EFFECTS OF HYPERBARIC OXYGEN THERAPY
ON PHYSIOLOGIC CHANGES IN RATS
FOLLOWING SIMULATED
MICROGRAVITY

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by

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ABSTRACT

EFFECTS OF HYPERBARIC OXYGEN THERAPY ON PSYSIOLOGIC CHANGES IN RATS FOLLOWING SIMULATED MICROGRAVITY

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The purpose of the present investigation was to examine the effects of hyperbaric oxygen therapy on: 1) bone mineral density (BMD; cortical and cancellous) of the mid-diaphysis of the femur, 2) mechanical characteristics (ultimate force (UF) and fracture force (FF)) of the mid-diaphysis of the femur, 3) bone mineral resorption and deposition, and 4) vasoconstrictive properties of the thoracic aorta following four weeks of hind limb suspension in five-month-old, male Sprague-Dawley rats. Forty rats were randomly divided into aging controls (AC, n =10), aging control with hyperbaric therapy (AC-HBO, n =10), hindlimb suspended (HLS, n =10), and hindlimb suspended
with hyperbaric therapy (HLS-HBO, n =10) groups for 4 wks. Groups receiving HBO were placed in a cage that was fitted for the animal hyperbaric chamber; HLS was maintained during HBO treatments. HBO groups were treated for 90 minutes, 6 d/wk (1×d) for a total of 24 treatments. After 28 d of HLS, animals were sacrificed under isoflourane anesthesia, thoracic aorta segments were isolated, and the femurs were removed for analysis. Cortical and cancellous BMD was determined using peripheral quantitative computed tomography. Bone markers for resorption and deposition were evaluated using commercially available assays to assess urinary content of deoxypyridinoline (DPD) and serum content of osteocalcin (OC), respectively. Mechanical testing of the femur was determined using a three-point bending test. Load-displacement curves were constructed to determine UF and FF. Maximal vasoconstriction (MAX) and the sensitivity (EC\textsubscript{50}) of aortic rings were determined in response to increasing concentrations of phenylepherine (PHE) administered cumulatively in vessel baths at 10×10^{-10}-10×10^{-4} mol/l. While HLS and HBO appeared to result in a decrease in BMD, these differences were not significant. In addition, HLS and HBO did not have significant effects on biochemical bone markers or mechanical strength. The maximal vasoconstriction response to PHE was decreased with HLS, however, it was decreased to an even greater extent in the HLS-HBO group. Hyperbaric oxygen therapy tended to decrease the levels of BMD and significantly decreased the vasoconstrictive ability of the thoracic aorta suggesting at this treatment level, HBO should not be used as an intervention during simulated spaceflight.
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CHAPTER 1

INTRODUCTION

The prospect of sending a man to Mars is getting closer to reality, however extreme duration space flight poses many physiological challenges. Exposure to microgravity elicits several physical responses such as, fluid shifts from the lower extremities to upper body, repositioning of certain organs, musculoskeletal unloading, and a lack of stimulus to the vestibular system (40, 52). These adjustments can lead to physiological adaptations that are detrimental to health such as decreases in mineral density in weight bearing bones (43) and orthostatic intolerance (15, 16, 37, 41, 54).

Significant decreases in bone mineral density (BMD) have been observed following spaceflight, however these changes have not been great enough to end a mission (12). There is concern that missions over a year in length will increase the risk of bone fractures later in life (12). Therefore, determining a means of reducing the effect of weightlessness on BMD is paramount to the success of long term space flight. It has been hypothesized that decreases in BMD are a result of a negative calcium balance, neural, hormonal, and mechanical factors, changes of stress on bone, and alterations in circulatory dynamics (43, 44, 55).

It is unclear which mechanism or mechanisms contribute to bone loss, thereby making it difficult to determine a treatment that would attenuate this decrease. Countermeasures that have been implemented to reduce the effects of microgravity are
calcium and phosphate supplementation, exercise regimens, and bisphosphonates. These steps, however, have met with little or no success. Research (10, 49) has suggested that the reduced blood supply to the bone may alter bone remodeling. Increasing the blood supply by lower body negative pressure coupled with 40 minutes of treadmill exercise six days a week has been shown to decrease bone resorption following 30 days of bed rest (49), however more research is needed to determine its effects on BMD.

Hyperbaric oxygen therapy (HBO) is a clinical treatment used most often in healing chronic wounds. Two primary mechanisms of action are hyperoxygenation and vasoconstriction. The mechanisms result from placing an individual under greater than atmospheric pressure and by breathing 100% oxygen. The data from numerous studies have demonstrated the efficacy in healing chronic non-healing wounds of both soft tissue and bone, however few studies have been conducted to determine the effects of HBO on changes in bone mineral density and no research has been done to determine the effects of HBO during simulated microgravity.

While on Earth, approximately 60% of bodily fluids are contained below the waist. During space flight a cephalic fluid shift occurs causing fluids to redistribute evenly throughout the body (41). This shifting of fluid causes the kidneys to increase diuresis resulting in a reduced circulating blood volume. A change in the baroreceptor reflex also occurs as a result of exposure to microgravity (37, 41, 54) which, along with decreased vascular responsiveness to sympathetic output, have been considered as mechanisms for decreased orthostatic responsiveness (15, 16). The combination of
these changes can lead to orthostatic intolerance and syncope when an astronaut returns to a 1g environment. While standing, blood volume is redistributed to the lower body and blood pooling occurs in the lower extremities. The resistance vessels are unable to constrict creating inadequate venous return, which, along with the lower blood volume and a decreased baroreceptor response results, in the inability to maintain blood pressure in the upright position.

Vascular responsiveness to hyperoxia and hyperbaric oxygen therapy has been investigated. Rousseau et al. (46) found that breathing 100% oxygen increases systemic vascular resistance. Hyperbaric oxygen therapy in dogs produced a 30% increase in systemic vascular resistance (4). No studies have examined vascular responsiveness after exposure to microgravity or simulated microgravity, therefore the effects of hyperoxia and hyperbaric oxygen therapy following microgravity and simulated microgravity need to be examined.

1.1 Purpose

The purpose of this study was to determine the effects of hyperbaric oxygen therapy on bone mineral loss, mechanical strength of bone, biochemical markers of bone remodeling, and vascular contractility after four weeks of simulated microgravity in adult rats.

1.2 Hypothesis

Hyperbaric oxygen therapy will attenuate the rate of bone mineral loss as a result of simulated microgravity, therefore minimizing the effects on bone remodeling and mechanical strength.
Hyperbaric oxygen therapy will enhance vasocontractility following simulated microgravity.

1.3 Definition of Terms

Remodeling: A process in which bone mineral is removed and then replaced.

Osteocyte: Mature bone cell that maintains the bone matrix.

Osteoblast: Cell that produces new bone matrix by making and releasing organic materials.

Osteoclast: Cell that removes bone matrix.

Resorption: The removal of bone matrix.

Deposition: The deposition of new bone matrix.

Cortical Bone: Dense outer layer of bone, also known as compact bone.

Cancellous bone: Inner layer of bone, adjacent to and less dense than cortical bone.

Microgravity: An environment where there is little gravitational force.

Hyperoxia: Oxygen breathing at levels greater than 21%.

Hyperbaric: Pressure greater than 1 atmosphere.

Ultimate Force: Highest load obtained at the instant of material damage.

Fracture Force: Load at which material experiences catastrophic failure or complete fracture of the bone.
1.4 Delimitations

No control group for treatments of 100% oxygen at ambient pressure was used.

No control group for 21% oxygen at 2.5 ATA was used.

1.5 Assumptions

Hindlimb suspension mimics physiologic responses incurred within a microgravity environment. Physiologic responses in HLS rats are similar to those incurred by humans during space travel. Mechanism for bone loss is due to decreased oxygen supply to the bone tissues. Aorta and femoral & carotid arteries responses are the same as peripheral vessels.

1.6 Limitations

Gravity is still present during our experiment. Unloading of only the hindlimbs will occur. Launch, landing, and transport to and from launching pad are not present. Rats are frequently handled. Rats may be able to put pressure on hind limbs intermittently while in the chamber cage.
CHAPTER 2
REVIEW OF LITERATURE

2.1 Bone Remodeling

Adult bone is a highly vascular, dynamic, and living tissue that is constantly undergoing remodeling. The rate at which bone remolds varies with age, nutritional status, activity level, and other environmental factors such as microgravity. The rate of remodeling also varies regionally within the skeleton and locally within the bone. For a young adult, approximately $1/5^{\text{th}}$ of the total skeleton is recycled and replaced annually (33).

