The Effects of 60 Days of Head Down Bed Rest on Vascular Health

by

Louis Mattar

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Author's Declaration

I hereby declare that I am the sole author of this thesis.

This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

This study was designed to test the hypothesis that 60 days continuous head down bed rest (HDBR), an Earth-based analogue of the effects of space flight, would elevate factors that increase vasoconstriction and would increase markers of vascular inflammation. The study incorporated countermeasures consisting of treadmill running within lower-body negative pressure and resistive "flywheel" exercise (exercise countermeasure, EX) or an increased protein intake of 0.6 g/kg body weight/day (dietary countermeasures, DIET) to determine whether these interventions might prevent the vasoconstrictor and inflammatory responses when compared to a control (CON) group. Markers of vascular health measured in the study include the vasoactive molecules angiotensin II (Ang II), endothelin-1 (ET-1), and nitric oxide metabolites (NO_{met}); and markers of inflammation including C-reactive protein (CRP), and the adhesion molecules E-selectin (E-sel), intracellular adhesion molecule-1 (ICAM), and vascular cell adhesion molecule-1 (VCAM). Twenty four women were housed at the MEDES clinic in Toulouse, France, as part of a large international study (Women International Space Simulation for Exploration, WISE) in which various experimental protocols and countermeasures were integrated into a single experimental design completed during two campaigns. Each 100 day campaign included 20 days of pre-testing (pre-HDBR), 60 days of bed rest (HDBR), and 20 days of post-testing (post-HDBR). The experimental countermeasures were applied only during the 60-day HDBR period. Following 60 days of HDBR, many changes occurred in the concentrations of the measured molecules. Specifically, the concentration of Ang II significantly increased in the CON and DIET groups (52.9%, $p = 0.014$; and 124.4%, $p < 0.0001$ respectively), but not in the EX group. Also, NO_{met}

decreased in all groups, with reductions in the EX and DIET groups ($p = 0.013$, and $p = 0.056$ respectively). Markers used to assess vascular inflammation increased following the HDBR. The increase in CRP in the CON and DIET groups and the decrease in the EX group from pre- to post-HDBR were not significant; however, the directional changes resulted in an interaction between group and HDBR ($p = 0.052$). The adhesion molecule E-sel was significantly increased in the DIET group ($p = 0.003$), and VCAM was significantly increased in the CON group ($p = 0.016$) with a smaller increase in the DIET group ($p = 0.08$). No changes in adhesion molecules were observed in the EX group. This study demonstrated that 60 days of HDBR by young, healthy, women caused changes in several different molecules that are beginning to emerge as risk factors for the development of cardiovascular diseases. Further, it was observed that regular, vigorous exercise during HDBR prevented these changes. These results suggest that future studies of this kind should directly monitor the effects of simulated space flight on vascular health in men and women to obtain a greater understanding of the adaptations that might occur during long term space exploration missions. HDBR can be considered an extreme model of physical inactivity and could be used to provide insight into mechanisms of disease processes associated with the sedentary lifestyle that is prevalent in Western society.

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Dedication

This thesis is dedicated to my father. You are the greatest man I have ever known, and your guidance has shaped me into the man I have become. The commitment that you have for your family and work are qualities that I strive to achieve myself. I can only hope that one day I will be as great as you.

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1 Literature Review

1.1 Head Down Bed Rest

Head down bed rest (HDBR) has been used as a model of simulated microgravity to determine the physiologic changes (deconditioning) that might occur with actual space flight. Prior to World War II there were few bed rest studies, and of the few there were, most examined the use of bed rest in the treatment of different chronic diseases (Barry *et al.*, 1998). Following the advent of manned space flights, scientists were interested in determining the reasons for the so-called orthostatic intolerance that often affected astronauts upon their return to earth. Orthostatic intolerance occurs when the cardiovascular system can no longer sustain adequate brain blood flow to maintain consciousness. Following prolonged bed rest, mal-adaptations occur that cause orthostatic intolerance.

Microgravity of space travel refers to the free-fall environment that places very little net gravitational force on an object. Bed rest studies mimic many of the physiological changes that occur in the microgravity of space because lying down places the body in a recumbent position with the loss of the normal head-to-foot gravity vector. Thus, the hydrostatic pressure that normally elevates blood pressure in the vessels below the heart, and that increases forces causing longitudinal compression on the spine and bones of the lower limbs, has been removed. Also, without the need to work against gravity, the muscular force acting on most of the bones in the body is reduced, as is the total energy expenditure (Convertino *et al.*, 1997). These changes allow the body to

adapt to stimuli that are similar to what astronauts experience in space (at a fraction of what it costs to send someone there).

One consistent finding regarding HDBR is that there is a loss of plasma volume that can be countered by exercise during bed rest (Greenleaf, 1997; Belin de Chantemele *et al.*, 2004). In fact, as little as one single bout of intense exercise before resuming an upright position can restore the lost volume (Convertino, 1997). Reduced plasma volume is the result of shifting of fluids between the intravasculature and extravascular spaces. Upon transition from a vertical to horizontal position part of the hydrostatic force in the vascular system, which is also the driving force for net filtration, is lost. As a result, net reabsorption is favored and fluid shifts towards the vascular compartment (Greenleaf, 1997). In an attempt to control plasma volume and tonicity, fluid is excreted by the kidney and a state of relative hypovolemia is established as soon as a few days after the beginning of HDBR (Bestle *et al.*, 2001; Trappe *et al.*, 2006). The new homeostatic set point of plasma volume usually occurs with a concomitant reduction in red cell volume so as to maintain the normal blood hematocrit (Convertino, 1997).

1.1.1 Physiological Responses (Deconditioning)

Bed rest following cardiovascular events or with many chronic diseases has, in the past, been a common prescription by many health care providers (Friedberg, 1966). It is now known that while rest is needed, extended bed rest for patients can be detrimental to the recovery process due to the deconditioning that can occur (Winslow, 1985). Deconditioning affects the cardiovascular system as much as it affects any other system of the body. When a person moves from a horizontal to vertical position (from lying to standing), blood begins to pool in the lower limbs due to gravity and the compliant nature

of the vasculature. In order to prevent a loss of consciousness due to lack of blood reaching the brain, the body makes adjustments to maintain mean arterial pressure (MAP). MAP is the product of cardiac output (Q) and total peripheral resistance (TPR) (*Equation 1*).

$$MAP = Q \times TPR \qquad \textit{Equation 1}$$

$$Q = HR \times SV \qquad \textit{Equation 2}$$

$$R \propto \frac{\eta \cdot L}{r^4} \qquad \textit{Equation 3}$$

MAP is mean arterial blood pressure (mmHg), Q is cardiac output (l/min), HR is heart rate (beats/min), SV is stroke volume (ml/beat), TPR is total peripheral resistance (mmHg/l/min), R is the resistance to flow (arbitrary resistance units), η is viscosity of the liquid (dyne-seconds/cm^2), L is the length of the tube (cm), and r is the radius of the tube (cm).

Equation 1 shows that MAP is the product of Q and TPR. The pooling of blood reduces the amount of blood returning to the heart (venous return). This causes a great reduction in stroke volume (SV) because with less blood returning to the heart, there is less to eject, and a decrease in Q results (*Equation 2*). This in turn causes a drop in MAP (*Equation 1*).

A drop in MAP causes unloading of the arterial baroreceptors which mediates an immediate reduction in vagal tone (causing an increase in HR) in an attempt to restore Q. The unloading of the arterial baroreceptors also increases sympathetic outflow (SNA) causing peripheral vasoconstriction, increased HR, and contractility via norepinephrines (NE) interactions with α-receptors on blood vessels and β-receptors in the heart. The peripheral vasoconstriction increases TPR in order to restore MAP (from *Equation 1*) and

the increased HR and contractility to try and compensate for Q. The overall result is maintenance of MAP (Rowell, 1993).

The events outlined above occur every time a person moves from a lying or sitting position to standing. If these actions are not employed, the amount of blood returning to the heart would quickly decrease, and the amount of blood reaching the brain would not be sufficient to maintain consciousness. Luckily, the orthostatic mechanisms outlined occur so often that they can be thought of as a form of orthostatic training for the blood vessels, nerves, and other tissues. In HDBR, these stimuli are no longer present. In the head-down position, blood is localized in the thoracic core, resulting in less blood in the periphery. This in turn results in blood readily returning to the heart, and no need for the orthostatic mechanisms outlined above. This lack of use by the peripheral blood vessels was seen by Shoemaker *et al.*, (1998a) who showed a decrease in the vasoreactivity of the forearm blood vessels following 14 days of HDBR. Shoemaker *et al.* (1998a) attributed some of the change to alterations in the SNA, which is also commonly seen in HDBR (Shoemaker *et al.*, 1998a; Shoemaker *et al.*, 1998b; Kamiya *et al.*, 2000a).

Changes in vascular tone result in changes in the diameter of the lumen of the blood vessel, and can have dramatic effects on blood pressure. Poiseuille's law (*Equation* 3) dictates that the resistance of a tube (in this case a blood vessel) is inversely proportional to the radius to the fourth power.

If the balance between vasoconstriction and vasodilation were tipped in favor of one side, even a small increase (or decrease) in the radius would have a large effect on the resistance in that vascular bed. Referring back to *Equation 1*, an increase in TPR (or R in *Equation 3)*, without a reduction in Q, would lead to an increase in MAP.

Therefore, small changes in the radius of a blood vessel (the vascular tone) can have a large impact on MAP.

Changes in the periphery are not the only adaptations that occur with HDBR. In the recumbent position, the heart does not have to pump blood against the same hydrostatic forces as it does in a normal (vertical) position. This results in the heart not having to pump as hard in order to move the same amount of blood, and as a result there is a reduction in ventricular muscle mass (atrophy). Following six weeks of HDBR Perhonen *et al.*, (2001) found an 8% drop in left ventricle mass as measured by MRI. Perhonen *et al.*, (2001) also found a 14% decrease in the left ventricular end-diastolic volume after just two weeks of HDBR. With the reduction in SNA, and therefore less NE to act on β-receptors, the contractility of the heart should also decrease.

Some of the changes seen in the cardiovascular system are due to altered physical stresses placed on the body by HDBR. In the same way that our bodies will adapt to demands placed on it by increased physical activity (exercise), our bodies will also adapt to the lack of demands placed on it by increased physical inactivity. Changes in blood flow and blood flow distribution can alter the stresses placed on the walls of the vasculature. This can change the expression of many genes that are important to the function of the vasculature (Malek *et al.*, 1999). Altered gene expression can change the concentration of various molecules in the blood that can act as markers of vascular health. Many of these vascular molecules are becoming recognized as potential risk factors for cardiovascular disease, and a lot of research is being done to determine if abnormal concentrations of these molecules can predispose a person to (or predict future) cardiovascular disease.

1.2 Emerging risk factors

One large area of research revolves around the search for factors in the blood that can act as early markers or flags for cardiovascular disease (Hwang *et al.*, 1997; Böger *et al.*, 1998; Blankenberg *et al.*, 2003; Vallance and Leiper, 2004). The rationale is that if a simple blood test can detect abnormal levels of these markers early (before the cardiovascular disease develops), prevention and treatment strategies can be implemented sooner. Many of these markers are found in normal healthy individuals, however, in patients with established cardiovascular disease the concentrations of these markers are thought to change (de Ferranti and Rifai, 2002; Hackam and Anand, 2003; Szmitko *et al.*, 2003a; Szmitko *et al.*, 2003b). It is the early changes in these markers that will allow researchers and health care practitioners to determine if a person is at risk for developing cardiovascular disease.

While looking for a marker that will allow doctors and researchers to predict cardiovascular disease seems promising, it may be more pertinent at this time to examine the health of the vascular system, and how the makeup of plasma can interact with the vessels to either promote or curb disease. Many of the aforementioned risk factors play important physiologic roles in the vasculature. When the concentration of these factors change, it may be an early indication of a much bigger problem. These factors fall into different categories, and two of the most important ones have been determined to be vasoactive molecules and inflammatory markers.

1.2.1 Vasoactive Molecules

Substances that cause blood vessels to either dilate or constrict are termed vasoactive substances and play a key role in regulating blood flow distribution, and blood pressure

(Kohno *et al.*, 1990; Skarphedinsson *et al.*, 1997; Kranzhofer *et al.*, 1999). Some common vasoactive substances include angiotensin II (Ang II), endothelin-1 (ET-1), norepinephrine (NE), and nitric oxide (NO). These molecules all have many actions (and target tissues), however, it is the interaction of these molecules with tissues of the vascular system that modulate vascular tone, cell growth, proliferation, and vascular health in general (Kranzhofer *et al.*, 1999; Williams, 2001; de Gasparo, 2002; Alonso and Radomski, 2003).

The classical view of Ang II pertains to its role as the effector molecule in the renin-angiotensin system (RAS), which is integral in the long term control of blood pressure (Hahn *et al.*, 1993). In this system, renin is released from the kidney in response to renal hypotension, reduced sodium delivery to the distal tubule, and reduced renal sympathetic innervation (RSNA) (Schweda and Kurtz, 2004). Renin then acts to convert angiotensinogen into Ang I. Angiotensin converting enzyme (ACE) then converts Ang I into the biologically active Ang II. Ang II stimulates the release of aldosterone from the adrenal cortex which causes the reabsorption of sodium, and therefore H_2O (Kato *et al.*, 1993). Ang II has two receptors: $AT_1$and AT_2. AT_1 is a member of the G-protein family of receptors; it is highly expressed on vascular smooth muscle cells and has been shown to mediate many of the additional physiological functions of Ang II (Sadoshima, 1998). These functions include vasoconstriction, cell growth, and proliferation. The AT_2 receptor is mainly found in fetal tissues, however, its expression decreases rapidly after birth. The functions of the AT_2 receptor are unclear, but they appear to antagonize the actions of the AT_1 receptor by inhibiting cell growth, inducing apoptosis, and causing vasodilatation (Touyz and Schiffrin, 2000).

