12-2010

Von Willebrand Factor Expression in Vascular Endothelial Cells of Cage Control and Antiorthostatic Cage Suspension Golden Hamster Ovaries.

Kristan Provchy
East Tennessee State University

Follow this and additional works at: http://dc.etsu.edu/honors

Part of the Medical Physiology Commons

Recommended Citation

Von Willebrand Factor Expression in Vascular Endothelial Cells of Cage Control and Antiorbostatic Cage Suspension Golden Hamster Ovaries.

Kristan Michelle Provchy
December 2010

A Senior Honors Thesis Submitted as a Partial Fulfillment of the University Honors Scholar Program, East Tennessee State University, Johnson City, TN

______________________________
Allan D. Forsman, Ph.D.       Date

______________________________
Bert C. Lampson, Ph.D.       Date

______________________________
Michael H. Stone, Ph.D.       Date
TABLE OF CONTENTS:

ABSTRACT .................................................................................................................................................3

INTRODUCTION ........................................................................................................................................3

MATERIALS AND METHODS ..............................................................................................................15

RESULTS ..............................................................................................................................................22

DISCUSSION .......................................................................................................................................26

REFERENCES .....................................................................................................................................31

FIGURES & TABLES ..............................................................................................................................34

ACKNOWLEDGEMENTS ....................................................................................................................45
Abstract:

The hamster estrous cycle lasts four days and is considered to be a physiological model for angiogenesis. Angiogenesis is the formation of new capillaries from preexisting vessels, and it occurs extensively during corpus luteum formation in the estrous cycle. Von Willebrand Factor (vWF) is a glycoprotein that is secreted uniquely in endothelial cells and megakaryocytes. It is frequently used as an endothelial cell marker and it is able to detect vessels within tissues when it is used in immunohistochemical staining techniques. This study explores von Willebrand Factor expression within Golden Hamster ovarian tissue. In particular, this study uses cage control and antiorthostatic cage suspension tissue. Antiorthostatic cage suspension is a model developed to mimic and study the physiological effects caused by microgravity, such as that experienced in space flight. It is hypothesized that simulated microgravity caused by antiorthostatic cage suspension would result in lower levels of vasculature and expression of vWF within ovarian tissue. Due to financial considerations, conclusive data was not obtained due to a lack of statistics. However, our study indicates that vasculature and vWF expression may be increased in antiorthostatic cage suspension tissue.

Keywords: von Willebrand Factor, vascular endothelial cells, immunohistochemistry, antiorthostatic, microgravity

Introduction:

The ovarian cycle of mammals has been extensively researched in the laboratory due to its repetitious nature as it relates to the cellular proliferation and differentiation that coincides with the follicular development, corpus luteum formation, and corpus luteum regression.
(Kaczmarek et al., 2005). These changes that occur within the ovary during the ovarian cycle require changes in the vascularization. This study focused on these vascularization changes within the hamster estrous cycle by visualizing an endothelial cell marker, von Willebrand Factor, that exists within the vascular endothelial cells of Golden Hamster (Mesocricetus auratus) ovarian tissue. We also conducted a comparative study by looking at the expression in vWF among cage control tissue and tissue that has been subjected to antiorthostatic suspension.

The Estrous Cycle:

The hamster estrous cycle lasts approximately four days and is comprised of two phases: the follicular phase and the luteal phase (Forsman and McCormack, 1992). The follicular phase begins with a primordial follicle within the ovary. A follicle is defined as a group of cells that surrounds an oocyte (Lawrence, 1995). Follicles are formed by specialized epithelial cells known as granulosa cells, and multiple follicles can be present during any given estrous cycle. A primordial follicle is comprised of a single layer of granulosa cells surrounding a primary oocyte. The follicle begins to develop in response to the gonadotrophin Follicle Stimulating Hormone (FSH) release by the anterior pituitary. As the follicle develops, the epithelial cells multiply and serve as a wall around the oocyte. Once this “wall” becomes six cells thick, the immediately surrounding stromal cells differentiate and become what is known as the theca. The follicle is now a secondary follicle that consists of both an outer thecal layer, which functions as the main source of estrogen, and an inner granulosa layer, which is the main source of progesterone (Perry, 1972). It is important to note that the theca of the continuously growing and maturing follicle is extremely vascular. The inner granulosa layer, however, is avascular (Forsman and McCormack, 1992; Kaczmarek, et al., 2005; Perry, 1972; Reynolds, et al., 1992). Separating the
two layers is a basal membrane called the membrane propria which serves as a barrier so that there is no vascular penetration into the granulosa layer until after ovulation (Perry, 1972).

