidants on vascular function has used FMD and an oral antioxidant combination (e.g. vitamin C, E, alpha-lipoic acid). The combination of water- and lipid-soluble vitamins are ideal for the reduction of both aqueous superoxide, peroxyl, alkoxyl radicals, and also scavenging peroxyl radicals as chain-breaking antioxidants (Bailey & Davies, 2001). Collectively, these specific studies (Ives et al., 2014; Wray et al., 2012; Wray et al., 2009) indicate that the use of this specific oral antioxidant supplementation improves FMD within the healthy aging, and diseased populations (Ives et al., 2014; Wray et al., 2012), while reducing FMD within healthy young participants (Wray et al., 2012). In contrast with these previous findings, however, in the current study there were no effect of antioxidants on peripheral vascular function assessed by FMD in healthy young adults at sea-level when using the same orally administered antioxidant combination. Methodological differences can be made between Wray et al., (2012) and our current study (e.g., sample size [n = 11 versus n

= 42. Importantly, the study by Wray and coworkers did not employ the same within day testing variability as the current project (e.g., pre-versus post measurements). In addition, in both young and, to a lesser extent, in old participants blood assays revealed an increase in superoxide dismutase (SOD), an enzyme and important antioxidant that catalyzes O_2^- into either O_2 or H_2O_2 (Domej et al., 2014), following the ingestion of the antioxidants compared to placebo. This finding provides indirect evidence of increasing the scavenging of free radical O_2^-, and also that H_2O_2 can act as a vasodilator as well as vasoconstrictor depending on concentrations (Lucchesi et al., 2005). Despite antioxidants having no effect on vascular function, the current study provided both between-day and within-day FMD, CBF, and cardiovascular measurements (e.g., pre-versus post; placebo versus antioxidants), at both sea-level and high-altitude, in order to strengthen the overall design and reduce variability. Moreover, some of the previous studies have incorporated the use of handgrip exercises in order to provide an additional stimulus to further increase vasodilation and oxidative stress (Donato et al., 2010 Richardson et al., 2007). Thus, the progressive increase in workload (and vessel diameter) during handgrip (Richardson et al., 2007), likely provides a very different stimulus and therefore might help explain the differences in findings with the current study. Nevertheless, for the first time, this current study also sought to determine the role of antioxidants in mitigating the decrements in vascular function observed in hypobaric hypoxic states (Frick et al., 2006; Frøbert et al., 2008; Lewis et al., 2014).

2.4.3 High altitude vascular and oxidative stress

Peripheral vascular function during exposure to hypoxia has been reported to be impaired (Frick et al., 2006; Frøbert et al., 2008; Johansson et al., 2014; Lewis et al., 2014; Rhodes et al., 2011; Vedam et al., 2009), although this is not a universal finding (Bruno et al., 2015; Frick et al., 2006; Iglesias et al., 2015; Rimoldi et al., 2012; Tremblay et al., 2017; Tymko et al., 2017). These differences in FMD at high-altitude could potentially be explained through the amount of exposure time to high-altitude i.e., acute (Bruno et al., 2015) versus chronic exposure (Lewis et al., 2014) which may influence the effect of the hypoxic stimulus. Also, the severity of the hypoxia (e.g. degree of altitude) may likely contribute to the observed changes in vascular function. For example, higher altitudes [5050m; (Lewis, et al., 2014)] compared to lower altitudes [1700m-3800m; (Frick et al., 2006; Tremblay et al., 2017;

Tymko et al., 2017)], possibly influencing the severity of the FMD response to the hypoxic stimulus. Lastly, the method of transportation to altitude may influence vascular function. For instance, in some studies, subjects trekked to high altitude (Lewis et al., et al., 2014), whereas in others they had a passive ascent via cable car or automobile (Bruno et al., 2015; Parati et al., 2013; Tremblay et al., 2017; Tymko et al., 2017).

Exposure to high-attitude has been shown to increase oxidative stress (e.g., H_2O_2) within lowlanders, and a reduction in antioxidant capacity (Bailey et al., 2009; Irarrázaval et al., 2017; Lewis et al., 2014). As previously stated, an increase in oxidative stress (c.f. $OONO^-$) can reduce NO bioavailability and impair peripheral vasodilatation (Moylan & Reid, 2007; Powers & Jackson, 2008). Despite an increase in oxidative stress at high-altitude, and antioxidants can augment FMD within populations that incur higher than normal oxidative stress (aging and diseased), acute antioxidant supplementation did not affect peripheral vascular function as assessed with FMD at high-altitude in the current study. Potentially, however, the acute antioxidants might not have had an effect due to the additional constraining influence of the marked elevations in sympathetic nerve activity on peripheral vascular function at high altitude (Duplain et al., 1999; Hansen & Sander, 2003).