The role of RAS in controlling blood pressure is of central importance in the development of many cardiovascular diseases, as hypertension is a major risk factor for cardiovascular disease (Unger, 2002). When Ang II binds to AT_1 many of the effects that are signaled appear to increase cardiovascular disease. For example, increased vasoconstriction can lead to hypertension, vascular smooth muscle activation (leading to cell proliferation and migration), and endothelial cell activation (causing increased expression of adhesion molecules–discussed later) (Kranzhofer *et al.*, 1999; Williams, 2001). Experimentally, exercise training protocols have been shown to increase the concentration of Ang II initially, however, the concentration usually returns back to baseline after a few weeks (Warburton *et al.*, 2004).

One of the most potent vasoactive substances known is the vasoconstrictive ET-1. ET-1 is produced in endothelial cells in response to humoral factors such as Ang II and arginine vasopressin, and is also controlled by shear and stretch factors acting on endothelial cells (Modesti, 1996). ET-1 has two receptors, ET_A and ET_B. Both receptor types are expressed on vascular smooth muscle cells, and when either has ET-1 bound, vasoconstriction occurs. ET_B is also expressed on endothelial cells and when ET-1 binds to this receptor it stimulates the release of NO (Modesti, 1996).

ET-1 production is enhanced in patients with moderate-to-severe hypertension and also in persons with developed atherosclerosis (Kohno *et al.*, 1990; Lerman *et al.*, 1995). Much like Ang II, ET-1 is thought to play a role in the development of hypertension, vascular smooth muscle cell activation, and endothelial cell activation (Schiffrin, 2001).

ET-1 not only acts as a vasoconstrictor, but like Ang II, it also stimulates vascular smooth muscle cell growth and cell adhesion (Alonso and Radomski, 2003). In this regard, increased concentrations of Ang II and ET-1 are thought to be harmful to the vasculature in that they can stimulate inflammatory processes, aid in vascular remodeling, and can influence the development of hypertension (Kranzhofer *et al.*, 1999; Damon, 2000; Duerrschmidt *et al.*, 2000; Cheng *et al.*, 2005).

NE is a neurotransmitter that is released by sympathetic neurons in response to various stimuli (e.g., exercise, Fight-or-Flight response). NE is released in such quantities that the amount measured in the plasma accounts for only 10-20% of the total NE released from the varicosities. The remaining 80-90% is either taken up by the neurons or the target tissue (usually vascular smooth muscle). Even though only 10-20% of the total NE makes it to the plasma, NE spill-over is usually proportional to SNA (Rowell, 1993).

NE is not usually thought of as being associated with cardiovascular disease; however, recent reports have found that elevated sympathetic outflow is associated with the development of hypertension and cardiovascular disease (Mancia *et al.*, 1999). Zoccali and colleagues (2002), found that plasma NE concentrations as a marker of SNA acted as independent predictors of all-cause mortality and cardiovascular morbid events in patients with end-stage renal disease. Although this finding is in a specific population, the association of NE concentrations, SNA, and cardiovascular disease is present. A review by Rozanski *et al.*(1999), explored the psychosocial factors that affect the development of cardiovascular disease. They recognized that in monkeys, chronic psychosocial stress was associated with increased cardiovascular disease, and a majority

of the documented cases were attributed to increases in SNA. Excess SNA leading to increased NE concentrations has been shown to increase smooth muscle cell proliferation via α-adrenoceptor which is a common feature of many cardiovascular diseases including atherosclerosis and coronary artery disease (Erami *et al.*, 2002).

Physical exercise in known to reduce resting SNA in both healthy and mildly hypertensive individuals (Meredith *et al.*, 1991). The reduction in SNA is thought to be partly responsible for the lowering of blood pressure that accompanies increased physical activity (Collins *et al.*, 2000).

NO is one of the most potent vasodilators produced in the vasculature. NO is made by the enzyme nitric oxide synthase (NOS) which has three distinct isoforms: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). NOS produces NO by the conversion of L-arginine and oxygen to L-citrulline and NO. One of the main stimuli for the production and release of NO is shear stress acting on the endothelium of the vascular wall. Shear stress is produced as blood flows along the wall of the blood vessel. The physical shear stress is internalized and causes the phosphorylation (and activation) of eNOS by many different protein kinases (Boo *et al.*, 2002; Li *et al.*, 2005). NO produced in the endothelium diffuses to the vascular smooth muscle where it binds and activates soluble guanylyl cyclase. Guanylyl cyclase causes an increase in the intracellular concentration of cyclic GMP which leads to relaxation of the muscle (vasodilatation) (Munzel *et al.*, 2003). In this experiment, NO metabolites (NO_{met}) in the form of nitrates (NO_3^-), and nitrites (NO_2^-) were measured. In the plasma NO can react with hemoglobin to form NO_3^-. NO can also interact with molecular oxygen to form NO_2^- and with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$).

$ONOO^-$ can then break down to either NO_3^- or NO_2^- depending on the surrounding conditions. NO also interacts with thiols in the presence of electron acceptors to form nitrosothiols which then break down to NO_2^-. NO_3^- and NO_2^- are the bi-products of NO metabolism and have been found to be a valid indicators of NO production (Kelm, 1999).

The effect of exercise (at least in animals), is to increase the activity of NOS (Roberts *et al.*, 1999). In previous bed-rest studies Rudnick *et al.*, (2004) found that the amount of eNOS isoform detected in skeletal muscle samples decreased after bed rest except for those subjects who had exercise countermeasures during HDBR. Exercise seems to have a protective effect on the expression of eNOS and therefore a protective effect on NO production.

In addition to being a potent vasodilator NO has the distinction of being anti-inflammatory, possessing anti-oxidant capabilities, and can prevent cell growth and proliferation (Jeremy *et al.*, 1999; Bult *et al.*, 1999; de Gasparo, 2002). With these actions, one would be inclined to assume that increased activation of NOS would be beneficial. On the contrary, excessive activation of NOS can cause NOS to become uncoupled, and as a result can lead to the formation of O_2^- instead of NO. This molecule in excess is harmful to the vasculature because it is a reactive oxygen species (ROS) that can directly damage tissue and quench (both enzymatic and non enzymatic) antioxidants (Wever *et al.*, 1998).

The anti-inflammatory, anti-oxidant, and anti-proliferative functions of NO are all cardio-protective (Jeremy *et al.*, 1999; de Gasparo, 2002). Panza and colleagues (1993) found that reduced forearm vasoreactivity in patients with essential hypertension was attributable to reduced NO release from the vascular endothelium. They found that using

endothelium dependent vasodilatory mechanisms (acetylcholine) caused a reduction in vasodilation compared to normotensive controls, while endothelium independent vasodilators (sodium nitroprusside) showed no difference between the groups.

While not directly vasoactive, the antioxidant capacity of the plasma and the molecule asymmetric dimethylarginine (ADMA) can have dramatic effects on the availability of NO (Vaziri *et al.*, 1999; Böger, 2003). Having a decreased antioxidant capacity in the plasma is an indicator of one of two things. Either the production of ROS has increased, or the amounts of antioxidants present in the system have decreased. If either of these events occurs, there could be a reduction in the availability of NO and therefore it is unavailable to elicit its protective functions (Vaziri *et al.*, 1999). ADMA is a structural isomer of L-arginine: it is known to inhibit NOS, and therefore decreases the production of NO (Böger *et al.*, 1998; Vallance and Leiper, 2004). Whatever the reason, with less NO, the vasoconstrictive forces of Ang II, ET-1 and NE may begin to dominate and hypertension (and cardiovascular disease) may result (Rajagopalan *et al.*, 1997).

The delicate balance of vasoconstrictors and vasodilators is crucial in maintaining the health of the vascular system. With either too much vasoconstriction or not enough vasodilation this balance is lost and the health of the vasculature is compromised.

1.2.2 Inflammatory Markers

Inflammation in the body is a natural response to infection or injury. The role of an inflammatory response is to clear infectious agents and repair damaged tissue (Barnes and Karin, 1997). In recent years, the inflammatory process has become a major focus in

the progression of many cardiovascular diseases including hypertension, atherosclerosis, and strokes (Griendling *et al.*, 2000; Yeh *et al.*, 2001). Infections leading to inflammatory processes in the vasculature are thought to be an initiating factor in many cardiovascular diseases (Kalayoglu *et al.*, 2002). Inflammatory markers are being examined to explore the link between inflammation and cardiovascular disease. The inflammatory markers include (but are not limited to) C-reactive protein (CRP) and the adhesion molecules E-selectin (E-sel), intracellular adhesion molecule-1 (ICAM), and vascular cell adhesion molecule-1 (VCAM) (Hwang *et al.*, 1997; Vallbracht *et al.*, 2002; Despres, 2004).

CRP is an acute phase protein that is released from the liver in response to tissue injury/infection. CRP release is stimulated by circulating cytokines (de Ferranti and Rifai, 2002). Cytokines are released from damaged cells and act as homing signals for the immune cells (Greaves and Gordon, 2005). The levels of circulating cytokines are modulated by the amount (and extent) of damaged tissue and by levels of adipose tissue (Mohamed-Ali *et al.*, 1997; Bastard *et al.*, 2000). CRP is used as a marker of general inflammation and has recently become one of the most intriguing molecules as it is being considered as an independent risk factor for cardiovascular disease (Ridker *et al.*, 2001). Work by Ridker and colleagues (2002) found that the serum levels of CRP were a better predictor of first cardiovascular events than LDL serum levels. Later work by Ridker's group (2005) and also by Nissen and colleagues (2005) found that lowering CRP levels actually improved (reversed) plaque formation in patients following an acute coronary event. Based on these findings, using CRP levels as an indication of inflammation may be useful in the diagnosis of cardiovascular disease and vascular health.

In order for the immune system to function properly, white blood cells (monocytes, lymphocytes, etc…) need to be able to leave the blood stream at sites of injury/infection. The precise location where the inflammatory cells are needed is marked by an increase in the concentration of cytokines in the area of tissue damage. In order for the immune cells to leave the circulation, some specific events must occur. Transmigration (as it is known) is a very complex process and there are a lot of aspects that are still being explored, however, there are specific steps that must occur for the immune cells to leave the vasculature. First and foremost there must be a chemical signal attracting the cells to the site (Springer, 1994). This chemical signal also serves to activate the endothelial cells lining the lumen of the blood vessel (Zhu *et al.*, 1999). When the endothelial cells become activated they begin to produce and express adhesion molecules that are vital for migration of immune cells into a tissue. E-sel, ICAM, and VCAM are all adhesion molecules that play roles in the transmigration process (Hwang *et al.*, 1997; Zouki *et al.*, 2000).

The next phase of migration involves rolling adhesions between the vessel wall and the immune cells (Lauffenburger and Horwitz, 1996). Rolling adhesions are regulated by the selectin family of molecules of which E-sel plays a major role. Cells continue to roll, until they reach an area of increased integrin expression. Integrins (ICAM and VCAM) create firm adhesions between the cell and the vessel wall and facilitate the transmigration process (Price and Loscalzo J, 1999).

Adhesion molecules are often sloughed off the endothelium and become dissolved in the plasma (Ponthieux *et al.*, 2004). These soluble versions of the adhesion molecules allow for their measurement in the plasma. With increasing concentrations in the plasma,

it can be assumed that there is increased expression and therefore increased inflammation (Vallbracht *et al.*, 2002; Galle *et al.*, 2003).

Adhesion molecule expression is increased in atherosclerotic lesions (Cybulsky and Gimbrone, 1991). This is thought to be due to endothelial activation by numerous factors that are also increased in the atherosclerotic lesion. Factors like modified LDL, ET-1, Ang II, and cytokines are all known to activate endothelial cells and increase adhesion molecule expression (Li *et al.*, 1993; Lerman *et al.*, 1995; Scholz *et al.*, 1996; Pastore *et al.*, 1999; Funk and Cyrus, 2001).

Exercise has been shown to decrease the amount of systemic inflammation (Ford, 2002). CRP has also been shown to be affected by the level of physical activity in that those with high levels of physical activity generally had lower levels of CRP (Albert *et al.*, 2004). It has also been found that moderate losses in weight due to regular exercise result in lower CRP levels (Okita *et al.*, 2004).

The concentrations of CRP, E-sel, ICAM, and VCAM are being used to determine the extent of not only systemic inflammation (Price and Loscalzo, 1999; de Ferranti and Rifai, 2002; Ponthieux *et al.*, 2004), but they are also beginning to be used to assess vascular health, and the risk of cardiovascular disease (Szmitko *et al.*, 2003a; Szmitko *et al.*, 2003b).

1.3 Previous Bed Rest/Space Flight Studies

In past bed-rest studies, many of the aforementioned factors have been investigated. The main goals for many of these studies was to assess the role of each of the molecules in the maintenance (or lack) of orthostatic tolerance (Buckey, Jr. *et al.*, 1996; Levine *et al.*, 1997; Bestle *et al.*, 2001). Due to the nature of the funding for many of the bed rest

studies (usually from international space agencies) the majority of past research has focused on how the body adapts to microgravity. A landmark space flight study was the 1998 Neurolab (STS-90) that was set up to carry out 26 experiments to examine the nervous systems response to microgravity. Of importance to this thesis are a group of studies that examined autonomic function, in particular altered vasoconstrictor responses (Levine *et al.*, 2002; Ertl *et al.*, 2002).

1.3.1 Vasoconstrictors

Many researchers attribute orthostatic intolerance to an impairment of vasoconstriction following prolonged microgravity exposure (Convertino and Sather, 2000; Kimmerly and Shoemaker, 2002). Many of them attribute this to a blunting of the SNA and therefore a reduction in circulating NE following microgravity exposure (Fritsch-Yelle *et al.*, 1994; Convertino, 1998). Reduced NE concentrations cause a decrease in the frequency of many of the signaling cascades that result in vasoconstriction. This reduction in use can cause the down regulation of many of the protein messengers that are integral to the normal function of the tissue (Bennett *et al.*, 1994)

Blunted SNA has been identified as a feature of prolonged bed rest (Shoemaker *et al.*, 1998b), however, other bed rest studies have indicated no change or even an increase in SNA (Shoemaker *et al.*, 1999; Kamiya *et al.*, 2000a). The Neurolab experiments that examined vasoconstrictor responses to space flight indicated that SNA increased during the 16-day mission (Levine *et al.*, 2002). The responses of other vasoconstrictors are not as clear.