As the follicle continues to develop, an antrum begins to form. This occurs when a rift appears within the granulosa layer initially forming a fluid-filled slit. As the follicle continues to grow and expand, the antrum enlarges and eventually accounts for most of the follicle’s volume (Perry, 1972). At this time, the theca layer is now fully developed and it consists of two separate capillary networks, one in the theca interna and one in the theca externa (Kaczmarek, et al., 2005). The theca externa is formed as a result of the outer stromal cells being compressed. The internal layer of the theca is the theca interna, which is made up of an envelope of condensed connective tissue known as the theca folliculi (FreeDictionary.com). Together, the theca interna and theca externa appear structurally as a “hollow ball” (Forsman and McCormack, 1992).

Up until ovulation, the follicle is secreting the hormone estrogen which causes the female to be in a sexually receptive state known as estrous. Estrous typically occurs right before ovulation. Near the end of estrous, Luteinizing Hormone (LH), another gonadotrophin from the anterior pituitary, causes ovulation. At the time of ovulation, the follicle ruptures and the oocyte is expelled (Perry, 1972).

After ovulation, the Luteal phase immediately begins. During this phase a temporary organ, the corpus luteum, forms from the remains of the ruptured follicle. It is during this time the blood flow to this tissue is at its highest (Forsman and McCormack, 1992). The granulosa cells begin to hypertrophy at a rapid rate and they luteinize and begin to secrete progesterone. Progesterone is the hormone that prepares the uterus to accept and nourish the developing embryo if fertilization were to occur. The blood vessels within the theca can now penetrate the membrane propria, and the newly formed luteal tissue begins to become highly vascularized.
(Perry, 1972; Kaczmarek, et al., 2005). The new vascularization starts from the vessels of the theca externa and the vessels begin to grow in an inward direction. This leads to a very complex network of blood vessels so that the initial “hollow ball” of the theca becomes a mature corpus luteum that is structurally similar to a “solid ball” (Forsman and McCormack, 1992). The mature corpus luteum has the greatest blood flow of any tissue in the entire body (Kaczmarek, et al., 2005). During this time, the microvascular endothelial cells are the most abundant and continuously proliferating cells within the developing corpus luteum (Kaczmarek, et al., 2005), and approximately 50% of the cells within the mature corpus luteum are these endothelial cells (Reynolds, et al., 1992). If no fertilization and subsequently no pregnancy occurs, the corpus luteum regresses rapidly and progesterone secretion ceases. At this time, FSH is again secreted from the anterior pituitary and follicular maturation can begin again. It is because of the hamster’s rapid growth and degeneration of its corpus luteum that makes the golden hamster particularly useful in laboratory studies as it is considered to be a physiological model for angiogenesis (Forsman and McCormack, 1992).

**Angiogenesis:**

The formation of the vascular networks that comprise the corpus luteum and allows for the increase in blood flow in the ovary results from a process known as angiogenesis. Angiogenesis is the formation of new capillaries by sprouting from pre-existing small blood vessels (Lawrence, 1995). Angiogenesis is important because it allows for hormone producing cells to obtain oxygen and other vital nutrients, in addition to allowing hormone transfer and substrate delivery to the target tissues (Kaczmarek, et al., 2005). This is important because the corpus luteum is an endocrine organ that secretes its hormones directly into the bloodstream. Angiogenesis is usually considered to be involved with pathological conditions such as tumor
growth, wound healing, retinopathies, and inflammatory processes. However, angiogenesis is a physiological and normal process in the female reproductive organs, such as the ovary (Augustin, et al., 1995).

Angiogenesis begins with capillary proliferation, which consists of three different stages: 1) fragmentations occur within the basal lamina of the existing blood vessel, 2) endothelial cells of the existing blood vessel migrate towards the angiogenic stimulus, and finally 3) the endothelial cells proliferate. Completion of the new blood vessel arises once a new capillary basal lamina is formed and the new capillaries are differentiated into arterioles and venules (Kaczmarek et al., 2005; Reynolds, et al., 1992). The fact that endothelial cells play such a crucial role in angiogenesis provides further rationale for why there are such a high percentage (50%) of them present within the corpus luteum. Often times angiogenic factors can be localized within these endothelial cells.

When it comes to angiogenic factors, there are two different types to be considered: 1) factors that act directly on vascular endothelial cells and 2) factors that act indirectly by means of mobilizing host cells to release endothelial growth factors (Folkman and Klagsbrun, 1987). Two very potent direct angiogenic factors are Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor-2 (FGF-2). These factors not only stimulate angiogenesis, but they also control the expression of the endothelial cell marker von Willebrand Factor (vWF). When the angiogenic factors VEGF and FGF-2 are present, they up regulate the vWF mRNA and protein levels within the endothelial cells. Von Willebrand Factor mRNA is derived from vascular endothelial cells and megakaryocytes and is a particularly useful endothelial cell marker. It can be used to detect early signs of angiogenesis because it detects the initial activation of the endothelium (Zanetta, et al., 2000).
**Von Willebrand Factor:**