2.4.4 Cerebrovascular function, ROS and antioxidants

The human brain itself is uniquely prone to oxidative stress, which is partly due to the brain being composed of polyunsaturated fatty-acid side chains (PUFAS) (Bailey et al., 2009). The neuronal membrane has shown to have a low antioxidant defense system, demonstrating the brain is vulnerable to redox mediated changes (Bailey et al., 2009). Previous work completed on the animal model, has shown that an increase in hydroxyl radicals can dilate cerebral pial arteries in mice (Rosenblum, 1983). Likewise, increasing extracellular superoxide dismutase, similar to superoxide dismutase but excreted into the extracellular space and anchors to cell surfaces, scavenge O_2^- and promote NO vasodilation within cerebral arteries of mice (Demchenko et al., 2002). In humans, consistent with the current findings, cerebrovascular vasodilation to isocapnic hypoxia was unaltered in healthy young participants after an intravenous infusion of vitamin C (3000mg) (Hartmann et al., 2015). However, a limitation of this latter study was that CBF was indexed via TCD and it is known that hypoxia can

dilate the MCA vessel diameter, and TCD only acquires velocity which can underestimate flow through the vessel (Fan et al., 2014; Lucas et al., 2011). To our knowledge this is the first study to assess integrative cerebrovascular function (e.g. flow, diameter, velocity and vascular reactivity to hypoxia and hypercapnia) following antioxidants or placebo. Despite our design, consistent with the vascular changes, we found no effect of antioxidants on cerebrovascular function. Whether such changes might occur with aging or chronic diseased state who suffer from a redox imbalance remains to be investigated.

2.4.5 High altitude, cerebrovascular function and antioxidants

Limited research has examined the role of oxidative stress and cerebrovascular function in hypoxia with antioxidant supplementation. There are well reported elevations in oxidative stress with normo- and hypobaric (Bailey et al., 2009; Irarrázaval et al., 2017). This increase in oxidative stress, likewise to peripheral vascular function, may have implications in the augmentation of cerebrovascular regulation at high altitude (Bailey et al., 2009; Lewis et al., 2014). gCBF increases during the initial arrival to high altitude, with progressively decreasing in flow over time at altitude , but remaining above sea-level values (Subudhi et al., 2014; Willie et al., 2014). Our gCBF data at high-altitude are consistent with these previous studies, with gCBF reducing to relative sea-level values following ~one week acclimatization. Despite these findings and the known increase in oxidative stress at high altitude, CBF remained unchanged at rest even after administration of antioxidants. Thus, potentially due to more powerful influence on the brain (arterial blood gases, metabolism, etc.), it seems under the conditions of the current experimental design, acute antioxidant administration do not influence CBF.

2.4.6 Strengths and limitations

The current study is the first to examine the effects of oral antioxidant supplementation on cerebrovascular regulation both at sea-level and at high-altitude, as well as peripheral vascular function. The current study was a double-blinded, placebo controlled novel experimental design. A Limitation of this study was our small sample size both at sea-level and high-altitude, as observed by post-hoc power calculations. Post-hoc calculation indicates a much larger sample size (n>344) would be needed to detect a significant change in (>1%)

in FMD at high-altitude. Likewise, to our peripheral vascular data, post-hoc power calculations revealed that a much larger sample size (n>66) would be needed to show a significant change in gCBF. At high-altitude, larger sample sizes were unfeasible due to constraints in available volunteers, and not interfering with other studies. Likewise, high altitude field studies come with variable challenges that may or may not interfere with testing results (e.g. AMS, increased SNA, subject physical conditions). Lastly, this study did not measure blood samples in order to track changes in oxidative stress both at sea-level and high-altitude, which was completed in previous studies (Bailey et al., 2009; Lewis et al., 2014). Logistically, this was unfeasible at altitude due to increased costs of storing, transportation, and analysis. Likewise, there was no significant change in vascular functionality both at sea-level and high-altitude, which, in retrospect, did not warrant blood samples.