Haruna *et al.*, (1997) examined the changes to the renin angiotensin system during 20 days of HDBR. They found that plasma renin activity and aldosterone were increased throughout bed rest, while Ang II levels were only increased in the first few days. The finding of no change in Ang II concentrations is contrary to the findings of Bestle and colleagues (2001), who found the Ang II concentrations doubled following 10 days of HDBR. Bestle *et al.*, attributed these changes to a reduced central blood volume and a reduction in the distension of the atria. Convertino *et al.,* (1998) found that Ang II infusion before and during 30 days of HDBR caused similar vasoconstriction responses, however, during HDBR the Ang II concentration during the infusion was higher than that seen before bed rest.

ET-1 in HDBR has been less well studied. Millet *et al.*, (2000) examined ET-1 concentrations during a 7-day bed-rest study and found that the ET-1 concentrations were unchanged following HDBR. Recent research in our lab revealed elevated ET-1 levels following 4 hours of HDBR (Fischer, unpublished 2006)

1.3.2 Vasodilators

NO_{met} have been shown to decrease following 14 days of HDBR in men (Kamiya *et al.*, 2000b). Although they did not directly measure NO (or NO_{met}), the data of Kamiya et al. (2000b) is supported by the findings of Shoemaker *et al.*, (1998a) who showed a blunted vasodilator response to ischemia following 14 days of HDBR. While this is not direct evidence of reduced NO formation, it does support the idea because of the blunted vasodilator response.

The antioxidant capacity of the body has been shown to decrease during space flight resulting in increased oxidative damage (Smith *et al.*, 2005). Fewer bed-rest

studies have looked at the effects on antioxidant capacity, however, one report has stated that the antioxidant capacity is decreased following long term bed rest (Maillet *et al.*, 2001). The effect of HDBR on the concentration of ADMA is unknown at this time.

1.3.3 Inflammatory Markers

While both bed rest and actual flight studies have been done to assess immune function, the majority of these only report on the levels of cytokines, immune cells, and adhesion molecule antigens on immune cells and the results are usually from experimental animals only (Grove *et al.*, 1995; Schmitt *et al.*, 2000; Sonnenfeld, 2002).

No study to date has examined the levels of CRP in association with HDBR. The only English studies relating bed rest and CRP levels examined the effect of intra-articular steroid use versus bed rest in response to rheumatoid arthritis (Sheeran *et al.*, 1993). The researchers found that CRP levels were lower in the intra-articular steroid group following three and five days of hospitalization, but these results are most likely due to the use of intra-articular steroids and can not be attributed to bed rest since there was no change in the control group.

Like CRP, the levels of circulating adhesion molecules have been studied in a limited capacity. As mentioned, much of the research has revolved around cytokine and immune cell changes with either simulated or actual microgravity (Grove *et al.*, 1995).

1.4 Overview of Current Study

1.4.1 Purpose

The purpose of the current study was to examine the effects of 60 days of HDBR on markers of vascular health, specifically vasoactive molecules and markers of

inflammation, and how countermeasures, in the form of increased protein intake (DIET) and resistive and treadmill running exercise (EX), could prevent these changes.

1.4.2 Hypothesis

This study was designed to test the hypothesis that 60 days of HDBR would cause changes in blood factors associated with increased vasoconstriction and markers of inflammation, and that exercise, but not nutrition countermeasures, during the bed rest would attenuate the response.

Specifically:

1) That 60 days of HDBR will increase the vasoactive molecules Ang II, and ET-1 and ADMA, but decrease NE, NO_{met}, and the antioxidant capacity.

2) That 60 days HDBR will increase markers of systemic inflammation: CRP and the adhesion molecules E-sel, ICAM, and VCAM.

3) Exercise is expected to prevent the changes outlined in 1) and 2) while DIET is expected to have no effect.

2 Methods

2.1 Subjects

The recruitment process for this experiment consisted of advertisements–on television, in newspapers, magazines, and on the internet–that offered women the opportunity to be part of this project. Twenty six hundred responses were generated and information packages were sent to those who inquired. The information packages consisted of an application, a questionnaire on the subject's way of life, education and professional experience, and a medical questionnaire on personal and family medical history. Three hundred and eighty eight of the packages that were sent out were completed and returned. Ninety nine individuals were invited for further medical/psychological screenings. From this pool of 99, 24 female subjects ages 25 – 40 were randomly assigned to one of three experimental protocols–diet (DIET), exercise (EX), or control (CON)–based on their predicted VO_2max and primary language spoken. Specific criteria for inclusion and exclusion can be found in appendix A. Control subjects received no intervention.

2.2 Experimental Protocols

The experimental protocol was established at International Working Group (IWG) meetings that consisted of the 15 principal investigators and representatives of the space agencies that supported the project: the European Space Agency (ESA), the Centre National d'Etudes Spatiales (CNES), the National Aeronautics and Space Administration (NASA) and the Canadian Space Agency (CSA). The experiment consisted of two campaigns that took place from February 2005 to March 2005, and September 2005 to November 2005, at the MEDES space clinic in the Toulouse Rangueil Hospital in

Toulouse, France. Each campaign examined 12 subjects over a 100-day period. The first 20 days consisted of baseline data collection (pre-HDBR), where each experimental team performed their specific tests on one pair of subjects each day. The pre-HDBR counted down from -20 until the start of head down bed rest (HDBR). HDBR consisted of the 60 days of bed rest and counted up from day 1 to 60. During HDBR each of the experimental groups performed their specific countermeasure, and strict adherence to a recumbent position was ensured by 24h/day monitoring by the MEDES staff. Following the HDBR, 20 days of recovery (post-HDBR) ensued. During post-HDBR, the experimental protocols that were performed in pre-HDBR were repeated.

2.3 Experimental Countermeasures

Due to the fact that the experimental countermeasures occurred during HDBR and the experimental protocols were done during the pre- and post-HDBR sections, there were different energy requirements in each phase. Nutrition was maintained by the MEDES staff according to the plan developed by the IWG as described below.

2.3.1 Diet Countermeasure (DIET)

2.3.1.1 Energy intake

During pre- and post-HDBR periods the energy intake of the subjects was matched to their TEE (Total Energy Expenditure) as determined in free living conditions. Exercise level was determined from TEE in free living conditions and RMR (Resting Metabolic Rate) as determined by indirect calorimetry. During HDBR, RMR and fat mass (by DEXA) were measured every 15 days. Caloric intake was corrected accordingly in the hopes of maintaining fat mass throughout the study.

The CON group received 110% of their RMR. The EX group received 110% of their RMR plus energy related to physical activity. This was accurately calculated and administered before each exercise session, maintaining the same energy (carbohydrate/fat)-protein relative composition of the entire diet. The DIET group received 110% of their RMR with the same energy (carbohydrate/fat)-protein composition throughout the experiment.

2.3.1.2 Protein Intake

During pre- and post-HDBR all experimental groups received 1 g of protein/kg/day. During HDBR the CON and EX groups maintained this protein intake (1 g/kg/day), however, the DIET group received 1.6 g of protein/kg/day to try and prevent the muscle wasting caused by the disuse associated with HDBR (Biolo *et al.*, 2005). Total daily energy was achieved by decreasing proportionally the carbohydrate and fat intake in the DIET group. In an attempt to prevent muscle wasting the leucine content in the diet was increased from the natural level of 8% to 20% of total amino acid content of the dietary protein by increasing the proportion of branched chain amino acids (Stein *et al.*, 1999). The increased protein was administered in the form of an added supplement given with the main meals.

2.3.2 Exercise Countermeasure (EX)

During the exercise sessions the subjects were monitored and supervised at all times. Each subject participated in two (more if necessary) orientation sessions to familiarize themselves with the equipment and all training and testing procedures.

2.3.2.1 Cardiovascular Training Program and LBNP

The EX group performed a daily regimen of 35 min of treadmill running at 1-1.2 times body weight followed by 10 min of static LBNP 3-4 times per week. This protocol was similar to that of Cao *et al.*, (2005) with the exception of 10 minutes of static LBNP instead of 5 minutes, and the protocol was performed 3-4 times/week, instead of 6 times/week. Subjects performed an interval exercise protocol (40–80% peak oxygen consumption) with both warm-up and cool-down sessions that consisted of walking at a comfortable pace.

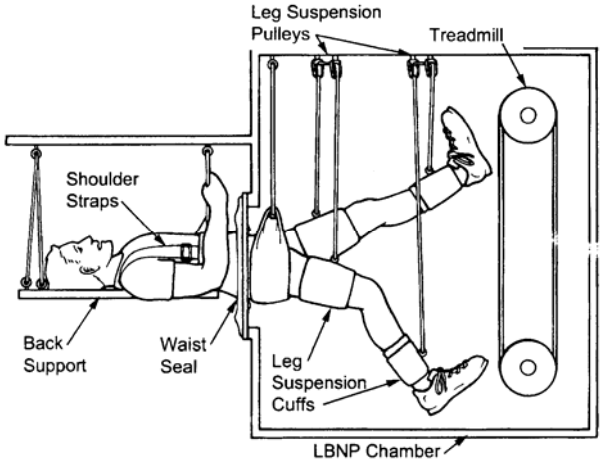

Figure 2.1 Schematic of the LBNP Treadmill
(From Smith *et al.*, 2003)

2.3.2.2 Resistance Training Protocol

Each subject in the exercise group performed warm-up and cool-down periods consisting of 10-15 minutes of stationary cycling at low resistance and slow speed, as well as stretching of the muscle groups involved in the resistance training.

Resistance training of the knee extensor and plantar flexor muscle groups was conducted on a flywheel ergometer that had been modified for bed rest. This is similar to the equipment described by Tesch *et al.,* (2005). The subjects trained both legs using

concentric and eccentric muscle actions; both actions together were considered one repetition. Each session consists of a series of warm-ups followed by the main exercise that included four sets of seven repetitions performed with maximal effort. Force and angle-specific force in each repetition were measured and simultaneously shown on a computer display for instant feedback.

2.4 Ethics

2.4.1 Ethics Committee

Before any experimental protocol began the coordinating investigator submitted the protocol, information form, and consent-of-participants document to the Comités Consultatifs de Protection des Personnes dans la Recherche Biomédicale (CCPPRB).

Once the CCPPRB approved the protocol, a written copy of the approval was forwarded to the "Agence Française de Sécurité Sanitaire des Produits de Santé" (AFSSAPS) for the declaration of intent. No experimental protocol could commence without written consent of the CCPPRB, acknowledgement of receipt of the declaration of intent by the AFSSAPS, and a signature from each contractual party involved.

2.4.2 Study Conduct

The study was performed in accordance with the ethical principles stated in the Declaration of Helsinki 1964, and all applicable amendments.

2.4.3 Information of Participants

The coordinating investigator, or a physician designated by him/her, collected consent from each participant. Prior to this the investigator or the physician informed each

participant of the objectives, benefits, risks, and requirements imposed by the study, and its specific details. The investigator or the physician also provided the participant with an information sheet in clear, simple language. A copy of the information sheet was given in the "Information for and consent of participants" document attached to the protocol. The study participant was allowed ample time to inquire about details of the study and to decide whether or not to participate in the study.

2.4.4 Informed Consent

Each participant gave written informed consent personally before entering the study. Two copies of the consent form were completed, signed and dated by the participant and the physician who conducted the informed consent. The physician gave one copy of the consent form to the participant and retained the other copy.

A copy of the consent form meeting the requirements of the Good Clinical Practice recommendations of the International Conference on Harmonisation (ICH) was given in the "Information for and consent of participants" document attached to the protocol.

2.5 Blood Collection and Separation

Blood samples were drawn by the MEDES staff between 7:00 and 7:30 am on days pre-HDBR-06 (six days before bed rest) and HDBR60 (on the last day of bed rest). Subjects were in a fasted state prior to blood collection. 14 ml of blood was collected and separated into 4, 6, 2.5 and 1.5 ml draws termed *Angiotensin II (Ang II), Main,* S*erum* and C*atecholamine (CATS)* respectively, each designed for the isolation of different blood factors.

2.5.1 Ang II draw

160 µL of EDTA and 80 µL of Bestatin were added to the 4 ml aliquot tube to isolate Angiotensin II. The sample was centrifuged at 2500 RPM at 4ºC for 10 min to separate the cell portion from the plasma. 2000 µL of plasma was isolated and stored at minus 80ºC until analysis.

2.5.2 Main draw

240 µL of EDTA was added to the 6 ml draw. The sample was centrifuged at 2500 RPM at 4ºC for 10 minutes to separate the cell portion from the plasma. The plasma was divided into aliquots of 1000 µL for Endothelin-1 (Endo-1), 200 µL for Nitric Oxide metabolites (NO_{met}), 100 µL for asymmetric dimethylarginine (ADMA), and 50 µL for each of: soluble intracellular adhesion molecule-1 (ICAM), soluble vascular cell adhesion molecule-1 (VCAM), and total antioxidant capacity (ANTIOX). With the plasma separated, the red blood cell (RBC) pellet was re-suspended with a 0.9% Sodium Chloride Injection, USP supplied by B|Braun (Center Valley, PA catalogue number 7983-03), and then prepared according to the method of Bøyum (Bøyum, 1964; Bøyum, 1968) for the isolation and separation of lymphocytes. The remaining RBC pellet was washed three times with 0.9% Sodium Chloride Injection, USP once more, and a sample of RBC was frozen for future analysis. All samples were stored at minus 80ºC until analysis. Excess plasma was saved and stored in the same manner.

2.5.3 Serum draw

Hematocrit was taken in triplicate from the 1.5 ml blood sample before it was allowed to clot at room temperature. The clot was loosened from the edge of the test tube with a wooden dowel and was then centrifuged at 2500 RPM at 4ºC for 10 min to separate the

cell portion from the serum. The serum was drawn off and separated into aliquots of 50 µL for each of C-reactive protein (CRP) and E-selectin (E-sel). The samples were stored at minus 80ºC until analysis. Excess serum was saved and stored in the same manner.

2.5.4 CATS draw

62.5 µL of EGTA/Glutathione was added to the 1.5 ml aliquot tube to isolate Norepinephrine (NE). The sample was centrifuged at 2500 RPM at 4ºC for 10 min to separate the cell portion from the plasma. Approximately 1250 µL of plasma was isolated and stored at minus 80ºC until analysis.