During remodeling osteoclast and osteoblast actions are coupled, bone resorption and deposition occur within the same area of the bone surface. Normally remodeling does not cause a large change in bone structure because the rates of resorption and deposition are close. However, after age 35 the osteoblast activity lags behind osteoclast activity resulting in a slow decrease in bone mass (6).

Remodeling of bone occurs in distinct phases where the old bone is removed then new bone is placed in the cavity. First, lining cells react to chemical signals which expose the bone. Mononuclear osteoclast precursors are attracted to the area by chemotaxis to congregate and fuse into multinuclear osteoclasts (6). Osteoclasts then dissolve the old bone and disappear. Next osteoblasts enter the cavity and deposit new bone matrix, which later mineralizes. After the new bone matrix is deposited some
osteoblasts die, some become buried in new bone and become osteocytes, while others transform into lining cells (6). These processes occur within both cortical and cancellous bone.

2.2 Regulation of Calcium Homeostasis

Calcium plays an important role in many physiological functions within the body such as muscular contraction, transmission of signals across neural junctions, blood clotting, and structural support (24). Serum levels of calcium lie within a narrow range and are closely regulated through feedback mechanisms. The skeleton acts as a reservoir where calcium can be stored or resorbed to maintain blood concentrations. Absorption of calcium through the intestine and excretion by the kidneys is also important mechanisms for maintaining the homeostatic balance of calcium. Moderation of serum concentrations of calcium is achieved through secretion of parathyroid hormone (PTH) and calcitonin. The parathyroid gland secretes PTH into the bloodstream when blood concentrations of calcium fall below normal. Parathyroid hormone stimulates osteoclast activity, increases intestinal absorption of calcium, and decreases the rate of calcium excretion by the kidneys (33). The thyroid secretes calcitonin into the bloodstream when blood calcium concentrations are above normal. Calcitonin inhibits osteoclast activity and increases the rate of calcium secretion through the kidneys (33).

2.3 Calcium Balance in Humans Following Microgravity

Travel within the microgravity environment of space causes many physiological responses that can be detrimental to astronauts’ health upon their return to earth. One
aspect that is of great concern is changes in bone deposition and resorption rates that can lead to losses in bone mineral density (BMD). Early human spaceflight studies have demonstrated increased calcium excretion through the urine (43, 44, 55) and feces (43, 44). During the Skylab II mission, crewmen consumed a controlled diet and collected urine and fecal excrement to determine changes in calcium balance during spaceflight. Urinary calcium increased steadily to approximately double the pre-flight values by day 28 while fecal calcium did not change significantly. The result was a mean calcium loss of 50 mg/day during the last 16 days of flight (55). To further demonstrate the changes in calcium metabolism, Rambaut et al. (44) combined data from several orbital spaceflight missions occurring over 28, 59, and 84 days in which diet was controlled and excrement was monitored. During the first 28 days calcium excretion in the urine increased to approximately 100% of pre-flight levels then leveled off for the remainder of the flight. Fecal calcium showed an initial decline at 14 days then increased steadily throughout the 84 day period to approximately 300 mg/day above pre-flight values (43). Calcium balance showed a steady decline throughout the 84 day period to a maximum deficit of approximately 300 mg/day (43).

It is hypothesized that a negative calcium balance is related to the decreases in bone mineral density observed as a result of microgravity. Bone mineral density has been shown to decrease at rates between approximately 1%-2% a month in specific bones (27, 58). The decreases in bone mineral density have been observed primarily in the weight bearing bones of the calcaneus, lumbar spine, and tibia (11, 43, 59).
2.4 Simulated Microgravity in Rats

Conducting research during space flight can pose many challenges. Space missions are limited in number and duration, require many man hours, and are expensive. The development of a ground-based model to simulate microgravity is important in the study of the effects of the microgravity environment incurred in humans during space travel. The benefits of ground based models are: (a) scheduling times for experiments are not limited to the number of space missions, (b) not as many precautions are needed during experimental manipulations on Earth, (c) ground-based experiments are more economical, (d) experimental duration is not limited to mission duration, (e) measurements can be made and tissue samples can be obtained at several points during the experiment, and (f) experiments can be extended or repeated on a regular basis (38).

The physiological responses to space travel include fluid shifts, repositioning of certain organs, musculoskeletal unloading, and lack of stimulus to the vestibular system (38). Human models have successfully simulated these effects by using bed rest and bed rest with a head-down tilt. Human ground-based models have their own limitations, therefore, animal models have been developed which successfully simulate the effects of space flight. A common animal model is the hindlimb suspended (HLS) rat model which has been approved by National Air and Space Administration (NASA). The HLS model requires that the rat’s hindlimbs be suspended so the body axis creates approximately a 30° angle with the floor. This allows for normal weight bearing on the forelimbs (38). The HLS model presents minimal stress to the animal based upon
hormonal levels and body weight measured (38). Suspension is achieved by using a tail traction harness which allows the rat freedom to move, eat, and groom.

2.5 Bone Mineral Changes in Rats Following Simulated Microgravity

Bone mineral loss during HLS in the rat model has been shown to occur in the femur, lumbar vertebrae, and tibia. Increased bone mineral deposition has been observed in the humerus, skull, and mandible. Arnaud et al. (1) suspended 6-month-old rats for 4 weeks and measured bone mineral content in situ of the whole body and the femur and skull separately using dual x-ray absorptometry (DEXA) technique. Bone mineral content, when corrected for body weight, was 14% and 7% lower in whole body and femur, respectively. There was no significant change in the skull bone mineral content (1). Roer and Dillaman (45) calculated the differences in wet, dry, and ash weights of the forelimbs, hindlimbs, and the skull during 1-, 2-, and 3-week HLS studies on 28-day-old rats. For the 3-week experiment, when corrected for body weight, the dry and ash weights of the femur and tibia were significantly lower ($p < 0.05$ for dry tibia; $p < 0.001$ for dry and ash femur, and ash tibia) in the HLS group. The dry weights of the forelimbs were greater ($p < 0.01$) in the HLS group, however there was no difference in ash weight, while the dry and ash weights of the skull and mandible were greater ($p < 0.001$) in the HLS group (45).

Dehority et al. (14) measured the bone weight using regression analysis in 6-month-old HLS and control rats in three separate experiments of 1-, 3-, and 5 week durations. There were significant decreases ($p < 0.05$) in the regression slopes for the fat-free weights of the femur and lumbar vertebrae but not for the humerus. Globus et
al. (21) measured changes in calcium in the humerus, mandible, tibia, and lumbar vertebrae after 15 days of HLS. The tibia and lumbar vertebrae of the HLS rats contained $86.2 \pm 2.5\%$ and $75.5 \pm 3.5\%$ (mean $\pm$ SD) calcium of the control values respectively. There were no differences in calcium content in the mandible and humerus between the HLS and control groups (21).

Bloomfield et al. (5) used peripheral computed tomography (pQTC) to compare the differences in BMD of the mid-diaphysis and proximal metaphysis of the tibia in six-month old rats after 7-, 14-, 21-, and 28-days of HLS. (Peripheral quantitative tomography separates cortical and cancellous bone mineral compartments and provides a real measure volumetric density (mg/cm$^3$).) When compared to 0-day control both the 28-day HLS and 28-control exhibited a similar increase in cortical BMD of the proximal tibia, +5.7 % and +4.0 % respectively. Cancellous BMD decreased by 21% after 28-days HLS in proximal tibia when compared to 0-day control. Greater BMD was observed at the tibial mid-diaphysis at days 14 – 28 of HLS. No significant difference was observed when BMD values were compared to 28-day control.