2.5 Synopsis

The findings from this study highlight that at both sea-level and high-altitude, acute antioxidant dosing does not alter FMD or cerebrovascular reactivity to CO_2 or hypoxia. Nevertheless, continued exploration of this methodology in both peripheral and cerebrovascular regulation at sea-level and high-altitude is recommended, due to the increase in oxidative stress and reduction in antioxidant capacity in pathologies such as chronic pulmonary obstructed disease (COPD) (Domej et al., 2014; Hartmann et al., 2015). Weather there are some adaptation to oxidative stress in lower lander or high-altitude natives also remains unknown.

2.6 Funding

This research was supported by an NSERC Discovery Grant and Canadian Research Chair in Cerebrovascular Physiology (PNA).

3 Chapter: Conclusion

3.1 Acute antioxidant administration had no effect on peripheral vascular function and cerebrovascular reactivity at both sea-level and high-altitude

The effects of oxidative stress and antioxidants on peripheral vascular function at sea-level has been well documented (Eskurza et al., 2004; Ives et al., 2014; Levine et al., 1996; Ting et al., 1996; Wray et al., 2012; Wray et al., 2009). These studies generally show an impairment within brachial FMD following acute antioxidants within the younger population (Wray et al., 2012), whereas it has shown improvement in older (Wray et al., 2012) and diseased populations (e.g., chronic obstructive pulmonary disease, diabetes, and coronary artery disease) (Ives et al., 2014; Levine et al., 1996; Ting et al., 1996). In contrast to these previous studies , very little work has been completed at sea-level on investigating the role of oxidative stress on cerebrovascular function (Hartmann et al., 2015), and no work on the effects of antioxidants at high-altitude on peripheral and cerebral vascular function. It is well documented that oxidative stress (e.g., superoxide dismutase [SOD], and hydrogen peroxide [H_2O_2]) is increased at high-altitude (Bailey et al., 2009; Irarrázaval et al., 2017; Lewis et al., 2014), and the cascading effect of oxidative stress which leads to form superoxide and hydrogen peroxide from the reduction of molecular oxygen, superoxide would then go on to interact with NO forming peroxynitrite (Cai, 2005; Moylan & Reid, 2007), which actively reduces NO bioavailability (Powers & Jackson, 2008). Since antioxidant administration improves endothelial function at sea-level in old and diseased populations, therefore, the notion that antioxidant administration would improve FMD at high-altitude was plausible. In addition, there has been little work completed on the influence of oxidative stress and/or antioxidants on resting cerebral blood flow and cerebrovascular reactivity at both sea-level and high-altitude. The use of cerebrovascular reactivity (e.g., hypercapnia [increase in arterial CO_2] and hypocapnia [decrease in arterial CO_2]) is an important measure due to the increased sensitivity of the brain to acute changes in $PaCO_2$, which will in turn create immediate changes to cerebral artery diameter and blood flow (Willie et al., 2012). For example, there has only been one study that investigated the effects of antioxidants on cerebrovascular (+1.5mmHg above resting $P_{ET}CO_2$) reactivity, they observed that Vitamin C had no effect on cerebrovascular reactivity (+1.5mmHg above resting $P_{ET}CO_2$) within young, old, and

diseased (Chronic obstructive pulmonary disease [COPD]) populations (Hartmann et al., 2015). In contrast to previous investigations, our data indicates that antioxidants did not alter FMD at both sea-level and high-altitude. Additionally, antioxidants did not alter cerebrovascular reactivity at sea-level, which agrees with previous findings (Hartmann et al., 2015), and interestingly, administration of antioxidants did not alter resting cerebral blood flow at high-altitude.