2.6 Blood Analysis

All of the blood analysis was performed in the Kinesiology Biochemistry lab (BMH 2401) at the University of Waterloo. An outline of the specific procedures can be found in appendix C. Quantification of the blood factors was performed using Enzyme-Linked Immunosorbent Assay (ELISA), Enzyme Immunoassay (EIA) Radioimmunoassay (RIA), spectrophotometry, and high performance liquid chromatography (HPLC).

Ang II was analyzed using an EIA purchased from SPI bio (Montigny le Bretonneux, France catalogue number – A05880).

ET-1 was analyzed using an EIA supplied by R&D Systems (Minneapolis, MN, USA catalogue number – BBE 5). Plasma NO_{met} were quantified using a nitric oxide analyzer (model 280i with software version 3.2, according to the procedures detailed in the manufacturers operating manual, Sievers Instruments, Inc., Boulder CO, USA). ADMA concentrations were assessed using an ELISA from ALPCO Diagnostics (Windham, NH, USA catalogue number – 017-EA201/96). ICAM was measured in the

plasma using an EIA purchased from R&D Systems (Minneapolis, MN, USA catalogue number – BBE 1B). The measurement of VCAM was done with an EIA from R&D Systems (Minneapolis, MN, USA catalogue number – DVC00). ANTIOX was measured using an ELISA purchased from ALPCO Diagnostics (Windham, NH, USA catalogue number – 30-5200).

CRP was quantified using an ELISA purchased from ALPCO Diagnostics (Whindham, NH, USA catalogue number – 030-9710s). E-sel concentrations were determined using and EIA from R&D Systems (Minneapolis, MN, USA catalogue number – BBE 2B). All of the instructions from the purchased assay kits were followed without deviation.

NE was quantified using HPLC from Waters Alliance using an HPLC 2690 (Milford, MA, USA). Samples were prepared following procedure in appendix C, and analyzed at a flow rate of 1.2 ml/min using a 15 cm C18, 5 μm particle size Supelco Supelcosil (Sigma-Aldrich Corporation, Bellefonte, PA, USA catalogue number – 58230U) and Waters Guard column module with Resolve C18 guard inserts (Milford, MA, USA catalogue number – WAT085824).

2.7 Plasma Volume

Plasma volume (PV) was assessed using a carbon monoxide (CO) re-breathing system as described by Burge and Skinner (1995). Subjects lay supine and were required to breathe through both an open and closed loop system; both systems were supplemented with 100% oxygen. The test started with 5 minutes of breathing from the open loop in order to flush nitrogen stores from the body (Burge and Skinner, 1995). Following the 5 minutes, the subject gave a signal indicating the end of a normal expiration. Upon this signal, the

subject was turned into the closed loop system via a three-way valve. Normal breathing was maintained for an additional 5 minutes to ensure that the subject was comfortable with the increased resistance of the closed loop, and was able to maintain normal resting tidal volume. The flow of oxygen to the closed loop was matched to the subject's metabolic demands by adding oxygen to a 5 L bag attached to the closed loop. The flow into the bag was maintained so as to not over- or under-inflate the bag. Following this period, a 3ml venous blood sample was drawn by a member of the MEDES staff into a dry heparanized syringe (Vital Signs Inc., NJ, USA). Venous blood was assessed (Nova CO-oximeter, Nova Biomedical, Mississauga, ON, Canada) to determine the %CO on the hemoglobin in the sample. Subjects were then given a priming dose of between 20 and 28 ml of 100% CO in order to raise the %CO bound to the hemoglobin to approximately 3-5%. Subjects continued to breathe in the closed system for 10 minutes after which time another venous blood sample was collected to measure the result of the priming dose. Upon the results of the priming dose, a test dose was administered to cause a change in the %CO bound by approximately 6.5%. This was done with a bolus injection of between 50 and 60 ml 100% CO. Following another 10 minute interval, the %CO bound was again measured by a venous blood sample, and the test %CO bound was assessed. The test dose typically gave a %CO bound of between 8% and 14%.

The line and stopcock used to administer the CO was flushed three times to ensure minimal contamination with room air. The subjects were continually reminded to keep a tight seal on the mouthpiece, but in the event of any feelings of light headedness they could come off the mouthpiece at any time. Calculations of blood volume, plasma volume and of red cell volume were calculated as per Burge and Skinner (1995).

2.8 Statistics

All data are presented as mean ± SD and percent change. Statistical analyses were performed using the Statistical Analysis Software program (SAS Institute, Cary NC, USA). Effects of diet, exercise, and a control group on the physiological and biochemical dependent measures before and after 60 days of HDBR were compared using a two-way repeated measures ANOVA with least squares difference post hoc analysis if required. A $p < 0.05$ was accepted as statistically significant. The interaction of HDBR and Group was also investigated in the ANOVA to determine the interaction between the two independent variables. Again, $p < 0.05$ was accepted as significant.

Correlations between Δ Ang II and Δ PV were analyzed using Sigma Stat for Windows v3.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted as $p < 0.05$. SAS was also used to assess power calculations for CRP. This analysis was used to assess the number of subjects that would be required per group in order to achieve significance.

3 Results

This study explored the relationship between 60 days of HDBR, and factors linked with cardiovascular disease. The subject's characteristics can be found in Table 3.1.

3.1 Weight and Plasma Volume

All 24 of the women completed the 60 days of HDBR, and all of the subjects lost on average 3 kg of body mass. The CON group lost the most weight with a 3.2 kg decrease ($p < 0.0001$), followed by the EX group with a loss of 3.0 kg ($p < 0.0001$), and finally the DIET group lost an average of 2.7 kg ($p < 0.0001$) (Figure 3.1). Absolute values of plasma volume were reduced following 60 days of HDBR, but the reduction varied between groups. The EX group had a decrease of 98.4 ml (3.4% decrease, $n = 7$, $p = 0.4$), the CON group had a decrease of 209.8 ml (7.8% decrease, $n = 8$, $p = 0.058$), and the DIET group had the largest decrease of 415.4 ml (13.9% decrease, $n = 8$, $p = 0.0007$) (Figure 3.2). When plasma volume was normalized by weight (ml/kg) there was no difference from pre- to post-HDBR (Figure 3.3).

Table 3.1 Subject Characteristics as Measured on pre-HDBR-20 by the MEDES Nursing Staff

Letter code	Group	Nationality	Age	Weight at inclusion (kg)	Height (cm)	Group Average Age	Group Average Weight at inclusion (kg)	Group Average Height (cm)
A1	Exercise	Czech	28	65.4	170			
B1	Exercise	Dutch	36	55.4	158			
C1	Exercise	French	35	56.9	172			
D1	Exercise	English	27	51.5	152			
E2	Exercise	French	36	58.6	163			
F2	Exercise	English	35	62	168			
G2	Exercise	French	33	52.3	164			
H2	Exercise	French	31	70.4	171	33 ± 3.6	59.1 ± 6.5	165 ± 7.0
E1	Control	Finish	29	57.4	163			
F1	Control	Polish	31	57.8	172			
G1	Control	French	32	58.7	170			
H1	Control	French	40	56	158			
A2	Control	French	38	61.2	166			
B2	Control	French	34	55.3	161			
C2	Control	French	34	53.6	157			
D2	Control	French	37	49.3	155	34 ± 3.7	56.2 ± 3.6	163 ± 6.2
I1	Diet	French	29	65.8	168			
J1	Diet	French	36	57.5	166			
K1	Diet	German	29	63.3	165			
L1	Diet	Polish	25	60.8	174			
I2	Diet	Finish	31	61.6	172			
J2	Diet	Finish	29	54.2	166			
K2	Diet	Finish	31	69.9	181			
L2	Diet	Swiss	25	60.9	170	29 ± 3.5	61.8 ± 4.8	170 ± 5.4
All Subjects						32 ± 4.1	59.0 ± 5.4	166 ± 6.8

Averaged data are mean ± SD

Alpha-numeric code refers to subject (A-L) and campaign (1 or 2)

Data was obtained by MEDES staff on pre-HDBR-20 (first day of each campaign)

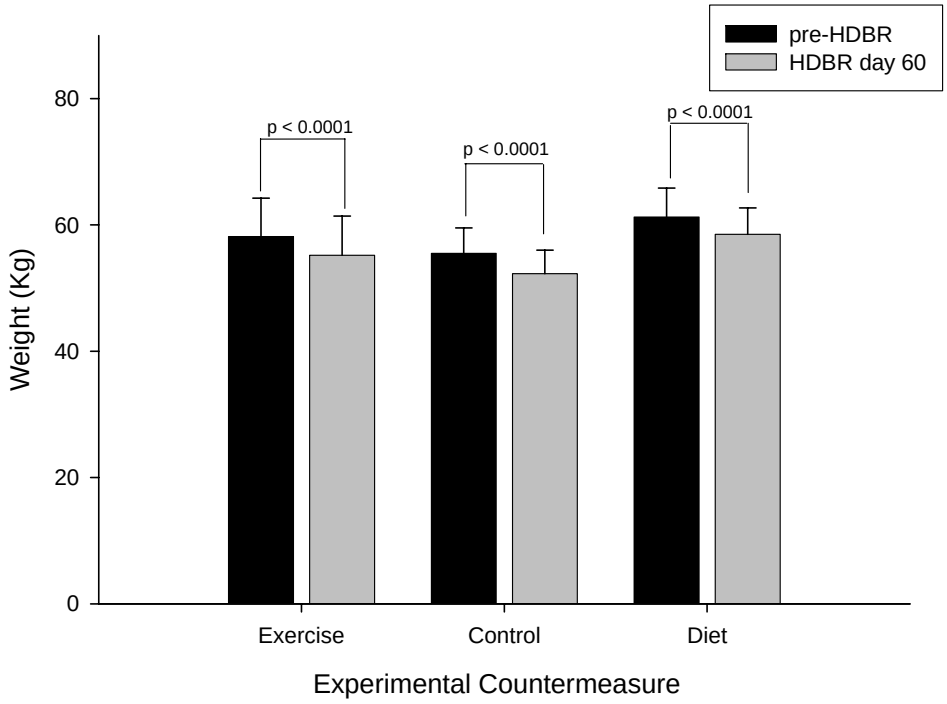

Figure 3.1 The effect of 60 days of HDBR on weight (kg)
Values are mean ± SD

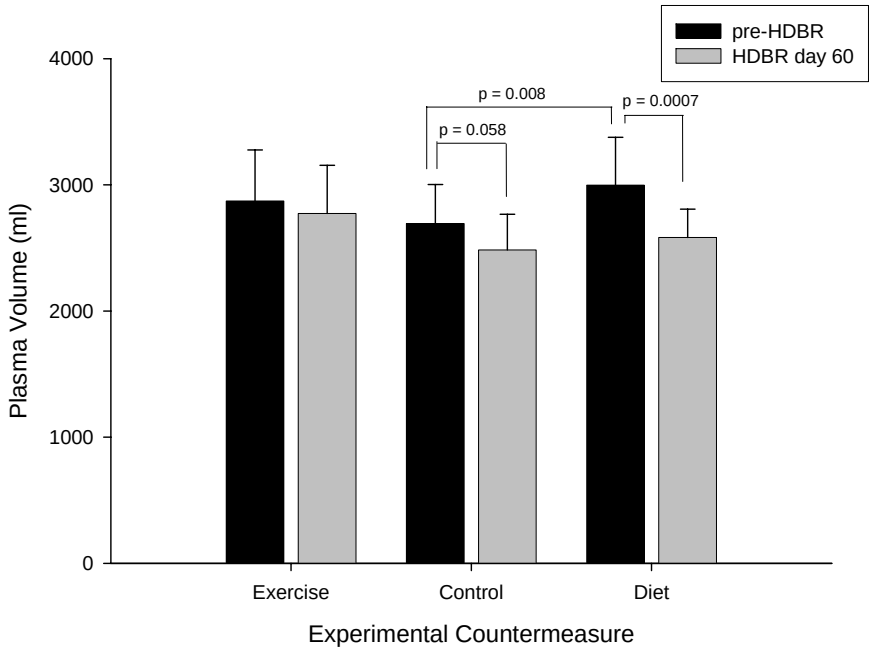

Figure 3.2 The effect of 60 days of HDBR on plasma volume (ml)
Values are mean ± SD

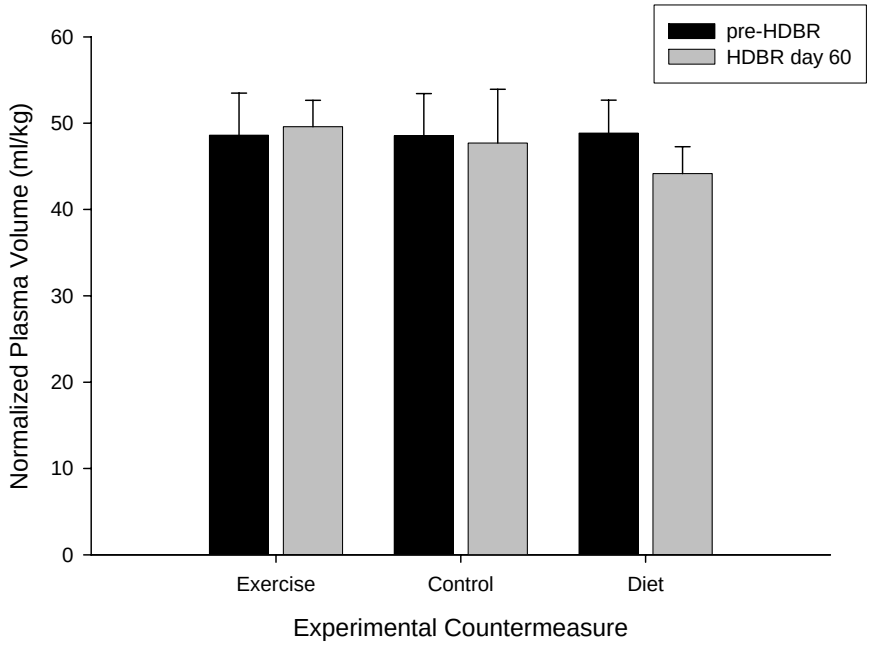

Figure 3.3 The effect of 60 days of HDBR on normalized plasma volume (ml/kg)
Values are mean ± SD