2.6 Bone Remodeling in Rats Following Simulated Microgravity

Mean periostial apposition rates (MAR) in adult rats (6-months-old) after 2 and 4 weeks of HLS were reduced by 65% and 85% respectively (14). In the same study mean periostial bone formation rate (BFR) of the tibiofibular junction (TFJ) and the tibia mid-shaft were decreased by approximately 80% ($p < 0.001$), and there were no significant changes in BFR in the humerus (14). In a separate study periostial MAR and BFR were 61% and 90% lower at 21 days HLS at 21 days in the tibial mid-
diaphysis; no significant differences in MAR and BFR were observed in the mid-tibia and humeral diaphysis (5). In cancellous bone, a decrease of 33% and 69% \((p < 0.05)\) in MAR and BFR respectively was observed after 2 weeks of HLS in adult rats (14). The percent surface area occupied by cancellous bone occupied by osteoblasts decreased by 66% \((p < 0.05)\), there were no significant changes in osteoclast numbers or cancellous bone volume (14). These results suggest that a decrease in BFR or osteoblast activity may account for the net bone loss seen during simulated microgravity.

2.7 Systemic and Local Factors Contributing to Bone Loss

The regional decreases in hindlimb mass and bone mineral density and the increases or stabilization of the skull and forelimb mass and bone mineral density may be attributed to an alteration in perfusion of bone as a result of the cephalad fluid shift during HLS rather than systemic factors that regulate bone mineral homeostasis (1, 14, 21, 45). In a study by Dehority et al. (14), changes in PTH were not observed after 5 weeks of HLS, however there were regional changes in MAR and BFR, therefore the data did not indicate that systemic factors contribute to bone loss (14). It is believed that if systemic factors influenced bone loss during simulated microgravity, then changes in BMD would appear in all areas of the body.

2.8 Biochemical Markers of Bone Resorption

Biochemical markers can be used as an indicator of the rate of bone turnover. During bone formation type I collagen is laid down then bonded together by pyridinium and pyrrolic cross-links for structural integrity (29). During resorption the collagen is
broken down by osteoclasts then pyridinoline (PYD) and deoxypyridinoline (DPD) are released. Pyridinoline and DPD are neither metabolized nor absorbed from the diet (18) and can be measured in the blood or urine. Low levels of resorption markers, such as DPD, reflect low rate of bone resorption and high levels of DPD reflect a high rate of bone resorption activity. A high rate of bone loss can lead to a decrease in bone mineral density and osteoporosis. Moderate correlations have been observed between high concentrations of bone resorption markers and bone mineral loss (18, 53). Caillot-Agusseau et al. 1998 and 2000 (8, 9) observed a 50 % and 54 % increase in DPD in cosmonauts during 21 and 180 days of spaceflight respectively. Kurokouchi et al. (30) measured changes in tartrate-resistant acid phosphatase (TRAP), a bone resorption marker, in five-week old rates during HLS. The results indicated a significant increase in TRAP at days one and three, but returned to pre-HLS level at day five and remained there throughout the duration of the study.

2.9 Biochemical Markers of Bone Deposition

Osteocalcin (OC) is a non-collagen protein excreted by exclusively osteoblasts into the bone matrix during bone deposition (56). Some osteocalcin escapes the bone matrix entering the bloodstream and is a good indicator of osteoblast activity (IMMUTOPICS). The literature suggests that bone formation is reduced during space travel and simulated microgravity. Caillot-Agusseau et al. (8, 9) measured changes in OC during 21 and 180 day space missions in cosmonauts. Osteocalcin levels were decreased by 18 % and 27 % during 21 and 180 days respectively when compared to pre-flight levels. Kurokouchi et al. (30) observed a decrease in OC levels from day
three to day 14 in 5-week old HLS rats. Patterson-Buckendahl et al. (42) measured weekly changes in OC levels for four weeks during HLS in 6.5 week-old rats. Osteocalcin for the control rats gradually decreased throughout the four week period. The HLS rats demonstrated a significantly greater decrease in OC than the control for week one and leveled out at week two but remained significantly lower than the control value. There was no significant difference in OC levels for weeks three and four and the rate of decrease in OC decreased in parallel between the two groups for the remaining two weeks of the experiment.

Biochemical markers for bone resorption or deposition should not be used to predict the rate of bone mineral loss or bone mineral deposition (3, 32) however they may be used to indicate whether bone loss is a result of excess resorption or decreased deposition rates.

2.10 Mechanical Properties of Bone

Cortical and cancellous bone is found in all bony structures. When found together, their structure is able to withstand high loads. Cortical bone is dense and surrounds the outer surfaces of bone. The cellular structures of cortical bone called lamella are oriented so that they run parallel to the bone surface enabling it to resist high compressive loads (34). Cancellous bone is primarily located at the ends of long bones and along the inner lining of bone. The structural framework of cancellous bone forms an open network of struts and plates that give it strength to resist stress from all directions and direct the stress towards the cortical bone (34). The amount of force a bone is able to absorb is dependent upon the volume of bone matrix, the type of bone
matrix (woven or laminallar), the degree of mineralization, and the structural arrangement (39). Disruptions in the remodeling of bone can lead to alterations in bone architecture and the removal of structural elements which lead to the loss of mechanical strength and fractures (6).

The deterioration of the struts and plates due to high rates of resorption weaken cancellous bone making it more susceptible to fractures. Hogan et al. (26) measured changes in the mechanical properties of cancellous bone in female rats that underwent an ovariectomy to stimulate bone loss. After five weeks there was approximately a 60% reduction in stiffness, energy absorbed, and maximal force in the proximal tibia of the ovariectomized versus sham rats. In the three point bending test of the femur a 10% increase in maximal force was observed in the ovariectomized rats when compared to sham. In a study by Bloomfield et al. (5) pQCT was used to measure changes in bone mineral density and differentiate between losses in cortical and cancellous bone in HLS rats. Mechanical testing was then performed to determine if there was a relationship between changes in compartmental bone density and the structural integrity of bone. The main finding was that there was an 18% increase in fracture force that corresponded to a 5.6% increase in cortical density of the diaphysis of the tibia.

2.11 Vasoconstrictor Properties Following Simulated Microgravity

Alterations in vasoconstrictor properties following simulated microgravity have been examined as a possible mechanism of orthostatic intolerance.
2.11.1 Aorta

Delp et al. (16) suspended Sprague-Dawley rats (age not reported) for 2 weeks to examine the vasoreactivity of the abdominal and thoracic aorta and to determine whether the influences were endothelium dependent or endothelium independent. The vasoconstrictors, KCl, norepinephrine (NE), arginine vasopressin (AVP), and CaCl₂ were used to test the responsiveness in vitro using force transducers. The vessel characteristics before treatment were similar between the HLS and control groups (P<0.05). Maximal contractile force in response to KCl and NE in vessels with the endothelium intact and denuded were lower in the HLS than control rats. Maximal contractile response to AVP, phenylephrine (PHE), and CaCl₂ of denuded vessels were also lower in HLS rats. Sensitivity to KCl of intact and denuded vessels was greater in HLS rats, however sensitivity to NE was not different in intact and denuded vessels. For denuded vessels, sensitivity to AVP and PE were not different between the groups. Sensitivity to CaCl₂ could not be determined because a plateau in vasoconstrictor response was not established. Results were similar between thoracic and abdominal aortas.

In a similar study Delp et al. (15) suspended 6-month-old Fischer 344/Brown Norway Rats for two weeks, then isolated the abdominal aorta and observed the vasoconstrictive properties in response to KCl, NE, and AVP. Maximal force production was significantly lower in HLS rats when induced by KCl, NE, and AVP. Sensitivity to NE and AVP was not different between the two groups and in contrast to the previous study, the sensitivity to KCl was lower in HLS rats.
Delp et al. concluded that HLS decreases in vascular force production were not due to endothelium dependent factors because the changes in force production in response to KCl still occurred with the endothelium removed. Delp et al. “attributes these changes to alterations in smooth muscle resting membrane potential due to alterations in regulating intracellular calcium concentration or the depressed ability of the smooth muscle contractile apparatus to produce force” (15, 16). The exact mechanisms of these changes are not fully understood. No explanation was given for the difference in the increased sensitivity of KCl in study 1 and decreased sensitivity to KCl in study 2.