3.2 Oxidative Stress: implications for disease; potential clinical use of antioxidants

As demonstrated previously, endothelial function is impaired in diseased populations, which may be due to increased oxidative stress (Ives et al., 2014). Additionally, it has been shown that increased oxidative stress may affect the brain, due to increased amounts of polyunsaturated fatty acid chains, increased cerebral iron stores (Haacke et al., 2005; Halliwell, 1992), and the lack of antioxidant defenses (Bailey et al., 2009). The overall increase of oxidative stress within the brain can reduce the synapse of the central nervous system (Halliwell, 1992), as well as, increase cerebral cell apoptosis (Bailey et al., 2009) Increases in superoxide (O_2^-) and H_2O_2, measured via EPR spin-trapping, has been shown within many clinical populations (Ives et al., 2014; Levine et al., 1996; Rossman et al., 2015; Ting et al., 1996; Wray et al., 2009). Chronic obstructive pulmonary disease (COPD) is as a progressive, and irreversible condition that is characterized by expiratory airflow obstruction (Chen & Mannino, 1999; Divo et al., 2012; Hansell et al., 2003). COPD patients also have an increase in inflammation, and oxidative stress, which contributes to the development of oxidant/antioxidant imbalance (Fischer et al., 2011). COPD patients are subjected to a reduced forced expired volume during the first second (FEV_1) and chronic/intermittent hypoxemia, which intermittent hypoxia had been shown to increase oxidative stress (Peng & Prabhakar, 2003; Pialoux et al., 2009). Increases in oxidative stress within COPD patients can also come from circulating and intrapulmonary leukocytes, and increased inhaled oxidants from cigarette smoke (MacNee, 2000). This latter evidence can potentially relate to the impaired endothelium function within COPD (Ives et al., 2014), arterial stiffness within COPD patients assessed via pulse wave velocity (Ives et al., 2014), as well as increased sympathetic nervous activity (SNA) (Heindl et al., 2001), for which increases in SNA had been previously established to cause vascular dysfunction (Dyson et al., 2006). Previous

work has shown that the use of acute antioxidants (using the same vitamins and dosage as the current study) will improve vascular function within those with COPD compared to healthy controls (Ives et al., 2014; Rossman et al., 2015). The improvement in vascular function within COPD patients after ingestion of antioxidants had been speculated to the overall improvement of NO bioavailability and superoxide dismutase due to the reduction in superoxide radicals (Ives et al., 2014).

The mentioned previous work had been completed using acute single trial doses of antioxidant supplementation study (Ives et al., 2014; Rossman et al., 2015). Studies using chronic antioxidant supplementation (28 days of either oral erdosteine, 300mg dose / day, oral placebo/day, or oral erdosteine 300mg and oral placebo/day) in COPD patients report a decrease in oxidative stress (as indexed by plasma levels of reactive oxygen species and 8-isoprostane) and an increase in FEV_1 (Dal Negro et al., 2015). Erdosteine is a mucoactive agent that contains both antioxidant and inflammatory effects and used as protection against oxidative stress (Vagliasindi & Fregnan, 1989). These positive influences on oxidative stress might be due to erdosteine capabilities of scavenging oxidative stress and offer protection against potential tissue damage (Dal Negro et al., 2015). Likewise, chronic six-month antioxidant supplementation of vitamin C has shown to increase total antioxidant capacity within COPD patients (Pirabbasi et al, 2016). Albeit, however, this study displays the effects of chronic antioxidant supplementation did not examine the effects on vascular endothelial function in both peripheral and/or cerebral vessels. Regardless, this is a stepping-stone to the introduction of antioxidants as a therapeutic treatment for COPD, and further research into the effects of chronic supplementation on the vascular mechanism(s) within COPD patients.

Diabetes mellitus is another disease that can incur vascular impairments and can affect the peripheral, cerebral and coronary vasculature creating early signs of atherosclerosis (Clark et al., 1995; Kannel & McGee, 1979; Nathan, 1993). Likewise, endothelial function has been shown to be impaired with patients who have diabetes mellitus (Johnstone et al., 1993; McVeigh et al., 1992; Ting et al., 1996; Williams et al., 1996). Previous work completed by Ting et al., (1996) showed that intravenous infusions of vitamin C can improve peripheral vascular function as assessed by forearm blood flow via venous occlusion strain gauge

plethysmography. Animal studies have shown that chronic dosing (two weeks) of ascorbic acid (100mg/kg) in mice will decrease blood brain barrier endothelial cell apoptosis and increase upregulation of glucose transporter-1 GLUT1, a glucose transporter located on the blood brain barrier (BBB) (Iwata et al., 2014). Therefore, chronic pre-treatment of ascorbic acid may potentially contribute to a therapeutic treatment of diabetes.