3.2 Baseline Differences

Many of the comparisons made in this thesis were within group comparisons, however, it must be stated that in a few of the measured variables there were significant differences seen in baseline values between the groups. The subjects were randomized according to their predicted VO_2max and primary language spoken. While we made every attempt to randomize the subjects, uneven distribution of other variables is unavoidable. A complete list of the baseline values of measured blood variables can be found in Table 3.2

Table 3.2 Baseline Values for Measured Blood Variables

Parameter	Group		
	Exercise	**Control**	**Nutrition**
Ang II (pg/ml)	21.5 ± 11.8	19.2 ± 11.9	14.8 ± 8.4
ET-1 (pg/ml)	0.59 ± 0.088	0.63 ± 0.14	0.65 ± 0.076
NE (pg/ml)	256.6 ± 132.3	229.1 ± 100.3	271.1 ± 133.8
NO_{met} (μmol/l)	62.5 ± 30.2*	32.1 ± 11.0	41.8 ± 16.9
ANTIOX (μmol/l)	271.7 ± 49.6	268.4 ± 38.6	289.2 ± 21.6
ADMA (μmol/l)	0.68 ± 0.13*	0.92 ± 0.084	0.83 ± 0.17
CRP (μg/ml)	0.251 ± 0.212	0.279 ± 0.202	0.157 ± 0.142
E-sel (ng/ml)	17.4 ± 5.1*	25.7 ± 10.9	25.5 ± 7.8
ICAM (ng/ml)	178.9 ± 35.4	164.7 ± 45.1	178.3 ± 35.4
VCAM (ng/ml)	553.1 ± 101.4	624.3 ± 85.8*	533.0 ± 112.2

Values are mean ± SD

* Indicates baseline values are significantly different from other groups ($p < 0.05$)

ANG II = angiotensin II, ET-1 = Endothelin-1, NO_{met} = nitric oxide metabolites, ANTIOX = total antioxidant capacity, ADMA = asymmetric dimethylargenine CRP = C-reactive protein, E-Sel = E-selectin, ICAM = soluble intracellular adhesion molecule-1, VCAM = soluble vascular adhesion molecule-1.

3.3 Vasoactive Molecules

Table 3.3 contains the values of the measured vasoactive molecules. The values are mean ± SD.

The most drastic change seen in any of the molecules measured occurred with the molecule Ang II. This molecule showed an increase in the CON and DIET groups of 52.9% ($p = 0.014$) and 124.2% ($p < 0.0001$) respectively, with no change seen in the EX group ($p = 0.80$) (Figure 3.3). ET-1 showed no changes in any of the groups (Figure 3.4). NE showed decreases in all groups, however, these decreases were not significant (Figure 3.5). Relationships between Δ Ang II and Δ PV did not show strong correlations in any of the experimental groups. In the EX group $r = -0.081$ ($p = 0.849$), in the CON group $r = -0.188$ ($p = 0.780$), and in the DIET group $r = 0.422$ ($p = 0.298$).

NO_{met} in the plasma showed decreases in all groups. The EX group decreased 37.7% ($p = 0.013$), the DIET group decreased by 42.1% ($p = 0.056$), and the CON group decreased by 17.3%, although this was not significant ($p = 0.53$) (Figure 3.6).

The antioxidant capacity of the plasma showed a trend towards a decrease in all groups, however, these changes were not significant (Figure 3.7). The concentrations of ADMA showed no changes in any of the groups (Figure 3.8).

Table 3.3 The Effect of 60 Days HDBR on the Concentration of Plasma Vasoactive Molecules

Variable	Group					
	Exercise		Control		Diet	
	Pre HDBR	Post HDBR	Pre HDBR	Post HDBR	Pre HDBR	Post HDBR
ANG II (pg/ml)	21.5 ± 11.8	20.6 ± 19.3	19.2 ± 11.9	29.3 ± 12.3*	14.8 ± 8.4	33.2 ± 17.8*
ET-1 (pg/ml)	0.59 ± 0.088	0.63 ± 0.10	0.63 ± 0.14	0.62 ± 0.093	0.65 ± 0.076	0.60 ± 0.090
NE (pg/ml)	256.6 ± 132.3	227.8 ± 83.7	229.1 ± 100.3	188.0 ± 79.8	271.1 ± 133.8	201.9 ± 118.2
NO_{met} (μmol/l)	62.5 ± 30.2	38.9 ± 17.6*	32.1 ± 11.0	26.5 ± 14.4	41.8 ± 16.9	24.2 ± 10.04*
ANTIOX (μmol/l)	271.7 ± 49.6	244.7 ± 33.8	268.4 ± 38.6	247.9 ± 26.2	289.2 ± 21.6	278.5 ± 20.3
ADMA (μmol/l)	0.68 ± 0.13	0.76 ± 0.11	0.92 ± 0.084	0.87 ± 0.14	0.83 ± 0.17	0.83 ± 0.22

Values are means ± SD

* Indicates significance of $p < 0.05$ for pre to post comparison.

ANG II = angiotensin II, ET-1 = Endothelin-1, NO_{met} = nitric oxide metabolites, ANTIOX = total antioxidant capacity, ADMA = asymmetric dimethylargenine.

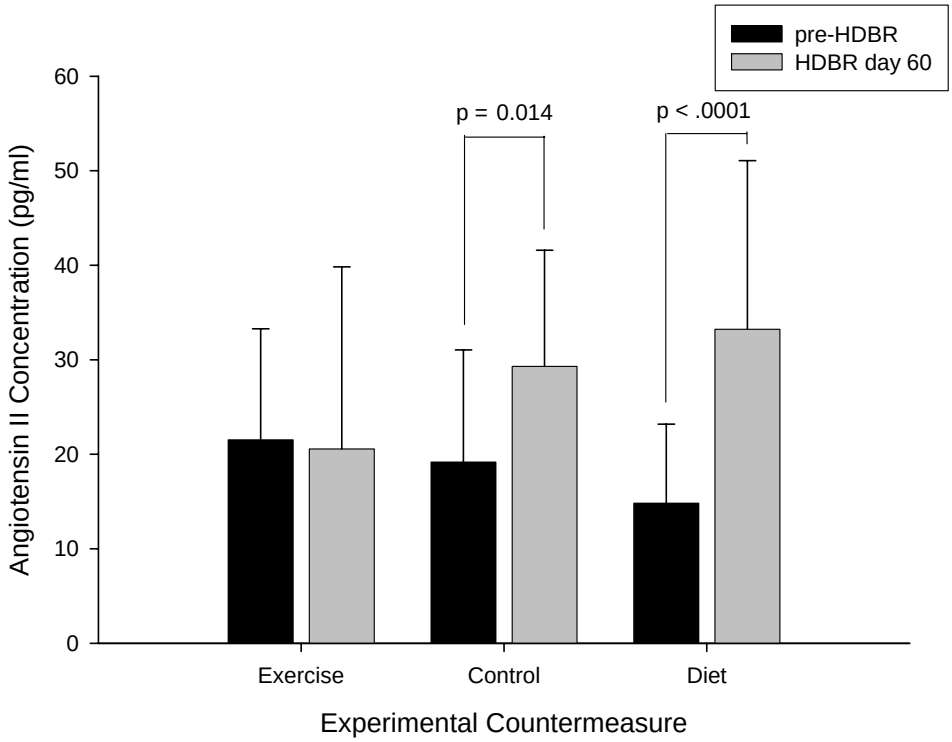

Figure 3.4 The effect of 60 days HDBR on the concentration of angiotensin II (pg/ml)
Values are mean ± SD

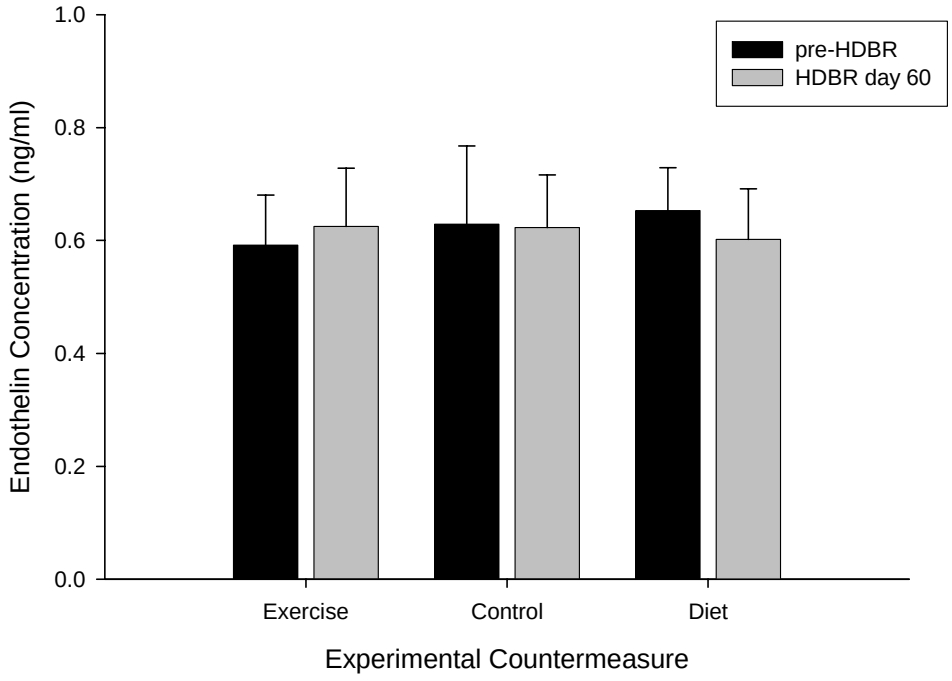

Figure 3.5 The effect of 60 days HDBR on the concentration of endothelin-1 (ng/ml)
Values are mean ± SD

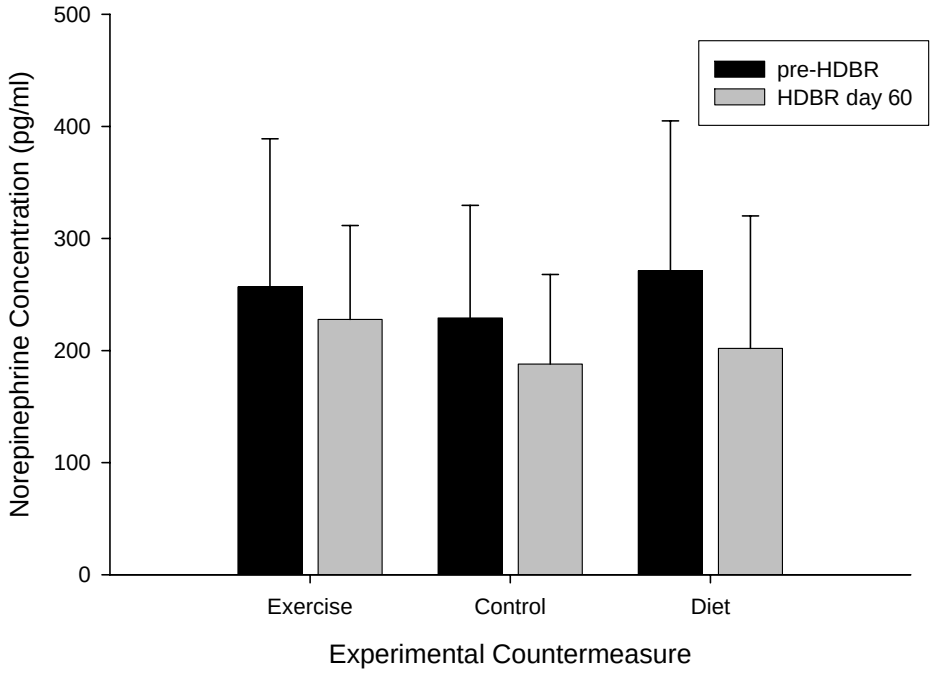

Figure 3.6 The effect of 60 days HDBR on the concentration of norepinephrine (pg/ml)
Values are mean ± SD

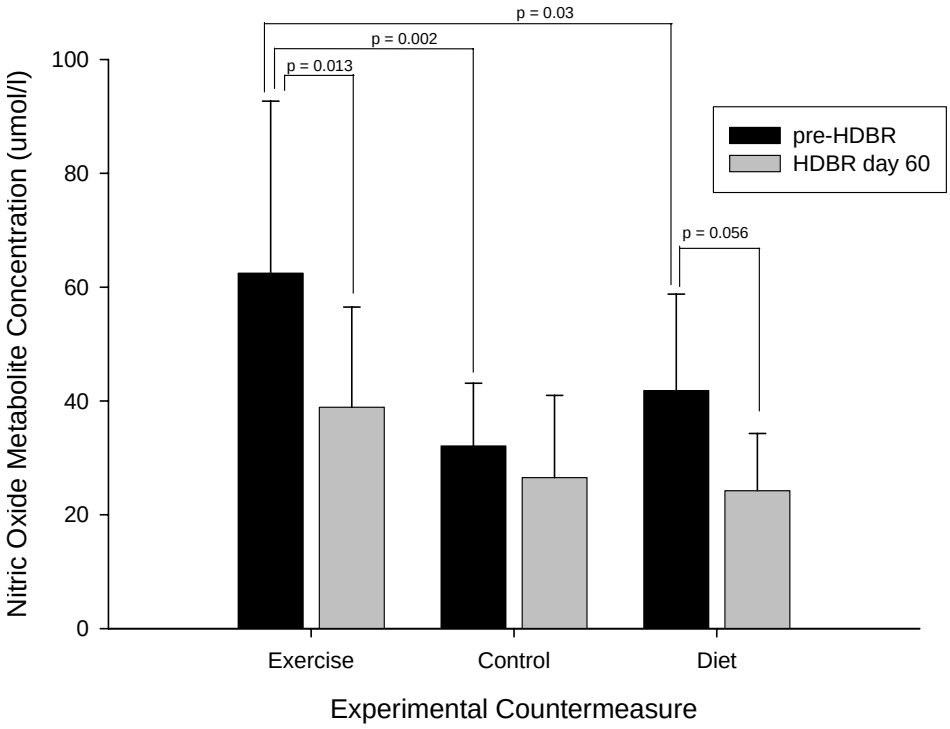

Figure 3.7 The effect of 60 days of HDBR on the concentration of nitric oxide metabolites (µmol/l)
Values are mean ± SD