2.11.2 Arterioles

Looft-Wilson et al. (31) suspended Sprague-Dawley rats (age unknown) for 4 weeks to study the effects of simulated microgravity on vasoconstriction in cannulated 2nd order mesenteric arteries in response to increasing concentrations of NE, KCl, and serotonin. Mesenteric arteries were used because they are important regulators of blood pressure through their effect on peripheral vascular resistance. The results of the first test showed no differences ($p > 0.05$) between HLS and control groups in response to PE, KCl, and serotonin. In a second test, cannulated 3rd order arteries were given chemicals in increasing concentrations administered in series: PE (vasoconstrictor), ACh (vasodilator), then KCl (vasoconstrictor). There were no significant differences between groups for the series of reactions. The results indicated that two weeks of HLS had no effect on VC response to PE, KCl, and serotonin.
2.12 Body Mass

It is not uncommon for astronauts to experience decreases in body mass during a space mission. An average decreases in body mass of 6.29 pounds in humans have occurred during space missions ranging from 9 to 30 days in duration (7, 17, 50). Stein et al. (50) and Drummer et al. (17) attributed the weight loss to a negative caloric balance.

Animal HLS studies have mixed results regarding changes in body mass. Studies ranging from 1 – 4 weeks HLS have demonstrated no change in body mass (5, 45) while other studies ranging from 2 - 4 weeks report a significant decrease in body mass (1, 15). Delp et al. reported a $56 \pm 10$ g (3 %) decrease in body mass in rats after 2 weeks HLS and Arnaud et al. reported a 4 % (actual values in grams where not reported) decrease in body mass after 4 weeks HLS.

2.13 Hyperbaric Therapy

At sea level ambient air is 20.94% oxygen and 0.04% carbon dioxide, the pressure of atmospheric air is 760 mmHg or one atmosphere absolute (ATA). According to Dalton’s Law, the sum of the partial pressures of the gases in a mixture equals the total pressure, therefore at 1 ATA the partial pressure of oxygen ($PO_2$) of the air we breathe is approximately 160 mmHg and the partial pressure of carbon dioxide ($PCO_2$) is approximately 0.304 mmHg. Oxygen is delivered to cells via the cardio-circulatory system in combination with hemoglobin. The initial $PO_2$ of air we breathe is approximately 160 mmHg before it enters our bodies. Once inside the lungs, the alveolar partial pressure ($P_AO_2$) decreases to 103 mmHg, due to the temperature and
humidification of the air within the lungs. The partial pressure of oxygen decreases to 98 mmHg in arterial circulation (PaO$_2$) where it is bound mostly to hemoglobin (98%), an oxygen binding compound within red blood cells. A small amount (2%) is dissolved in the plasma. In the capillary region oxygen diffuses across the capillary wall into the interstitial fluid where the partial pressure of oxygen decreases to 40 mmHg by the time the blood reaches the end of the capillary. After diffusion into the interstitial fluid, oxygen diffuses toward a nearby cell where it must cross the cellular membrane, move through the cytoplasm and into the mitochondria, where is it utilized, reaching a final partial pressure of 2-3 mmHg.

Our cells require oxygen to meet its metabolic demands to perform their respective activities or functions. A reduction in oxygen supply can limit or inhibit normal cellular activity and can even cause cell death or necrosis. Several conditions can limit the amount of oxygen that reaches the tissues which include but are not limited to a decrease in PO$_2$ experienced at high altitudes, anemia, or ischemia. Insufficient oxygen availability to tissues can inhibit cellular functions. Hyperbaric oxygen therapy (HBO) has been used as a countermeasure in conditions where there is low tissue oxygenation resulting from reduced or disrupted blood flow.

Hyperbaric oxygen therapy provides a way to increase the normal PaO$_2$ of 100 mmHg to levels greater than 2000 mmHg. Breathing 100% O$_2$ at increased pressures increases the amount of O$_2$ dissolved in the plasma. The O$_2$ is carried in solution to the tissues and provides a tremendous gradient from the capillaries to the tissues that increases the perfusion distance of the oxygen two to four times above normal (48).
The use of hyperbaric therapy for certain clinical conditions to increase oxygen delivery to tissues has been shown to restore or enhance cellular functions and speed healing.

To gain a better understanding of how much hyperbaric therapy can increase the amount of oxygen dissolved in the blood consider that at 1 ATA approximately 197 ml of oxygen is bound to hemoglobin per liter of blood and 3.0 mL of oxygen per liter of blood is dissolved in plasma. At 3.0 ATA with 100% the amount of oxygen bound to hemoglobin remains essentially unchanged, but the amount of oxygen dissolved in plasma increases to 60 ml·L⁻¹ of blood (20).

2.13.1 Bone

Bone tissue responses to varying concentrations of oxygen have been examined in vitro. Shaw et al. (47) examined the effects of 5, 20, 35, 65, and 95% oxygen on embryonic cartilage explants. At oxygen concentrations of 5%, little or no formation of osteoid occurred and at 35% osteogenesis was optimal. Osteogenesis at 95% oxygen was less than what was observed at 35%, increased chondroclasia and osteoclasia at 65% and 95% oxygen were observed. Therefore, it appears that bone tissue is broken down more rapidly at higher oxygen concentrations. Similar results have also been reported in other studies that have looked at varying oxygen concentrations on bone tissue in vitro. Gray et al. (23) and Goldhaber (22) both observed increased rate of bone resorption with increasing concentrations of oxygen. It was suggested that the increase in oxygenation increased cell activity by raising cellular metabolic activity. No studies have looked at the effects of HBO oxygen therapy on bone remodeling following simulated microgravity.
2.13.2 Vessels

Acute responses to hyperoxia and HBO therapy have been examined. Rousseau et al. (46) measured the changes in systemic vascular resistance (SVR) using echocardiography while breathing 100% oxygen compared to breathing room air. Systemic vascular resistance was significantly increased when compared to air-breathing (14.1 ± 1.1 to 18 ± 1.3 mmHg·L⁻¹·min⁻¹, p < 0.05). Changes in blood flow to the calf were measured using venous occlusion plethysmography, while breathing oxygen at increasing intervals (11kPa (medical air), 20 kPa, 40 kPa, and 56 kPa (100%)). There was a significant reduction (p < 0.05) in blood flow between each concentration when compared with baseline and between 20 kPa and 56 kPa. Blood flow was 1.3 ± 0.4, 1.1 ± 0.5, 1.0 ± 0.5, and 0.9 ± 0.5 ml·min⁻¹·100 ml⁻¹ tissue for 11kPa (medical air), 20 kPa, 40 kPa, and 56 kPa (100%), respectively. In their experiment, Rousseau et al. (46) demonstrated that hyperoxia increased SVR in vivo, therefore it was concluded that breathing increasing concentrations of oxygen reduces blood flow to the calf. The physiologic mechanisms for these results could not be concluded.

Berry et al. (4) measured changes in SVR and blood flow in vivo during HBO therapy using 100% oxygen at 2 ATA for 90 minutes in dogs. Systemic vascular resistance was significantly elevated (p < 0.05) after 30 minutes and remained elevated during the remainder of the treatment. After returning to room air SVR remained elevated for up to 30 minutes post treatment. Blood flow to the vertebral, coronary, renal, mesenteric, and hepatic arteries remained unchanged. Carotid blood flow decreased from 96 ± 8 ml·min⁻¹ to 79 ± 3 ml·min⁻¹ and remained depressed (75 ± 3
ml·min⁻¹) up to 30 minutes post treatment. Total organ blood flow remained unchanged as well as regional vascular resistance. Berry et al. (4) suggested that blood flow is redistributed from peripheral vascular beds to major organs during hyperbaric hyperoxia and that this compensates for decreased cardiac output in order to preserve blood flow to the major organs.

Tahepold et al. (51) measured vascular responsiveness in isolated rat thoracic aorta immediately and 24 hours following 60 minutes of breathing 95% oxygen. These values were compared to room air breathing (normoxic) controls. The contractile response to PHE was attenuated in vessels harvested immediately post treatment, but not in the vessels harvested 24 hours later. For vasodilation, the immediate group had an earlier relaxation response to Ach and SNP and increased maximal relaxation when compared to the normoxic group. The 24 hour post group had a more pronounced relaxation response to SNP when compared to normoxic. It was suggested by the authors that the mechanisms for these responses warrant further examination. No studies have examined the effects of HBO oxygen therapy on vascular responsiveness following simulated microgravity.
CHAPTER 3

METHODS

3.1 Animals

Five month old, male, Sprague-Dawley rats were separated into four groups with similar mean weights. The experimental groups consisted of hindlimb-suspended (HLS) (n = 10) and hindlimb-suspended undergoing hyperbaric oxygen therapy (HLS-HBO) (n = 10). The controls groups consisted of aging controls (AC) (n = 10) and aging controls undergoing hyperbaric oxygen therapy (AC-HBO) (n = 10). While all groups were fed the same diet, Harlan Teklad (2018, 18 % protein) rat diet (Harlan Holdin Inc., Wilmington, DE), the experimental groups were free fed and the control groups were fed the previous day’s average consumption of the experimental groups. Individual food and water consumption were measured and recorded daily. Body weight was measured and recorded at the beginning of the experiment and once weekly thereafter and on the 28th day. All HLS rats’ abdominal and genital areas were cleaned daily and Vaseline (Chesegorrough-Ponds USA Co., Greenwich CT) was applied to prevent urine burn. All procedures were approved by the University’s Animal Care and Use Committee prior to the initiation of the experiment.