3.3 Methodological limitations

3.3.1 Oral antioxidants versus intravenous infusion

In the present study, orally administered antioxidants were used, using the exact same dosing strategy previous studies have reported (Ives et al., 2014; Richardson et al., 2007; Wray et al., 2009). These assay's included assessment of oxidative stress via electron paramagnetic resonance (EPR), which allows for direct detection of oxidative free radicals in intact cells and tissue samples, and is often considered the gold standard and only current technology for detecting free radicals (Davies, 2016). EPR spin-trapping was used to quantify an index for ROS during previous studies using a similar antioxidant dosage as the current study (Richardson et al., 2007). Pharmacokinetics of the orally administered antioxidants determined the exact peak concentrations 90 mins from dose #1 which includes 500 mg of vitamin C, 400IU of vitamin E, and 300mg of alpha-lipoic acid, and 60 mins from dose #2 which includes 500mg of vitamin C, 200IU of vitamin E, and 300mg of alpha-lipoic acid of the oral antioxidants within the blood plasma (Donato et al., 2010; Ives et al., 2014; Richardson et al., 2007; Rossman et al., 2015; Wray et al., 2012; Wray et al., 2009). On the other hand, intravenous infusion may potentially allow for a more effective method to administer dose of antioxidants, since you bypass the metabolism process and infuse right into the blood plasma, as well as potentially reduced time to reach peak concentrations levels (Caruana & Marshall, 2015; Crecelius et al., 2010; Hartmann et al., 2015; Jablonski et al., 2007; Ranadive et al., 2014; Richards et al., 2015; Ting et al., 1996; Trinity et al., 2016).

Previous work using intravenous or intra-arterial infusion has shown an increase SOD, which scavenges superoxide radicals and increases NO bioavailability (Trinity et al., 2016).It has been shown that using intravenous infusions offers a much greater infusion rate, and allow

for a greater increase in plasma serum levels (Fritz et al., 2014). Regardless, the use of intravenous infusion of vitamin C always has the potential of overdosing , and there is conflicting evidence that high-doses of intravenous vitamin C infusion might also yield a pro-oxidative state and further aggravate oxidative catalysis (Halliwell, 1992; Mühlhöfer et al., 2004; Podmore et al., 1998). For example, it has been shown that high-dose vitamin C infusion (500mg) can either increase pro-oxidative properties and induce cell damage (Cai et al., 2001; Podmore et al., 1998), or present with no biochemical markers of pro-oxidative properties (Mühlhöfer et al., 2004), as assessed using 8-oxoguanosine – a marker of DNA damage from oxygen radicals (Podmore et al., 1998). Regardless of these mixed findings. the use of either intravenous infusion or orally administered antioxidants should be further investigated within the context of the current study.

3.3.2 Venous blood samples

In the current study, venous blood samples were not obtained, therefore, it was not possible to quantify an individual's antioxidant capacity and oxidative stress levels before administering the antioxidant combination. The procurement of blood samples would have improved the mechanistic insight in the current study, and determine if antioxidants were having effect on overall antioxidant capacity.

3.3.3 Variability in measures

In the current study, we observed a high variability for FMD at both sea-level and high-altitude. The between-day coefficient of variation of the FMD at sea-level was 26.1%. At high-altitude the between-day coefficient of variation of the FMD was 28.4%. The high variability within the FMD measure may potentially have influenced the effect outcome of the antioxidants. Reasoning for the high variability in FMD at sea-level may be contributed to the inexperience in scanner that was used, and at high-altitude it may be due to many different environmental stresses that is seen throughout a high-altitude field study (e.g., increased SNA, dehydration, sickness, trekking).

3.3.4 Antioxidants and blood brain barrier permeability

As previously stated vitamin C is an essential antioxidant known for scavenger oxidative stress, and that being an water-soluble antioxidant it can scavenge aqueous superoxide anions (Bailey & Davies, 2001). In diseased conditions such as diabetes (Iwata et al., 2014) and stroke (Allahtavakoli et al., 2015) there is an increase in oxidative stress within the brain, which can lead to cerebral ischemic injury (Iwata et al., 2014). Cerebral ischemic injury can cause apoptosis within endothelial cells located in the blood brain barrier (BBB), and have a reduction of GLUT1 in the BBB (Iwata et al., 2014). Increases in oxidative stress have been shown to cause an increase in BBB permeability (Lagrange et al., 1999). It has been shown that chronic supplementation of ascorbic acid or in other words vitamin C, can cause upregulation of GLUT1 and decrease pro-inflammatory cytokines and apoptosis of endothelial cells within the BBB (Iwata et al., 2014). Likewise, ascorbic acid has been shown in rats to decrease BBB disruption that is normally caused by stroke (Allahtavakoli et al., 2015). Previous work has also examined lipid-soluble antioxidants, which act as essential free-radical poly-fatty acid chain breaking antioxidants, and the BBB. Likewise, to water-soluble antioxidants studies have shown that lipid-soluble antioxidants such as lipoic-acid has stabilized BBB permeability within rats displaying signs of multiple sclerosis (MS), an autoimmune disorder of the central nervous system (Schreibelt et al., 2006). Albeit, all of this work has been completed within animal models (Allahtavakoli et al., 2015; Iwata et al., 2014; Schreibelt et al., 2006), and to our knowledge no work has been done within the human model measuring the effects of antioxidants on the permeability of the BBB. Regardless, the previous work might elucidate the effect of antioxidants on the permeability of the BBB within the human model.