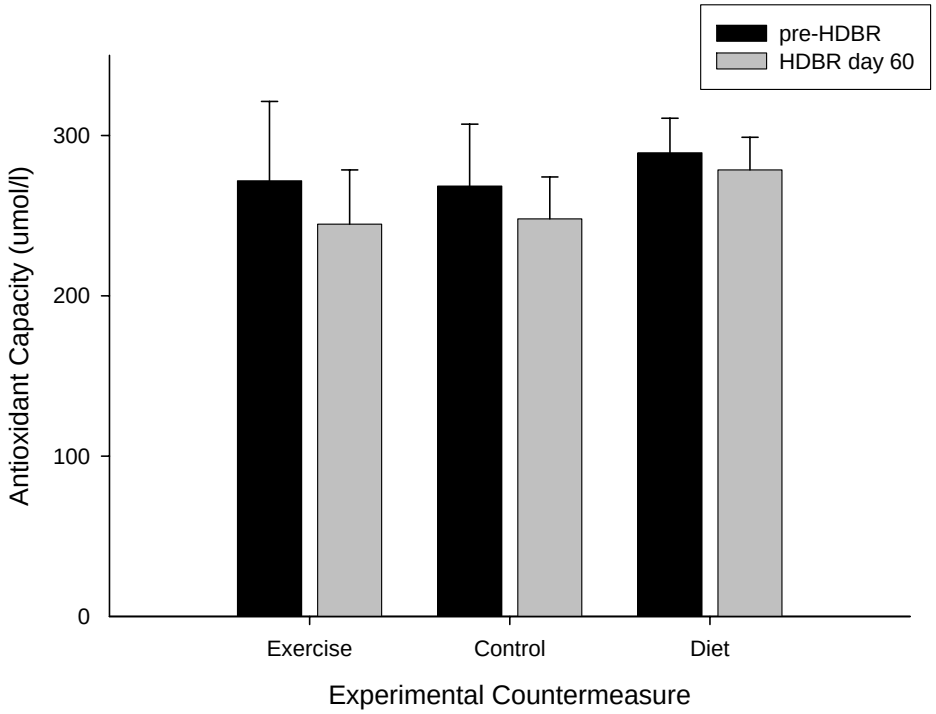

Figure 3.8 The effect of 60 days of HDBR on the antioxidant capacity of the plasma (µmol/l)
Values are mean ± SD

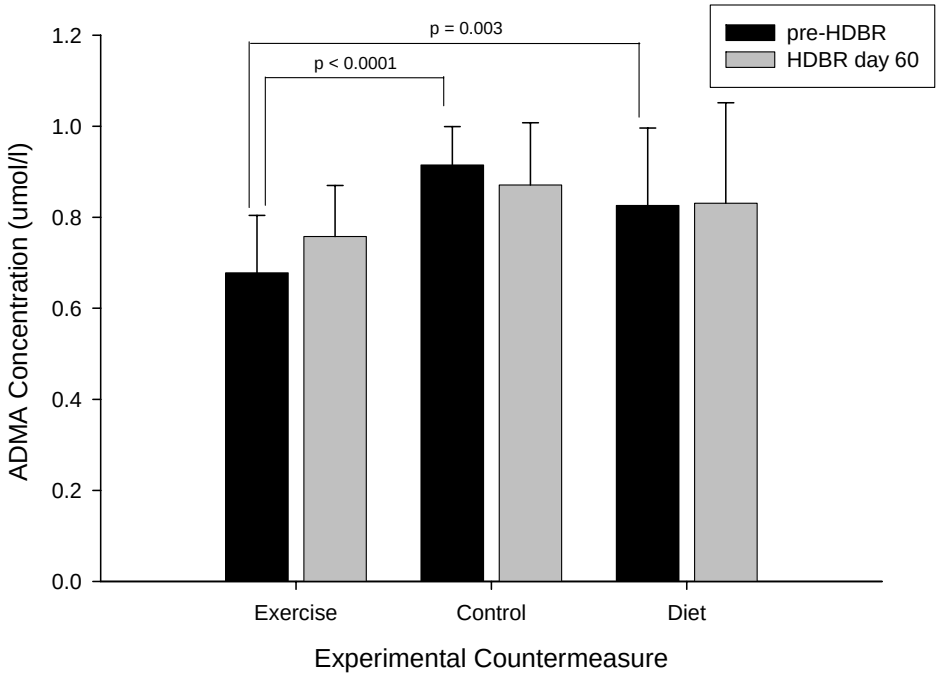

Figure 3.9 The effects of 60 days HDBR on the concentration of asymmetric dimethylarginine (µmol/l)
Values are mean ± SD

3.4 Inflammatory Markers

Table 3.4 contains the values of the measured inflammatory markers. The values are mean ± SD.

There was a non-significant decrease in the serum concentration of CRP in the EX group dropping by 56.3% ($p = 0.12$). The CON group showed an increase of 60.1% ($p = 0.063$), and the DIET group increased by 47.6% ($p = 0.40$). The directions of the changes in the EX group (increasing), and the CON and DIET groups (decreasing), resulted in a significant HDBR x Group interaction effect ($p = 0.05$) (Figure 3.10).

Power analysis on CRP showed that the numbers required to achieve significance were well beyond those that were in this experiment. The EX group would have needed 19 subjects, the CON group would have needed 39 subjects, and the DIET group would have needed 48 subjects to achieve a power of 0.85 based on the group means and standard deviation of the differences of the mean.

ICAM showed no significant changes in any of the groups (Figure 3.11), while E-sel and VCAM showed significant changes. However, these changes occurred in different groups. E-sel showed an increase of 18.2% ($p = 0.003$) in the DIET group, and VCAM showed an 8.0% increase ($p = 0.016$) in the CON group, and a non-significant 6.5% increase ($p = 0.08$) in the DIET group. The EX group showed no changes in either molecule (Figure 3.11).

Table 3.4 The Effect of 60 Days of HDBR on Markers of Systemic Inflammation

Variable	Group					
	Exercise		Control		Diet	
	Pre HDBR	Post HDBR	Pre HDBR	Post HDBR	Pre HDBR	Post HDBR
CRP (μg/ml)	0.251 ± 0.212	0.110 ± 0.154	0.279 ± 0.202	0.448 ± 0.416	0.157 ± 0.142	0.232 ± 0.185
E-SEL (ng/ml)	17.4 ± 5.1	16.9 ± 5.2	25.7 ± 10.9	25.1 ± 9.6	25.5 ± 7.8	30.1 ± 9.5*
ICAM (ng/ml)	178.9 ± 35.4	181.9 ± 25.2	164.7 ± 45.1	166.3 ± 50.8	178.3 ± 35.4	187.7 ± 33.7
VCAM (ng/ml)	553.1 ± 101.4	574.7 ± 88.3	624.3 ± 85.8	674.0 ± 98.2*	533.0 ± 112.2	567.8 ± 119.4

Values are means ± SD

* Indicates significance of $p < 0.05$ for pre to post comparison.

CRP = C-reactive protein, E-Sel = E-selectin, ICAM = soluble intracellular adhesion molecule-1, VCAM = soluble vascular adhesion molecule-1.

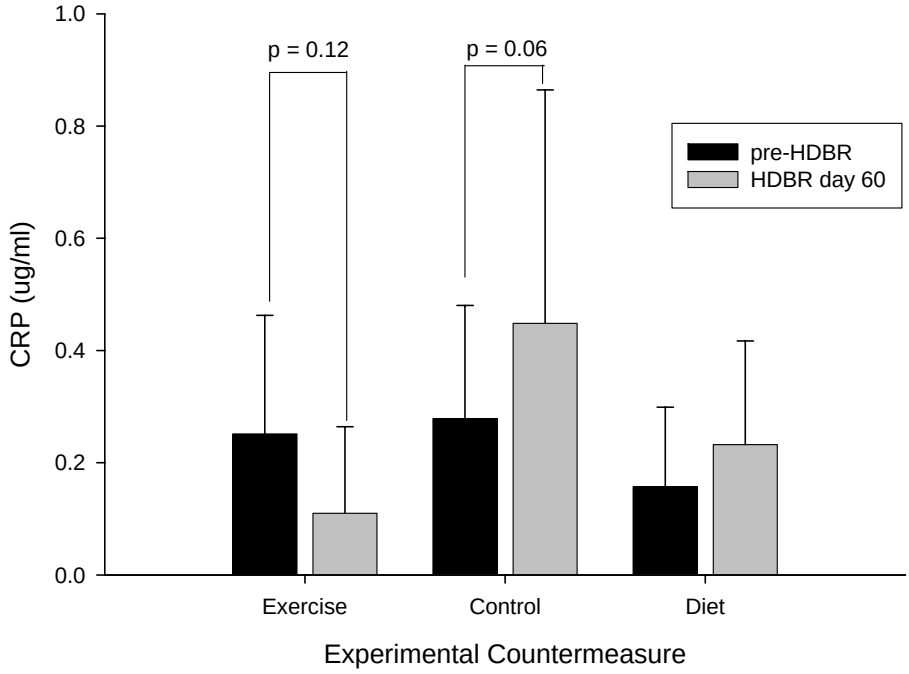

Figure 3.10 The effect of 60 days of HDBR on the concentration of C- reactive protein (ng/ml)
Values are mean ± SD

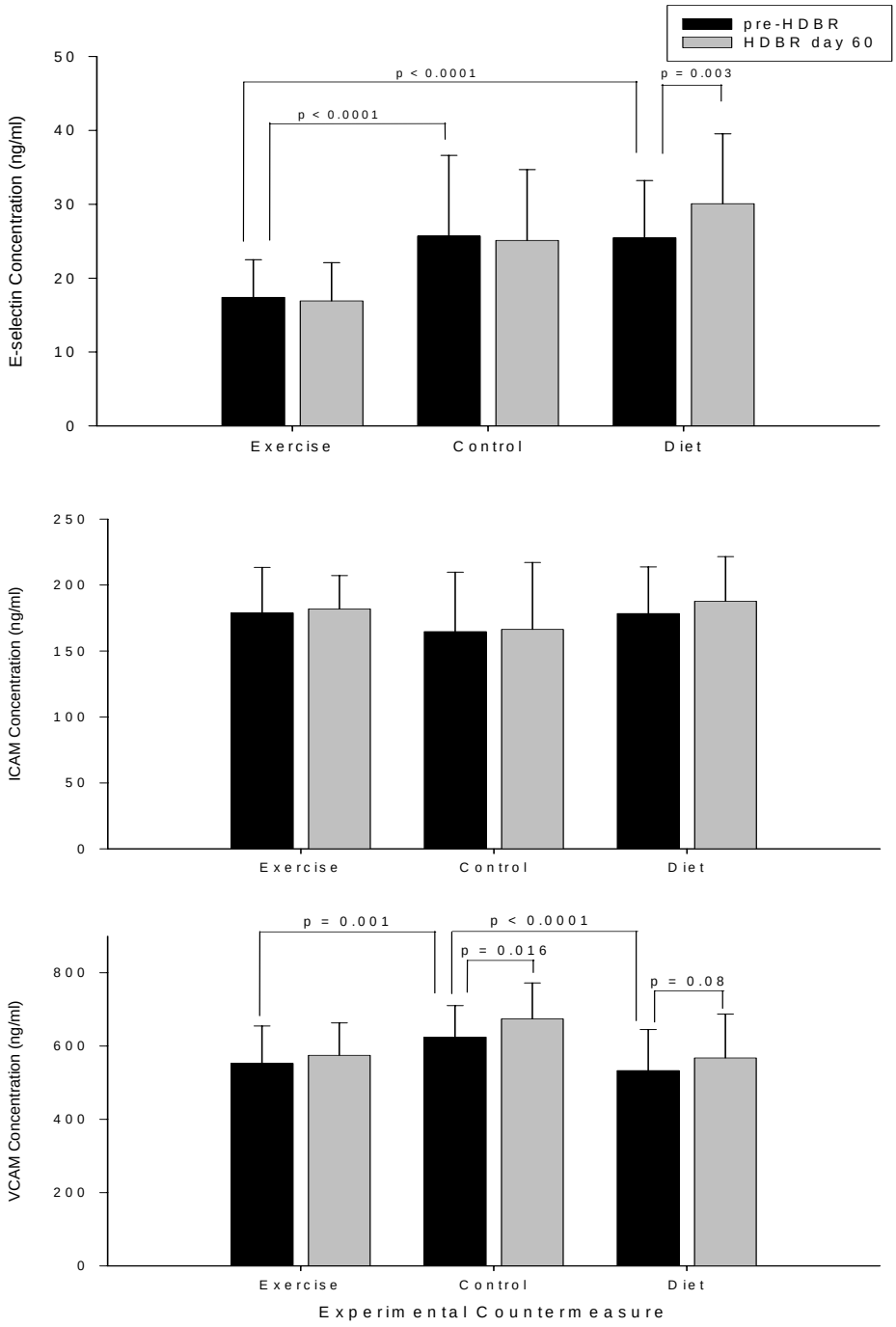

Figure 3.11 The effect of HDBR on the concentration of the different adhesion molecules (ng/ml)
Values are mean ± SD

4 Discussion

This study was the first long-term bed-rest study that examined the physiological changes that occurred in women. While studies have been done that compare the responses of men and women, they have been relatively short in duration (14 – 17 days) (Convertino *et al.*, 1977; Convertino, 1998).

With respect to the hypotheses outlined:

1) Following 60 days of HDBR, Ang II increased as hypothesized. HDBR had no effect on the concentrations of ET-1 or ADMA.

NE and the antioxidant capacity showed non significant decreases in all groups and NO_{met} showed significant decreases in the EX and DIET groups.