Rats were given one week to acclimate to their new environment prior to the beginning of the experiment. The cages were 18 inches cubed and made out of clear acrylic and aluminum bracing. A swivel hook was suspended from a line that was
placed across the top at the middle of the cage. The tail harness of the rat was attached to the swivel to allow the animal free range of the cage. After the acclimatization period the experimental groups were fitted with their harness (Figure 1). A combination injection of Ketamine (60 mg/Kg) and Tranquived (5 mg/Kg) drugs was given IP to sedate the rats while fitting the tail harness.

The tail was prepared for the application of the harness by cleaning it with soap and water and drying with a hairdryer. Then acetone was rubbed over the tail to remove oils and scales and then it was sprayed with PDQ skin adhesive (Cramer Products, Inc. Gardner, KA). The tail harness (Figure 3.1) consisted of cloth tape approximately 30 cm in length, folded along the long axis so the tacky areas were no longer exposed. The tape was then trimmed along its length to approximately ¾ of its width. The tape was then folded in half along the short axis and a paperclip was anchored with a staple at the folded end and secured into place with a bobby pin and tape. Marine Goop (Eclectic Products, Inc., Pineville LA) was applied along the insides of the cloth tape then adhered along the lateral sides of the tail at approximately the middle 3/5 of the tail. Zinc oxide ointment was applied to the base of the tail to deter chewing and Vaseline was applied. After the rats awakened they were returned to their cage and their tail was suspended from the swivel hook with the paperclip. All limbs were in contact with the floor of the cage. The following day, day 1 of the experiment, the rats were suspended so their hindlegs did not contact the floor and the long axis of their body created approximately a 30° angle with the floor of their cage (Figure 3.1). The animals in the
HLS groups remained in that position for 28 days. Tail suspension was also maintained during the HBO treatments.

![Figure 3.1 Hindlimb suspended rat in cage.](image)

At the end of the 28 days the rats were sedated using Isoflurane (1.0-1.5%). While sedated, blood and urine samples were collected. The thoracic cavity was opened, exposing the heart which was perfused externally with cold saline. The thoracic aorta and femur were dissected, weighed, and then frozen to be used for further analysis.

### 3.2 Hyperbaric Treatment

HLS-HBO and AC-HBO underwent hyperbaric oxygen therapy six days a week for four weeks. The treatment was 1.5 hours in duration at a depth of 2.5 ATA (22.5 psi gauge) with 100% oxygen. Rats were placed in a cage made out of acrylic and
aluminum bracing which allowed for continual HLS. The cage was designed to fit in the hyperbaric chamber (Gulf Coast Hyperbarics, Panama City, FL) and accommodates six rats (3 HLS, and 3 non-HLS) (Figure 3.2). Acrylic dividers were used to keep the rats separated. The chamber was flushed for 30 seconds and pressurized at a rate of 2.5 psi/min to 22.5 psi gauge (2.5 ATA), using 100% oxygen, and maintained for 1.5 hours. Compression and decompression time added 10 minutes to each side of the treatment. Oxygen was allowed to vent at a rate of approximately 5-7 L/min, in order to prevent carbon dioxide buildup in the chamber, while maintaining pressure. At the end of the treatment the pressure was brought back to ambient level at a rate of 2.5 psi/minute. Afterward the rats were removed from the chamber and returned to their cages.

Figure 3.2 Hyperbaric treatment cage.
3.3 Peripheral Quantitative Computed Tomography

Peripheral Quantitative Computed Tomographic (pQTC) scans were performed ex vivo on the femur mid-diaphysis using a Stratec XCT Research-M device (Norland Corp., Fort Atkinson, WI). This pQCT device has a scanning fan beam thickness of 0.5mm and minimal voxel resolution of 70µm, minimizing the partial volume effect common in earlier QCT models. Daily machine calibration was performed using a hydroxyapatite standard cone phantom to ensure measurement precision throughout the study. Thawed femurs were placed in a 1 mol/L PBS-filled vial to maintain proper hydration during the course of the scan, after which time they were returned to the -80°C freezer. A single scan of each femur centered at 50% of the bone length was used for analysis. Scan speed was set at 5.0 mm/sec with a voxel resolution of 0.07 × 0.07 × 0.50mm.

Analyses were performed using STRATEC software (version 5.40B). The analysis for mid-diaphyseal bone (contour mode 3, peel mode 1) was then applied to each femur.

Peripheral Quantitative Computed Tomography (pQTC) protocols were implemented at Texas A&M University, College Station, TX under the direction of Susan Bloomfield, PhD.

3.4 Biochemical Bone Markers

The determination of bone deposition and bone resorption was conducted using commercially available kits. Bone resorption rate was analyzed using a Metra, DPD EIA kit (Quidel Corp., San Diego, CA) to measure urinary DPD crosslinks. The DPD
kit utilized a competitive enzyme assay utilizing a monoclonal anti-DPD antibody to capture DPD (REF METRA). DPD was corrected for urinary concentration of creatine.

Bone resorption rate was analyzed using an Immutopics, Rat Osteocalcin Immunoassay kit (Immutopics, Inc., San Clemente, CA). Osteocalcin was measured using a two-site immunoradiometric assay.

Bone marker analysis was conducted at the University of Texas Southwestern Medical Center at Dallas, Texas under the direction of Dr. Joseph Zerwekh.

3.5 Mechanical Testing

Mechanical testing of the mid-diaphysis of the femur was determined using a three-point bending test on an Instron 1125 machine. Sites of testing were matched to pQCT sampling sites: femoral mid-diaphysis (50% of total bone length). Prior to testing, anteroposterior (AP) and mediolateral (ML) surface diameters at each testing site were measured. Femurs (thawed to room temperature) were placed lateral side down on metal pin supports located ±9mm from the mid-diaphysis testing site. For all tests, quasistatic loading was applied in displacement control at 2.54 mm/min to the upper bone surface until fracture occurred. The small displacements of the servo-controlled Instron were monitored by a linear variable differential transformer, and the applied force was measured with a 4.45 kilonewton (kN) load cell at 445 N maximum load. Load and displacement outputs were digitized to a personal computer at 10Hz using NOTEBOOK PRO software (version 8.01, Laboratory Technologies Corp., Wilmington, MA).
Load-displacement curves were later analyzed with TABLECURVE 2.0 (Jandel, San Rafael, CA). Fracture force (FF) was defined as the highest load obtained at the instant of material damage. Ultimate force Load at which material experiences catastrophic failure or complete fracture of the bone. The same investigator visually checked all load-displacement curves to assure that consistent criteria were used in designating ultimate load.

Mechanical testing protocols were implemented at Texas A&M University, College Station, TX under the direction of Harry A. Hogan, PhD.

3.6 Vascular Responses

Vascular responsiveness to the vasoconstrictor PHE was examined. Rings of the thoracic aorta were cut into 1-2 mm lengths and mounted on two stainless steel wires that were passed through the vessel lumen. One wire was attached to a fixed end and the other to a force transducer so that the vessels could be stretched in order to produce 1.5 g of tension. The resting tensions of the vessels were allowed to stabilize for 30-60 minutes before testing. The vessels suspended in a modified Krebs-Henseleit tissue bath of the following composition (mM): NaCl 115, NaCO\textsubscript{3} 20, KCl 4.0, K\textsubscript{2}HPO\textsubscript{4} 0.9, MgSO\textsubscript{4} 1.1, and glucose C\textsubscript{6}H\textsubscript{12}O\textsubscript{6} bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2} to achieve a pH of 7.4 and temperature was maintained at 37°C. Constriction of arterial rings was determined in response to increasing concentrations of PHE (10\textsuperscript{-10} - 10\textsuperscript{-4} mol/l). Constriction response was expresses as a percentage of KCl-induced preconstriction. Data was collected on Power Lab equipment (AD Instruments, Colorado Springs, CO) using Chart 5 Software (AD Instruments, Colorado Springs, CO) and concentration-
response curves were produced using Table Curve software (AD Instruments, Colorado Springs, CO) to obtain the values for EC$_{50}$ and maximal vasoconstriction.