3.4 Future studies

3.4.1 Cerebral endothelial assessment

Regardless that the current study had shown no effect of antioxidants on peripheral vascular function and cerebrovascular reactivity and function at both sea-level and high-altitude, further research on the influence on oxidative stress on cerebrovascular reactivity is needed. The current study methodologically contrasts previous work that has been completed

assessing the effects of antioxidants on cerebrovascular reactivity (Hartmann et al., 2015), presenting cerebral blood flow and artery diameter using duplex ultrasound. It has been shown within animal literature that oxidative stress causes vasodilation within mice cerebral pial arteries (Demchenko et al., 2002; Rosenblum, 1983). Regardless, the work that has been completed showing vasodilation of the cerebral pial vessels due to oxidative stress was only assessed within the animal model, it is still speculative the overall effects of oxidative stress on human cerebral arteries. The current study has provided some insight on the effects of antioxidants on cerebrovascular reactivity to both increases in $PaCO_2$ and decreases in PaO_2 at sea-level, as well as resting cerebral blood flow at high-attitude. Further work within this methodology is required potentially assessing the role of different types of antioxidants being either water or lipid – soluble, as well as using a much greater CO_2 stimulus (Willie et al., 2012).

3.4.2 Peripheral and cerebral vascular assessment in clinical populations

It has been previously established that clinical populations have increased vascular dysfunction (e.g., COPD, diabetes, hypertension, coronary disease), and have increased oxidative stress (Ives et al., 2014; Rossman et al., 2015; Ting et al., 1996). Likewise, it has been previously established that patients with spinal cord injury also have increased vascular dysfunction as assessed through femoral FMD (Totosy de Zepetnek et al., 2015). In contrast, there has been little work properly establishing the effects of oxidative stress on cerebrovascular function within the clinical population (Hartmann et al., 2015). The cause of this dysfunction may potentially be influenced by increases in oxidative stress, but this has not been fully investigated. Such a study in clinical populations would be a double-blinded crossover trial involving both placebo and antioxidants, with pre-and post-femoral and brachial FMD measurements, that is used for vascular assessment, as well as cerebrovascular assessments. Blood samples would ideally be taken before and after antioxidant dosage, which will assess oxidative stress and antioxidant capacity before and after administration of drugs. The use of antioxidants (water and lipid – soluble vitamins) on oxidative stress may elucidate the confounding mechanisms displayed in pathologies augmenting a hypoxic stimulus on the human body.

3.4.3 Lipid versus water-soluble antioxidants

As previously mentioned there is a difference between water and lipid soluble vitamins. Water-soluble vitamins (e.g., vitamin C) are ideal for the reduction of aqueous superoxide, peroxyl, alkoxyl radicals (Bailey & Davies, 2001). While lipid-soluble vitamins (e.g., vitamin E) are effective for scavenging peroxyl radicals as chain-breaking antioxidants (Bailey & Davies, 2001). This current study used both water and lipid-soluble vitamins (e.g., vitamin C, E, and alpha-lipoic acid), for which the efficacy was already proven (Richardson et al., 2007). Whereas other studies have just used water-soluble vitamins (e.g., vitamin C) (Caruana & Marshall, 2015; Crecelius et al., 2010; Eskurza et al., 2004; Hartmann et al., 2015; Jablonski et al., 2007; Kirby et al., 2009; Levine et al., 1996; Ranadive et al., 2014; Richards et al., 2015; Teppema et al., 2005; Ting et al., 1996; Trinity, et al., 2016). To our knowledge there has been no work completed just using lipid-soluble vitamins (e.g. vitamin E). Further investigation should be completed measuring the efficacy of lipid-soluble compared to water-soluble antioxidants, within populations with increased oxidative stress.

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