2) Many of the markers of vascular inflammation that were hypothesized to increase following 60 days of HDBR were significantly elevated when compared to baseline values. CRP showed non significant changes in all groups, however the directions of the changes (decrease in the EX while increasing in the CON and DIET groups) led to a significant HDBR by Group interaction. E-sel showed a significant increase in the DIET group but not in the CON and EX groups, while ICAM showed no significant change in any group. VCAM showed a significant increase in the CON group, and a non-significant increase in the DIET group, with no change in the EX group.

3) As outlined in 1) and 2) exercise was able to attenuate changes that occurred with the exception of NO_{met}, while DIET (for the most part) was ineffective at preventing any changes.

The results of this experiment indicate that 60 days of HDBR can cause shifts in the blood borne markers that collectively indicate changes in vasoconstrictor and inflammatory responses suggesting an environment of reduced vascular health.

4.1 Vasoactivity

The delicate balance between vasoconstrictors and vasodilators is one of the main determinants of vascular tone. If the balance is tipped in favor of one side over the other, vascular tone can be altered and the health of the vasculature can be compromised.

4.1.1 Angiotensin II

One of the main findings of this study is the large increase in Ang II in the CON and DIET groups, with no change seen in the EX group. This finding is supported by Bestle *et al.*, (2001) who found a doubling of Ang II following 10 days of HDBR. The Bestle paper attributed the increase to central hypovolemia causing reduced pressure on the atria of the heart; this mechanism does not explain the sustained increase following such a long period of time. Since the main function of Ang II is to maintain fluid balance via aldosterone release and increased re-absorption of sodium in the kidney, Bestle's explanation may be relevant in short term maintenance of plasma volume. Following 60 days of HDBR, the increase seen in the concentration of Ang II in the CON and DIET groups may have been an attempt to return plasma volume back to normal levels. This is because of the sustained reduction in plasma volume that was observed in the CON and DIET groups. The EX group showed no reduction in plasma volume, and therefore there was no reason for Ang II to be increased in that group.

An interesting feature of Ang II that needs to be mentioned is its role in inflammation. Increasing evidence is linking Ang II to many processes in the inflammatory pathway, including increased permeability, leukocyte infiltration, and tissue remodeling (Cheng *et al.*, 2005). While increasing the permeability of the vasculature can have its disadvantages, it is the increased leukocyte infiltration and tissue remodeling that are critically important in vascular health.

Ang II can stimulate the production of adhesion molecules (both selectins and integrins (ICAM and VCAM)) through redox sensitive pathways. Ang II increases the production of ROS and this leads to the expression of nuclear factor kappa B (NF-κB) and other redox sensitive messengers (Suzuki *et al.*, 2003; Nakashima *et al.*, 2006). NF-κB is a transcription factor that is necessary for the production of many immune molecules including the aforementioned adhesion molecules as well as cytokines and other factors that activate various cell types (endothelial cells, vascular smooth muscle) (Suzuki *et al.*, 2003). Since we saw increases in Ang II in the same groups that showed increases in the adhesion molecules the increases in the adhesion molecules may have been in response to the increased Ang II concentrations.

4.1.2 Nitric Oxide Metabolites

The decrease in NO_{met} seen in all groups is contrary to what was hypothesized. We expected to see a decrease in the CON and DIET groups, but expected exercise to preserve plasma concentrations of NO_{met}. The decrease in NO_{met} has been seen in previous bed rest studies by Kamiya *et al.*, (2000b) who gave two explanations for the decrease in NO_{met}. Firstly, the lack of physical exercise may reduce NO expression. The

reasoning for this explanation is due to the fact that an increase in physical activity is known to increase NO production through phosphorylation (activation) of eNOS (Sessa *et al.*, 1994; Roberts *et al.*, 1999), so a reduction in physical activity may reduce NO production because of less eNOS activation. Secondly, they proposed that hypovolemia may have reduced the shear stress in the blood vessels, therefore reducing the formation of NO. NO production is stimulated by shear sensitive mechanisms, and a reduction in these mechanisms may reduce NO production. The decrease seen in the EX group was surprising, and does not fit with the explanations given by Kamiya *et al.* (2000b).

The changes seen in the concentration of NO_{met} can not be attributed to changes in the antioxidant capacity or the concentration of ADMA. Since there were no significant changes in either factor it can be assumed that neither affected the production of NO_{met}.

The data given for Ang II and NO_{met} support the idea that 60 days of HDBR causes changes in the balance between vasoconstrictors and vasodilators that can change the normal functioning conditions of the vasculature.

4.2 Inflammation

Inflammation is a normal and expected part of the healing process. While it is welcomed in most circumstances, other times it can have dramatic and negative impacts on an individual's health (Stokes *et al.*, 2002; Galle *et al.*, 2003). More and more cardiovascular diseases have been linked to inflammatory processes, and a growing number of researchers are looking at the link between inflammation and exercise as a way to manage disease (Ford, 2002; Nicklas *et al.*, 2004).

While exercise is known to have beneficial effects on markers of systemic inflammation, the effects of HDBR on these same markers had not been explored prior to this study. Experiments by Laufs and colleagues (2005) showed that in rats, inactivity caused endothelial dysfunction, increased oxidative stress, reduced eNOS activity, and increased atherosclerotic plaque formation. The disease atherosclerosis is now considered to be, in part, an inflammatory disease. Elevated levels of CRP are associated with the development of atherosclerosis, and in an atherosclerotic lesion, endothelial cells are activated, increasing the expression of adhesion molecules and cytokines (Libby *et al.*, 2002; Blankenberg *et al.*, 2003).

4.2.1 C-reactive Protein

While CRP has been linked to many disorders that are associated with inactivity (Ridker *et al.*, 2001), this is the first report on the effect of long term bed rest on the levels of CRP. With the significant HDBR and Group interaction the directions of change for all of the experimental groups becomes very meaningful. An apparent increase in the concentration of CRP in the CON and DIET groups following HDBR indicates that the amount of systemic inflammation may have increased. This has increased meaning when you consider that the EX group showed a tendency to decrease the concentration of CRP, indicating that the amount of systemic inflammation seemed to have decreased in this group. Previous research into the link between exercise and CRP levels confirms that exercise is able to reduce levels of CRP (Okita *et al.*, 2004; Obisesan *et al.*, 2004; Albert *et al.*, 2004). When the magnitudes of the changes seen in CRP are examined in relation to the clinical values that are becoming indicative of cardiovascular disorders, the values

seen here fall within the lowest quintile of risk (≤ 0.49 μg/ml). While this does not indicate increased risk for the development of cardiovascular disease, the changes can be associated with the amount of inflammation in general (Ridker *et al.*, 2002).

While reduced CRP levels are associated with weight reductions (Okita *et al.*, 2004), it has also been found that the amount of adipose tissue may play a role in modulating cytokine levels, which influence the release of CRP (Mohamed-Ali *et al.*, 1997; Bastard *et al.*, 2000). The EX group maintained their thigh and most of their calf volume, while the CON and DIET groups lost 21 ± 1% and 24 ± 2% volume in the thigh respectively ($p < 0.05$ for both) (Burd *et al.*, 2006). It can be assumed that the majority of this volume is due to maintenance of muscle mass. With the CON and DIET groups losing such a large amount of muscle mass, the proportion of adipose tissue relative to lean mass increased. This increased ratio may lead to higher production of cytokines that can stimulate the release of CRP. With the maintenance of leg volume in the EX group, the lean mass to adipose ratio would be maintained and the increased CRP would not occur. Also the exercise itself is thought to have a positive effect on CRP levels (Despres, 2004; Okita *et al.*, 2004).

Another reason for the increase seen in the levels of CRP in the groups that did not perform any exercise could be due to the increased Ang II levels in the same groups. As mentioned earlier, Ang II is emerging as a major mediator of inflammation. Increased Ang II levels have been associated with increased vascular smooth muscle cell activation and increased cytokine production (Kranzhofer *et al.*, 1999; Cheng *et al.*, 2005). These increases are stimuli for the production and release of CRP form the liver (Jialal *et al.*, 2004).

Due to the variable nature of CRP and the wide range of physiologic concentrations that can be achieved, power analysis reveals that the group sizes were too small to achieve significance. Thus, if the changes were to be seen in CRP, larger groups were needed (see section 4.3 Limitations).

4.2.2 Adhesion Molecules

The increases seen in the DIET and CON groups for the adhesion molecules E-sel and VCAM respectively, indicate that there may have been an increase in the amount of inflammation (Hwang *et al.*, 1997; Libby and Ridker, 1999). In spite of the fact that the increases seen occurred in different groups (DIET for E-sel, and CON for VCAM), the fact that VCAM approached significance in the DIET group, and that there was no increase seen in the EX group, helped to support the idea that HDBR may increase systemic inflammation and exercise can protect against it (Ford, 2002; Despres, 2004).

4.3 Limitations

The large number of research groups involved in this study meant that some groups were unable to measure some variables in the manner that they wanted. For example, with so many groups involved, the quantity of blood that was available for each group, and the time available with the subjects was severely limited. This meant that the project was limited to 14 ml of blood taken on one day before bed rest and one day during bed rest. The original proposal was to take the same samples on consecutive days in order to get a more accurate average. This was not possible, because with so many groups requesting blood samples, it was necessary to restrict sampling to remain under the limit set by the ethics committee.

Another limitation of the study is the fact that blood samples were only taken before (pre-HDBR-06) and at the end of bed rest (HDBR60). This two point comparison prevented us from observing the timeline of the changes. Ideally, it would have been beneficial to collect samples from the subjects during the bed rest so that a profile of how each molecule changed could be established. This would have given us the chance to see if any of the changes were in the process of either increasing or decreasing in magnitude at the end of the bed rest, or if the molecules that showed no change had in fact done so and had returned back to baseline values for the final sample.

When looking at CRP levels, according to the power analysis preformed, the EX, CON, and DIET groups, would have needed 19, 39, and 48 subjects respectively to achieve significance. These large numbers reflect the variable nature of this molecule and were unattainable in this experiment.

With respect to variables measured, it would have been beneficial to measure more markers of both fluid regulation and inflammation. The measurement of plasma renin activity (or perhaps aldosterone) would have given a better indication of why Ang II was increased: for fluid regulation or for other reasons. Similarly, measuring other markers of inflammation, specifically pro- or anti-inflammatory cytokines, would give a better indication of the inflammatory state of the body.

4.4 Conclusion and Summary

Sixty days of HDBR in women caused a shift in many cardiovascular risk factors towards a state of increased risk. These changes include a propensity for increased vasomotor tone, as a result of a higher concentration of Ang II, and a decreased concentration of

NO_{met}. Exercise during the bed rest prevented the increase in Ang II, but had no protective effect on the NO_{met}. Increased protein intake by the DIET group did not have any important effects on vasoactive molecules compared to the CON group.

Sixty days of HDBR also increased the release of CRP and adhesion molecules expression in the non-exercising subjects. Increased protein intake had no effect on these values. Exercise seemed to protect the body from increased inflammation during prolonged bed rest.

Appendix A

Specific Inclusion and Exclusion Criteria of the Subjects

Acceptance criteria

- Healthy female volunteer (see below the description of medical tests and laboratory analysis),
- Age 25 to 40,
- Non smokers,
- No alcohol, no drug dependence and no medical treatment,
- Height less than 185 cm,
- No overweight nor excessive thinness with BMI (weight Kg/ height m^2) between 20 and 25,
- Regular menstrual cycles,
- No personal nor family past record of chronic or acute disease or psychological disturbances which could affect the physiological data and/or create a risk for the subject during the experiment,
- Active and free from any orthopedic, musculoskeletal and cardiovascular disorders,
- Subject who accepts to vaccinated against flu during the autumn preceding the hospitalization,
- Subjects must be volunteer for psychological investigations,
- Subject to be covered by a Social Security system,
- Free of any engagement during four consecutive months.

Specific exclusion criteria

- Having given blood (more than 300ml) in a period of three months or less before the start of the experiment,
- Subject already participating in a clinical research experimentation
- Subject who is in the exclusion period in the Healthy Volunteers National Register of the French Ministry of the Health
- Poor tolerance to blood sampling,
- Past record of orthostatic intolerance,
- History of hiatus hernia or gastroesophageal reflux,
- Cardiac rhythm disorders,
- History of inguinal hernia,
- History of vestibular disease,
- History of central or peripheral nervous system disease,
- History of active claustrophobia,
- History of severe hypersensitivity,
- Known or suspected pregnancy,
- Status post-partum or post-abortion within a period of 3 months prior to the start of the study,
- Breastfeeding or within 2 months after stopping breast feeding prior to the start of the study,
- Use of hormonal contraceptives in a period of two months or less before the start of the experiment,
- Use of IUD at the beginning of the study,

- Completely sedentary or extremely fit subjects,
- Fractures or tendon laceration within one year,
- History of genetic muscle and bone diseases of any kind.
- Osteosynthesis material
- Chronic back pains,
- Past records of thrombophlebitis,
- Presence of metallic implants,
- Special food diet,
- Refusal to give permission for her general practitioner to be informed of her participation in the trial,
- Belonging to one of the following categories: incarcerated persons, patients in an emergency situation,
- Unlikely to co-operate in the study, and/or poor compliance anticipated by the investigator, or unable to cooperate because of a language problem,
- Subject who has received more than 3800 Euros within 12 months for being a research subject,

Appendix B

Assay Procedure Summaries

Angiotensin II – SPI-BIO (#A05880)

Sample Preparation

1 Pre-wash phenyl cartridges with 1 ml of methanol, followed by 1 ml of water
2 Pass 2 ml of plasma through the cartridge and then wash with 1 ml of water
3 Elute absorbed Angiotensin peptides with 0.5 ml of methanol
4 Evaporate methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen
5 Add 0.5 ml of Enzyme ImmunoAssay (EIA) buffer, vortex and centrifuge at 3000 g for 10 min at +4^{o}C

Assay Procedure

6 Prepare all reagents according to kit instructions
7 Rinse plate five times with wash buffer (300 µl/well)
8 Set up plate (i.e. similar to one suggested)