Vascular response testing was conducted at the University of North Texas Health Science Center, Forth Worth, TX under the direction of Joan Carroll, Ph.D.

3.7 Statistical Analyses

Group differences were determined using four separate 2 × 2 multiple analysis of variance (MANOVA) (HLS [with vs. without HLS] × HBO [with vs. without HBO]), see figure 3.3 for the experimental design. A type I error level was pre-set at 5% ($p \leq 0.05$) and, because there were five analyses, a Bonferroni adjustment was used to protect for the inflation of alpha. The adjustment was $p \leq 0.01$. The analyses were: a) cortical BMD and cancellous BMD in the mid-diaphysis of the femur, b) ultimate load and ultimate failure of the mid-diaphysis of the femur, c) osteocalcin and DPD, and d) thoracic aorta EC$_{50}$ and maximal vasoconstriction in response to PHE. Vascular data were analyzed using four-parameter (minimum, maximum, EC$_{50}$, slope) nonlinear regression. If a significant effect was present follow-up ANOVAs were preformed. Analyses were performed using SPSS Version 11.5 (SPSS Inc., Chicago, IL). Due to the exploratory nature of this study, significant pairwise comparisons were acknowledged when no significant effect occurred with the MANOVA.
\[ IV_2 = HBO \]

\[
\begin{array}{cc}
0 & 1 \\
0 & \text{AC}_{(0,0)} & \text{AC-HBO}_{(0,1)} \\
1 & \text{HLS}_{(1,0)} & \text{HLS-HBO}_{(1,1)}
\end{array}
\]

Figure 3.3 Experimental design scheme.

All data sets met the following criteria for the assumptions of a MANOVA; dependent variables were independent of each other, independent variables were categorical, dependent variables were based upon continuous and interval scales, and the sample size was adequate for the number of analyses performed. The group sizes were unequal due to the unexpected death of two animals and unresponsive aortas. However, SPSS uses analyses that are robust to violations of the assumption of equal group sizes. Due to the relatively small and unequal group sizes the statistical power of the analyses using a MANOVA became limited. Kolmogorov-Smirnov’s test was used to test for multivariate normality of the data, \( p < 0.05 \) indicated a failed test. Data sets for BMD, mechanical testing, vascular testing and DPD met the criteria for normality (\( p > 0.05 \)) (see table 3.1). Osteocalcin failed to meet the assumption for multivariate normality. Levene’s test for homoscedasticity was used to test for homogeneity of variances of the data, \( p < 0.05 \) indicated a failed test. All data sets for BMD, mechanical testing and vascular testing exhibited equal group variances (\( p > 0.05 \)) (see table 3.1). Bone markers failed to meet the assumption for equal group variances. The MANOVA results data sets that violated the assumptions were treated with caution.
Table 3.1 Results for MANOVA Assumptions Testing.

<table>
<thead>
<tr>
<th>Assumption</th>
<th>BMD</th>
<th>Bone Markers</th>
<th>Mechanical Testing</th>
<th>Vascular Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multivariate Normal Distribution (Kolmogorov-Smirnov)</td>
<td>0.20 (Canc)</td>
<td>0.002 (DPD)*</td>
<td>0.06 (FXF)</td>
<td>0.13 (MX)</td>
</tr>
<tr>
<td>Homoscedasticity (Levene)</td>
<td>0.65 (Canc)</td>
<td>0.05 (DPD)*</td>
<td>0.503 (FXF)</td>
<td>0.16 (MX)</td>
</tr>
<tr>
<td></td>
<td>0.95 (Cort)</td>
<td>0.04 (OSTEO)*</td>
<td>0.444 (FMX)</td>
<td>0.15 (EC50)</td>
</tr>
</tbody>
</table>

*p > 0.05 indicates the assumption is upheld. * Failed the assumption for corresponding test.

Changes in body mass pre- and post-28 day experimental period were compared using a 2 × 2 repeated measures analysis of covariance (ANCOVA) (HLS [with vs. without HLS] × HBO [with vs. without HBO]) design. A type I error level was pre-set at 5% (*p* ≤ 0.05), because there five analyses a Bonferroni adjustment was used to protect for the inflation of alpha. The adjustment was *p* ≤ 0.01.
CHAPTER 4

RESULTS

One rat each from the HLS and HLS-HBO groups died during the experimental period, therefore the total $N$ was reduced by two ($N = 38$) and the $n$ for the two HLS groups were each reduced by one (HLS, $n = 9$ and HLS-HBO, $n = 9$). The group sample sizes were further reduced during the experimental procedures for bone marker testing due to inadequate dilution of samples resulting in data beyond the measurable range. Group sample size was also reduced for vascular testing due to damage to the vessel during isolation and preparation.

4.1 Bone Mineral Density

Bone mineral densities for the cancellous and cortical compartments of the mid-diaphysis were measured. The average values for cancellous and cortical BMD are located in table 4.1 and are shown graphically in figures 4.1 and 4.2. The $2 \times 2$ MANOVA on BMD (HLS [with vs. without HLS] $\times$ HBO [with vs. without HBO]) using Pillai’s Trace revealed no overall significant difference ($F (6, 68) =1.54$, $p = 0.179$). However, due to the exploratory nature of the analysis any significant pairwise comparisons ($p < 0.039$) were of interest. The pairwise comparison for cortical BMD between HLS and AC-HBO was significant ($p = 0.014$, $\bar{x}_{HLS} = 1419.70 \pm 14.67$ mg/cm$^3$, $\bar{x}_{AC-HBO} = 1437.07 \pm 16.70$ mg/cm$^3$).
Table 4.1  Bone Mineral Density

<table>
<thead>
<tr>
<th></th>
<th>Cancellous (mg/cm³)</th>
<th>Cortical(mg/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (n = 10)</td>
<td>395.66 ± 44.59</td>
<td>1430.82 ± 12.55</td>
</tr>
<tr>
<td>AC-HBO (n = 10)</td>
<td>378.46 ± 63.08</td>
<td>1437.07 ± 16.70</td>
</tr>
<tr>
<td>HLS (n = 9)</td>
<td>370.13 ± 45.40</td>
<td>1419.70 ± 14.67*</td>
</tr>
<tr>
<td>HLS-HBO (n = 9)</td>
<td>383.42 ± 65.32</td>
<td>1427.12 ± 14.03</td>
</tr>
</tbody>
</table>

Group mean ± SD for each dependent variable. * Significant pairwise comparison with AC-HBO, p = 0.014.

Figure 4.1 Cancellous bone mineral density.
4.2 Biochemical Markers of Bone Remodeling

Deoxypyridinoline and OC were measured to determine bone resorption and formation rates respectively. The average values for DPD and OC are located in table 4.2 and are shown graphically in figures 4.3 and 4.4. The $2 \times 2$ MANOVA on bone markers (HLS [with vs. without HLS] $\times$ HBO [with vs. without HBO]) using Pillai’s Trace revealed no overall significant difference ($F(6, 56) = 0.818$, $p = 0.561$). There were no significant pairwise comparisons for bone markers ($\alpha > 0.039$).

<table>
<thead>
<tr>
<th>Group</th>
<th>DPD (nmole/mmol Cr)</th>
<th>Osteocalcin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (n = 8)</td>
<td>$18 \pm 4$</td>
<td>$69.00 \pm 24.77$</td>
</tr>
<tr>
<td>AC-HBO (n = 10)</td>
<td>$18 \pm 4$</td>
<td>$72.50 \pm 23.82$</td>
</tr>
<tr>
<td>HLS (n = 8)</td>
<td>$17 \pm 3$</td>
<td>$93.50 \pm 51.05$</td>
</tr>
<tr>
<td>HLS-HBO (n = 6)</td>
<td>$17 \pm 2$</td>
<td>$91.50 \pm 13.79$</td>
</tr>
</tbody>
</table>

Group mean $\pm$ SD for each dependent variable.
Figure 4.3 Deoxypyridinoline (nmol/mmol Cr).