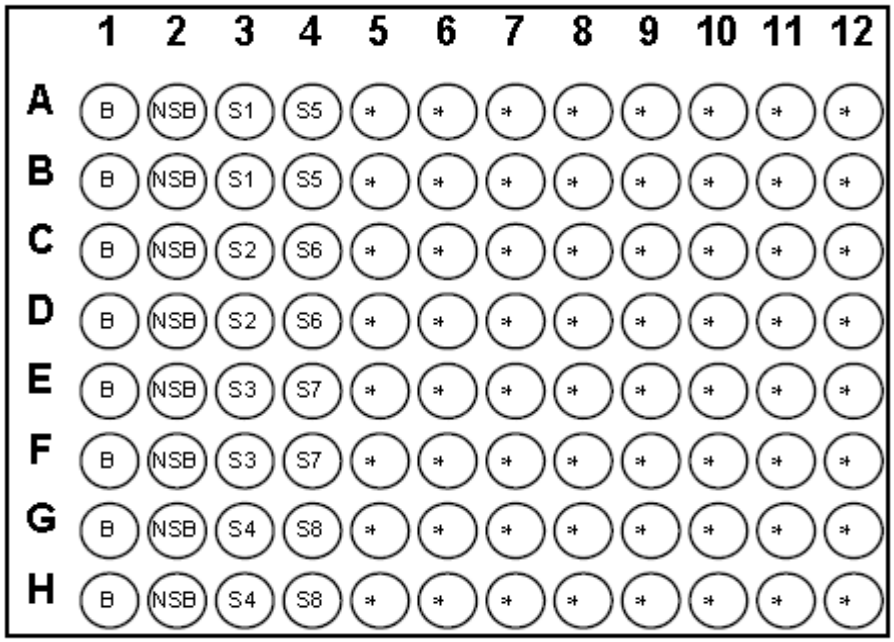

Figure C.1 Plate setup for angiotensin II
(Similar setup used for other molecules)

B : Blank
NSB : Non-Specific Binding
S1-S8 : standards 1-8
* : samples or Quality controls

9 Add 100 µl of EIA buffer to Non-Specific Binding (NSB) wells

10 Add 100 µl of Angiotensin II standard to appropriate standard well (S1 to S8)
11 Add 100 µl of Sample to appropriate wells (*) (at least in duplicate)
12 Incubate for 1 h at room temperature with gentle agitation
13 Add 50 µl of gluteraldehyde to each well (except blanks) and incubate for 5 min at room temperature with gentle agitation
14 Add 50 µl of borane-trimethylamine to each well (except blanks) and incubate for 5 min at room temperature with gentle agitation
15 Wash each well five times with wash buffer (300 µl/well)
16 Add 100 µl of anti-Angiotensin II IgG tracer to each well (except blanks)
17 Cover plate with plastic film and incubate overnight at +4^{o}C
18 Wash each well five times with wash buffer (300 µl/well)
19 Add 300 µl of wash buffer and incubate for 10 min at room temperature with gentle agitation
20 Wash each well five times with wash buffer (300 µl/well)
21 Add 200 µl Ellman's reagent (to all wells) and incubate in dark for 1 h on an orbital shaker
22 Read plate between 405 and 414 nm (yellow colour)

Endothelin-1 – R&D Systems (# BBE 5)

Sample Preparation
1 Add 1 ml of plasma and 1.5 ml of extraction solvent (acetone:1 N HCl:Water (40:1:5)) to a polypropylene tube
2 Mix by inversion
3 Centrifuge for 20 min at 2000 g at 2 – 8^{0}C
4 Decant supernatant into polypropylene tube
5 Dry down supernatant in centrifugal evaporator (min 4 h at 37^{o}C)
6 Reconstitute pellet in 0.25 ml sample diluent and vortex for 30 s
7 Assay immediately or store reconstituted samples overnight at 2 – 8^{o}C

Assay Procedure
8 Prepare all reagents according to kit instructions
9 Add 100 µl of diluted Conjugate to each well
10 Add 100 µl of Standard, Control, or reconstituted sample to each well (in triplicate)
11 Cover plate with a plate sealer provided and incubate at room temperature for 1 h
12 Aspirate or decant contents of wells and wash six times with Wash Buffer
13 Add 100 µl of Substrate to each well
14 Cover plate with new plate sealer provided and incubate at room temperature for 1 h
15 Add 100 µl of Stop Solution to each well
16 Read plate at 450 nm with a wavelength correction set at 620 or 650 nm

VCAM-1 – R&D Systems (#DVC00)

Sample Preparation
1 Dilute sample 20-fold (i.e. 20 µl sample + 380 µl Calibrator Diluent RD5P (1x)

Assay procedure
2 Prepare all reagents according to kit instructions
3 Add 100 µl of sVCAM-1 Conjugate to each well
4 Add 100 µl of Standard or diluted sample to each well (in triplicate)
5 Cover plate with a plate sealer provided and incubate at room temperature for 1.5 h
6 Aspirate or decant contents of wells and wash four times with Wash Buffer

7 Add 100 µl of Substrate to each well
8 Cover plate with new plate sealer provided and incubate at room temperature for 20 min **(Protect from light)**
9 Add 50 µl of Stop Solution to each well
10 Read plate at 450 nm with a wavelength correction set at 540 or 570 nm
11 Correct for dilution

ICAM-1 – R&D Systems (#BBE 1B)

Sample Preparation
1 Dilute sample 20-fold (i.e. 15 µl sample + 285 µl Sample Diluent)

Assay procedure
2 Prepare all reagents according to kit instructions
3 Add 100 µl of sICAM-1 Conjugate to each well
4 Add 100 µl of Standard, Control or diluted sample to each well (in triplicate)
5 Cover plate with a plate sealer provided and incubate at room temperature for 1.5 h
6 Aspirate or decant contents of wells and wash six times with Wash Buffer
7 Add 100 µl of Substrate to each well
8 Cover plate with new plate sealer provided and incubate at room temperature for 30 min
9 Add 100 µl of Stop Solution to each well
10 Read plate at 450 nm with a wavelength correction set at 620 or 650 nm
11 Correct for dilution

E-Selectin – R&D Systems (#BBE 2B)

Sample Preparation
1 Dilute sample 20-fold (i.e. 25 µl sample + 475 µl Sample Diluent)

Assay procedure
2 Prepare all reagents according to kit instructions
3 Add 100 µl of sE-Selectin Conjugate to each well
4 Add 100 µl of Standard, Control or diluted sample to each well (in triplicate)
5 Cover plate with a plate sealer provided and incubate at room temperature for 1.5 h
6 Aspirate or decant contents of wells and wash six times with Wash Buffer
7 Add 100 µl of Substrate to each well
8 Cover plate with new plate sealer provided and incubate at room temperature for 30 min
9 Add 100 µl of Stop Solution to each well
10 Read plate at 450 nm with a wavelength correction set at 620 or 650 nm
11 Correct for dilution

CRP – ALPCO Diagnostics (#30-9710s)

Sample Preparation
1 Dilute sample 100 fold (i.e. 10 µl sample to 990 µl dilution buffer)

Assay procedure
2 Wash plate five times with wash buffer (250 µl/well)
3 Prepare all reagents according to kit instructions
4 Add 100 µl of calibrator and samples into each well (in triplicate)
5 Incubate for 1 h shaking on a horizontal mixer at room temperature
6 Aspirate and wash plate five times with wash buffer (250 µl/well)

7 Add 100 µl prediluted Peroxidase-labeled CRP antibody to each well
8 Incubate for 1 h shaking on a horizontal mixer at room temperature
9 Decant contents and wash plate five times with wash buffer (250 µl/well)
10 Add 100 µl TMB substrate solution to each well
11 Incubate for 5 to 10 min at room temperature **in the dark**
12 Add 50 µl Stop Solution to each well and mix shortly
13 Read plate at 450 nm
14 Correct for dilution

ADMA – ALPCO Diagnostics (#17-EA201/96)

Sample Preparation (Acylation)

1 Add 20 µl standard A – F, Control 1 & 2, and samples into appropriate wells of Reaction Plate
2 Add 25 µl Acylation Buffer to all wells
3 Add 25 µl Equalizing Reagent to all wells
4 Mix reaction plate for 10 sec
5 Prepare Acylation reagent freshly and add 25 µl to all wells, mix **immediately**
6 Incubate for 30 min at room temperature on an orbital shaker
7 Dilute 1.5 ml prepared Equalizing Reagent in 9 ml distilled water, mix and add 100 µl to each well
8 Incubate for 45 min at room temperature on an orbital shaker

Assay Procedure

9 Remove 50 µl of prepared standard A – F, Control 1 & 2, and samples from the reaction plate and add it to corresponding well of the coated plate
10 Add 50 µl of Antiserum into all wells and shake shortly on an orbital shaker
11 Cover plate and incubate overnight at 2 to 8^{o}C **in the dark**
12 Aspirate or decant contents of wells and wash five times with wash buffer (250 µl/well)
13 Add 100 µl enzyme conjugate into all wells
14 Incubate for 60 min at room temperature on an orbital shaker
15 Aspirate or decant contents of wells and wash five times with wash buffer (250 µl/well)
16 Add 100 µl of Substrate into all wells and incubate for 20 to 30 min at room temperature on an orbital shaker
17 Add 100 µl Stop Solution to all wells
18 Read plate at 450 nm (with a reference between 570 and 650 nm)

NO Metabolites

Analysis was performed as aseptically as possible with all glassware, pipette tips, and syringes rinsed repeatedly with nitrogen-free water before and between uses. Serial dilutions of high purity sodium nitrate obtained from BioShop Inc., (Burlington, ON. Canada, catalogue number – SON 001) were dissolved in nitrogen-free water to use as standards were made new each day. HPLC grade ethanol was obtained from Fisher Chemicals (Fairlawn, NJ, USA) was mixed 1:1 with EDTA prepared plasma samples in microtubes stood at 4^{o}C for 30min before being centrifuged at 14000g for 5min. The supernatant was recovered and used for analysis. Sample preparation was always preformed on the day of measurement. A blank 2:1 ethanol and nitrogen-free water was also prepared each day for analysis. Blank, standard, or sample injection into a chamber containing 95^{o}C vanadium (III) chloride from Sigma-Aldrich (St. Louis, MO, USA catalog number – 20827-2) saturated in 1M hydrochloric acid initiated the conversion of nitrate, nitrite, and S-nitrosocompounds to an acidic NO vapor.

After neutralization by bubbling through aqueous sodium hydroxide, the NO gas was passed through a 0.2µm pore size polypropylene filter from Whatman Inc. (Clifton, NJ, USA catalog number – 6725-5002) and reacted with ozone in a small reaction cell at a pressure ranging from 4-6mmHg. Emission from electronically excited nitrogen dioxide, generated from the chemiluminescent reaction between NO and ozone, is in the red and near-infrared region of the electromagnetic spectrum, and was detected by a thermoelectrically cooled (-12^{o}C) photomultiplier tube outfitted with an optical filter that transmits only wavelengths above 600nm, avoiding the detection of other compounds undergoing reaction with ozone and emitting light at shorter yet otherwise detectable wavelengths.

A standard curve was established prior to and following injection of plasma samples on each day to ensure that the sensitivity of the analyzer remained constant. All samples were analyzed using the calibration curve generated prior to collection. Following calibration each day, a sample blank and the plasma samples were injected in triplicate. If the triplicates were not consistent, replicates of the sample were injected until consistent peaks were observed.

Norepinephrine

Standard preparation

A. Concentrated standard
   1 Weigh 1 mg Norepinephrine, Epinephrine, Dopamine to make 5 ml with 0.5 PCA

B. Concentrated internal standard
   2 Weigh 5mg 3,4-dihydroxybenzylamine (DHBA) and add 0.1 PCA to make 5 ml

Working standard

3 To 955 µl 0.1 M PCA add 21.5 µl A and 21.5 µl B (from above), mix well
4 To 976 µl 0.1 M PCA add 21.5 µl of the above mixture (#3) and mix well
5 To 976 µl 0.1 M PCA add 21.5 µl of the above mixture (#4) and mix well

Working internal standard

6 To 968 µl 0.1 M PCA add 27 µl of B (from above), mix well
7 To 968 µl 0.1 M PCA add 27 µl of above mixture (#6) and mix well
8 To 968 µl0.1 M PCA add 27 µl of above mixture (#7) and mix well

Sample preparation (extraction)

9 To a 1.5 ml polypropylene tube add 10 mg acid washed alumina
10 Add 400 µl 2.0 M tris + 2% EDTA, pH 8.7 (fresh weekly)
11 Add 50 µl DHBA
12 Add 1 ml EGTA/Glutathione plasma **or** 1 ml working internal standard to each tube
13 Mix by inversion (avoid foaming) for 30 to 60 min
14 Discard supernatant and wash 4 times with dH_2O (ensure inside of tube is completely rinsed of plasma)
15 Add 100 µl of 0.1 M PCA, vortex then centrifuge at 12000 g for 10 min at +4^{o}C
16 Transfer 90 µl of supernatant to vial for, analysis with Waters 2690 alliance HPLC

Total Antioxidant Capacity – ALPCO Diagnostics (#30-5200)

Reagent Preparation

1 Reagent 1 is prepared by adding 5 ml of reaction buffer A and 10 µl of peroxide solution (this is **dilution 1**)
2 Add 100 µl of dilution 1 to 4.9 ml of reaction buffer A (this is **reagent 1**)
3 Reagent 2a is prepared by adding 5 ml of reaction buffer A + 100 µl of reaction buffer B + 5 µl of enzyme solution
4 Reagent 2b is prepared by adding 5 ml of reaction buffer A and 100 µl reaction buffer B

The reason for two reagent B is due to the fact that the self-absorption of the sample and the reaction products produced by the added hydrogen peroxide. Therefore it is important to measure the sample with and without the addition of enzyme

5 Add 10 µl of sample, calibrator and control into wells (in duplicate)
6 Add 100 µl of reagent 1
7 Incubate for 10 min at 37°C
8 Add 100 µl of reagent 2a and reagent 2b respectively
9 Incubate for 5 min at room temperature
10 Add 50 µl of Stop Solution
11 Read plate at 450 nm
12 Calculate total aniox. capacity using the following equation:

$$\text{total aniox. capacity } (\mu\text{mol/l}) = 392 - (392 - \text{Conc. Calibrator}) * \frac{\Delta\text{OD sample}}{\Delta\text{OD Calibrator}}$$

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