Figure 4.4 Osteocalcin (ng/mL).
4.3 Mechanical Properties of Bone

Three point bending tests of the femur were performed to determine ultimate load and fracture force of the femur. The average values for FF and UL are located in table 4.3 and are shown graphically in figures 4.5 and 4.6. The $2 \times 2$ MANOVA on mechanical strength (HLS [with vs. without HLS] × HBO [with vs. without HBO]) using Pillai’s Trace revealed no overall significant difference ($F (6, 68) = 1.19$, $p = 0.324$). No significant pairwise comparisons were observed.

Table 4.3 Mechanical Properties

<table>
<thead>
<tr>
<th></th>
<th>Fracture Force (N)</th>
<th>Ultimate Failure (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (n = 10)</td>
<td>166.51 ± 43.25</td>
<td>186.3±28.84</td>
</tr>
<tr>
<td>AC-HBO (n = 10)</td>
<td>195.58 ± 32.24</td>
<td>204.18±29.06</td>
</tr>
<tr>
<td>HLS (n = 9)</td>
<td>169.93 ± 33.84</td>
<td>185.42±36.17</td>
</tr>
<tr>
<td>HLS-HBO (n = 9)</td>
<td>186.96 ± 22.09</td>
<td>192.05±22.47</td>
</tr>
</tbody>
</table>

Group mean ± SD for each dependent variable.

Figure 4.5 Ultimate force (N).
4.4 Vasoconstrictive Properties

Sensitivity (EC$_{50}$) and maximal vasoconstriction were measured in response to increasing concentrations of PHE. The average values for EC$_{50}$ and MAX are located in table 4.4 and are shown graphically in figures 4.7 and 4.8. The 2 × 2 MANOVA on vascular responsiveness (HLS [with vs. without HLS] × HBO [with vs. without HBO]) revealed an overall significance (F (6, 58) = 3.825, $p = 0.003$, power = 0.947). Follow-up ANOVAs indicated no significant difference (F (3, 29) = 0.612, $p = 0.613$) among EC$_{50}$ and a significant difference among the maximal vasoconstriction (F (3, 29) = 10.935, $p < 0.001$, power = 0.997). Post hoc analysis revealed a significant difference in maximal vasoconstriction between AC and HLS ($p = 0.031$, $\overline{x}_{AC} = 97.66 ± 25.09 \%$, $\overline{x}_{HLS} = 59.97 ± 27.58 \%$), AC and HLS-HBO ($p < 0.001$, $\overline{x}_{AC} = 97.66 ± 25.09 \%$, $\overline{x}_{HLS-HBO} = 32.99 ± 13.81 \%$), and AC-HBO and HLS-HBO ($p = 0.008$, $\overline{x}_{AC-HBO} = 72.95 ± 25.93 \%$, $\overline{x}_{HLS-HBO} = 32.99 ± 13.81 \%$).
Table 4.4  Vasoconstrictive Properties

<table>
<thead>
<tr>
<th>Group</th>
<th>EC$_{50}$ (mol)</th>
<th>MAX (% constriction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (n = 9)</td>
<td>3.58×10$^{-7}$ ± 2.42×10$^{-7}$</td>
<td>97.66 ± 25.09*</td>
</tr>
<tr>
<td>AC-HBO (n = 10)</td>
<td>3.50×10$^{-7}$ ± 1.18×10$^{-7}$</td>
<td>72.95 ± 25.93**</td>
</tr>
<tr>
<td>HLS (n = 6)</td>
<td>4.74×10$^{-7}$ ± 2.99×10$^{-7}$</td>
<td>59.97 ± 27.58</td>
</tr>
<tr>
<td>HLS-HBO (n = 8)</td>
<td>4.28×10$^{-7}$ ± 1.71×10$^{-7}$</td>
<td>32.99 ± 13.81</td>
</tr>
</tbody>
</table>

Group mean ± SD for each dependent variable. * Significantly different compared with HLS and HLS-HBO, $p < 0.039$ for post hoc analysis. ** Significantly different compared with HLS-HBO, $p = 0.008$ for post hoc analysis.

Figure 4.7 EC$_{50}$ (mol)
Figure 4.8 Maximal vasoconstriction (% maximal vasoconstriction). Expressed as a percentage of maximal preconstriction by KCl. * Significantly different compared with HLS and HLS-HBO, $p < 0.039$ for post hoc analysis. ** Significantly different compared with HLS-HBO, $p = 0.008$ for post hoc analysis.

4.5 Body Mass

Body mass on day one and on day 28 of the experimental protocol was measured. The mean mass (g) for each experimental group is located in table 4.5 and is shown graphically in figure 4.9. The repeated measures ANOVA revealed a significant difference in post-experimental mass between groups ($F(3, 33) = 12.456, p < 0.001$, power = 0.997). Post hoc analysis revealed that the mass of HLS and HLS-HBO were significantly less than AC and AC-HBO rats ($p < 0.01$). There were no significant differences between HLS and HLS-HBO or between AC and AC-HBO ($p > 0.01$)
Table 4.5 Body Mass (g)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (n = 10)</td>
<td>423 ± 34</td>
<td>454 ± 41</td>
</tr>
<tr>
<td>AC-HBO (n = 10)</td>
<td>432 ± 27</td>
<td>460 ± 35</td>
</tr>
<tr>
<td>HLS (n = 9)</td>
<td>433 ± 35</td>
<td>415 ± 37</td>
</tr>
<tr>
<td>HLS-HBO (n = 9)</td>
<td>440 ± 31</td>
<td>409 ± 24</td>
</tr>
</tbody>
</table>

Group mean ± SD for each dependent variable.

Figure 4.9 Body mass (g) at day 28 of HLS. * Significantly different than AC and AC-HBO ($p < 0.01$).
CHAPTER 5

DISCUSSION

Effective countermeasures to the microgravitational alterations in BMD decreases and orthostatic intolerance have yet to be identified. Ascertaining countermeasures to eliminate or minimize these responses is essential to protect the health and well-being of astronauts during and after their missions. The purpose of this investigation was to determine if HBO therapy would attenuate or eliminate decreases in BMD and orthostatic response resulting from simulated microgravity. To our knowledge, this was the first study conducted to examine the effects of HBO therapy on physiologic changes due to simulated microgravity.

5.1 Bone Mineral Density

An average decrease in BMD of 1-2 % per month in microgravity and simulated microgravity environments has been reported in the literature (27, 58). Previous studies have reported decreases in total BMD (1, 14, 21, 45), however they neither separated the different types of bone into compartments nor isolated a specific section of the bone. The BMD measurement technique, pQCT, is able to differentiate between cortical and cancellous bone compartments which allow precise measurement of the areas of bone mineral changes. It has been reported that the decreases in total BMD in previous studies could be attributed to a decline in cancellous bone (5).
Bloomfield et al. (5) reported a significant decrease in cancellous BMD (by 21%) at the proximal tibia after 28 days HLS and a significant increase in cortical BMD (by 5.6%) at the metaphysis of the proximal tibia. The MANOVA results of the present investigation indicate that HLS and HBO did not significantly affect cortical or cancellous BMD at the mid-diaphysis of the femur following of the 28 day experimental period. It appeared that HBO and weight bearing resulted in a slight increase in cortical BMD. A pairwise comparison indicated that cortical BMD was significantly reduced in the HLS group when compared to the AC-HBO group. Adding HBO to HLS resulted in a slight increase in cortical BMD over HLS alone. Further examination is required to confirm these results.

5.2 Biochemical Markers of Bone Remodeling

Bone continually undergoes a remodeling process where bone mineral is removed or resorbed then new bone mineral is deposited within the cavity. The uncoupling, increase in bone resorption, a decrease in bone deposition or the combination of the two processes, can lead to a decrease in BMD. Deoxypyridinoline and osteocalcin are commonly measured to determine alterations in bone resorption and deposition respectively.

Previous space and land-based studies have observed increases in bone resorption and decreases in bone formation markers. Caillot-Agusseau et al. (8, 9) observed a 50% and 54% increase in DPD in cosmonauts during 21 and 180 days of spaceflight respectively. Kurokouchi et al. (30) measured an increase in tartrate-resistant acid phosphatase (TRAP) in five-week old rats at days one and three, but this
returned to pre-HLS levels at day five and remained within pre-HLS values during remainder of the 14 of days HLS. Caillot-Agusseau et al. (8, 9) measured changes in OC during 21 and 180 day space missions in cosmonauts. Osteocalcin levels were decreased by 18% and 27 % during 21 and 180 days, respectively when compared to pre-flight levels. Kurokouchi et al. (30) observed a decrease in OC levels from day three to day 14 in 5-week old HLS rats.

There were no significant changes in PYD and OC among the groups in the present study, therefore biochemical bone markers did not indicate that uncoupling of bone formation and resorption had occurred. These results are consistent with the observations that there were no significant changes in bone markers therefore any significant changes BMD were not expected.

5.3 Mechanical Properties of Bone

Disruptions in the remodeling of bone can lead to alterations in bone architecture and the removal of structural elements which lead to the loss of mechanical strength and fractures (6). This is a primary concern when planning long term space travel such as a mission to Mars where travel time to and from the planet will take about three years. A fracture that occurs in space could compromise the health of the individual, the crew, and the mission.

The deterioration of the struts and plates due to high rates of resorption weaken cancellous bone making it more susceptible to fractures. Hogan et al. (26) measured changes in the mechanical properties of cancellous bone in female rats that underwent an ovariectomy to stimulate bone loss. In a three point bending test of the femur, a 10%
increase in maximal force was observed in the ovariectomized rats when compared to sham. In a study by Bloomfield et al. (5) pQCT was used to measure changes in bone mineral density and differentiate between losses in cortical and cancellous bone in six-month old rats after 28 days of HLS. Mechanical testing was then performed to determine if there was a relationship between changes in compartmental bone density and the structural integrity of bone. The main finding was that there was an 18% increase in fracture force that corresponded to a 5.6% increase in cortical density of the mid-diaphysis of the tibia. The results of the present study indicate HLS and HBO did not alter the strength of the shaft of the femur as determined by the three point bending test. These results correspond to the maintenance of BMD at the end of the experimental period.

5.4 Hyperbaric Therapy and Bone

The rationale for the use of HBO as a countermeasure to reduce the rate of bone demineralization was based on the research supporting the use of HBO in non-union and delayed union fractures (13, 57). It was hypothesized that by increasing oxygen delivery to the bone tissue with HBO, balance in the bone remodeling process would be maintained. Within microgravity and simulated microgravity a cephalad fluid shift occurs. It has been demonstrated that the perfusion pressure of blood to the femur is compromised (10). Hyperbaric oxygen therapy increases the delivery of oxygen through its dissolution in the plasma. On reaching the tissues, the increased concentration gradient increases the perfusion distance of oxygen. It was hypothesized
that these two factors combined would compensate for the decreased perfusion pressure to the hind limbs that occurs with HLS.

5.5 Vasoconstrictive Properties

The regulation of orthostasis upon arrival to Earth after space travel is compromised and can lead to syncope. Buckey et al. (7) observed that nine of 14 astronauts were unable to complete a 10 minute stand test after returning from 9-14 days of space travel. Buckey et al. (7) found that there was a greater vasoconstrictive response in the astronauts who were able to complete the stand test versus the non-finishers. Research has suggested that the cephalad shift of bodily fluids may elicit physiologic responses that alter the baroreceptor reflex (37, 41, 54). However, decreased vascular responsiveness to sympathetic output has also been proposed as a contributing factor to orthostatic intolerance (15, 16).

It has been demonstrated that hyperbaric therapy increases SVR. Berry et al. (4) observed an increase in SVR following 90 minutes of HBO therapy using 100 % O₂ at 2 ATA. The SVR continued even after returning to ambient air and remained elevated for 30 minutes. Oxygen acts as an alpha adrenergic agent resulting in vasoconstriction therefore it was concluded that SVR would be increased during HBO treatments (35, 36). The vessel response post treatment was a matter of speculation. We hypothesized that the intermittent vasoconstrictive response to HBO might result in a more vasoconstrictive state between HBO treatments. The results indicate that the opposite response occurred. Hindlimb suspension in the present study resulted in a decreased maximal vasoconstrictive response to PHE of the isolated thoracic aorta when
compared to control rats, a 39% and 54% decrease in MAX was observed between AC and HLS, and AC-HBO and HLS-HBO respectively. These results are consistent with previous studies examining the effect of HLS on maximal vasoconstriction of the abdominal and thoracic aortas (15, 16). A further reduction in maximal vasoconstriction occurred in the aortas of the HLS-HBO group. A 66% decrease in MAX occurred between AC and HLS-HBO. Our results of decreased vasoconstriction in response to PHE are supported by the findings of Tahepold et al. (51).

There were no differences in the sensitivity (EC$_{50}$, the mean effective agonist concentration that produces 50% of maximal vasoconstrictive response) to PHE between HLS and control rats. Previous studies have also reported no changes in sensitivity to PHE after HLS (15, 16). Hyperbaric therapy had no affect on sensitivity to PHE between any of the experimental groups.

5.6 Body Mass

The HLS model to simulate microgravity has been successfully used in numerous studies. The HLS model presents minimal stress to the animal based upon hormonal levels (14, 38) and body weight measures (5, 21, 38, 45). However, several HLS studies ranging from two to five weeks in duration have demonstrated significantly lower body weight in the HLS groups when compared to controls (14, 15, 25). Body mass of the HLS and HLS-HBO rats in this study were significantly lower than both control groups. The decreases in body mass may have been due to a normal physiologic response to simulated microgravity, such as decreased appetite leading to a
decrease in food consumption, decreased muscle mass due to disuse and reduction in blood volume.

5.7 Conclusions

Twenty-eight days of hindlimb suspension and 90 minutes of hyperbaric oxygen therapy treatment at 2.5 ATA using 100 % oxygen did not have a significant effect on cortical or cancellous bone mineral at the mid-diaphysis of the femur. Biochemical bone markers for bone remodeling did not indicate significant changes with HBO. Deoxypyridinoline and osteocalcin levels were maintained indicating that rates of bone resorption and deposition were maintained. Furthermore no alterations in bone mechanical strength were observed. The absence of a significant effect due to HLS in the present study was not consistent with previous studies. No studies, however, have examined the effects of HBO on bone parameters. Therefore, there are no bone data to compare the HBO results with from this experiment. To confirm these results, future research should to be conducted that examines the effects of HBO and HLS on bone. Altering the HBO treatment frequency and duration, increased sample size, and the addition of hyperoxia and pressure control groups should be considered in future investigations.

Under the conditions of the current study we could not recommend the use of HBO as an intervention to offset detrimental effects of microgravity on bone. However, other pressure and oxygen doses might show positive results. When compared to humans, rat’s arterial blood pO₂ is approximately twice as great as humans (19). Klemetti et al. (28) found that reducing HBO therapy treatment time in rats to 60
minutes from 90 minutes (the standard clinical treatment time in humans) for seven
days on surgically damaged mandibles significantly restored blood flow to the bony
surgical site. Barth et al. (2) found that once daily HBO therapy 5 day a week was more
effective at repairing surgically damaged bone tissue than a twice daily regimen.

Maximal vasoconstriction response to PHE was decreased with HLS and was
further reduced in both HBO groups. The effect of HBO on maximal vasoconstriction
could further attenuate the orthostatic response in astronauts returning from space.
Therefore, under the conditions of this study HBO is not recommended as a
countermeasure to the orthostatic intolerance experienced after space travel. However,
future studies that vary the dosage of the HBO treatment (duration and depth) may
have different results.
REFERENCES


BIOGRAPHICAL INFORMATION

The author received her Bachelor’s degree in exercise science in 1998 from The University of Texas at Arlington. She began her graduate studies in exercise physiology in the fall of 2003. While pursuing her degree she was a graduate teaching assistant and participated in several research activities involving electromyography, mechanomyography, stretching, and muscle strength. Laurie plans to pursue a career in secondary or higher level education or work in a research laboratory. Laurie’s research interest is in sport performance and spinal cord injury.