Von Willebrand Factor is a glycoprotein with a multimeric structure. Von Willebrand Factor can have a mass of anywhere between 500 and 10,000 kilodaltons (kDa). Electron microscopy has shown it to be either a filamentous structure or a loosely coiled molecule. Within the multimeric structure of vWF, there are a series of oligomers that can contain a wide range of subunits. The number of subunits possible ranges from between two and one hundred. Within these subunits, there is a relatively high carbohydrate content with approximately 10-19% of the total mass of a mature subunit being carbohydrates. The most significant characteristic about vWF’s structural composition is that it has a high cysteine content. This is essential for vWF because it links all of the subunits into a high-order structure. This complex molecular structure of vWF allows for the potential of numerous contact sites with the platelets and subendothelial structures (Ruggeri and Ware, 1993).

Von Willebrand Factor is produced solely by the endothelial cells and megakaryocytes (Pusztaszeri, et al., 2006; Ruggeri and Ware, 1993; Wagner, et al., 1982; Zanetta, et al., 2000). The vWF is found within the endothelial cells and subendothelial matrix of the vessel wall, and is found in the blood in both the plasma and the platelets (Ruggeri and Ware, 1993). It has also been identified in the vascular lumen (Pusztaseri, et al., 2006). The vWF protein has several functions. It is required for the normal arrest of bleeding that occurs during hemostasis, or tissue injury, by aiding in the binding of platelets to the subendothelium. It also serves as the carrier for coagulation factor VIII within the circulating blood, in which the two proteins form a factor VIII/vWF complex. Von Willebrand Factor also is crucial for the adhesion of the endothelial cells to the vessel basal lamina, in addition to serving as a bridge between the components of the
vessel wall and specific receptors on the platelet surface (Pusztaseri, et al., 2006; Ruggeri and Ware, 1993; Zanetta, et al., 2000).

While vWF is expressed uniquely in the endothelial cells and megakaryocytes, it is important to note that not all endothelial cells express von Willebrand Factor. In addition to this, expression levels of vWF can differ significantly among the endothelial cells of different murine tissues. Expression levels of vWF can also differ when it comes to venous and arterial endothelial cells of the same tissue type. In fact, vWF has been found to be expressed in higher concentrations on the venous side, rather than the arterial side, of the capillary circulation (Zanetta, et al., 2000). In order, veins have the strongest expression of vWF, followed by the arteries, arterioles, capillaries, and venules (Pusztaszei, et al., 2006).

The synthesis of von Willebrand Factor is controlled at the transcriptional level within the endothelial cells and megakaryocytes (Zanetta, et al., 2000). After synthesis, vWF is stored in specific organelles: Weibel-Palade bodies for endothelial cells and the α-granule for the megakaryocytes and platelets (Pusztaszei, et al., 2006; Ruggeri and Ware, 1993; Wagner, et al., 1982; Zanetta et al., 2000). Weibel-Palade bodies originate from the Golgi Apparatus and are rod shaped organelles that derive uniquely in endothelial cells (Ruggeri and Ware, 1993; Wagner, et al., 1982). Weibel-Palade bodies contain another protein known as β-selectin. β-selectin is a cell adhesion molecule that is postulated to be involved in the interactions of leukocytes with the endothelial cells and platelets (Ruggeri and Ware, 1993).

There are two different pathways that regulate the secretion of vWF. The first is the constitutive pathway that is directly linked to synthesis. The other is a regulated pathway in which there is storage in the appropriate organelles and then there is release after stimulation. Von Willebrand Factor within megakaryocytes generally is expressed via the regulated pathway.
Endothelial cells, on the other hand, typically secrete vWF through the constitutive pathway. However, when agonists such as histamines, estrogens, thrombins and fibrins are present, the endothelial cells secrete the stored vWF in a regulated fashion (Ruggeri and Ware, 1993).

**Immunohistochemistry:**

Because vWF is an endothelial cell marker, it is commonly used to identify vessels within tissue sections because vessels are lined with endothelial cells. This is done with immunohistochemical staining techniques. Immunohistochemistry was first developed in 1941 by Coons, et al. when they identified a technique for detecting cellular antigens in tissue sections. Since then, immunohistochemistry has served as a fundamental tool in anatomic pathology for research and diagnostic purposes (Cregger, et al., 2006; Ramos-Vara, 2005). The fundamental concept behind immunohistochemistry is the visualization of antigens within tissue sections by means of specific antibodies binding to the antigens, followed by a colored histochemical reaction.

An antigen is characterized as any substance capable of specific binding to an antibody or a T-cell receptor (Lawrence, 1995). Antigens are proteins, and as such, can have many different isoforms (structures). However, a common problem among antigens located within fixated tissue is that the fixation can modify the tertiary structure of the antigen. This is problematic as it may make the antigens undetectable by antibodies that should have specificity for them. Therefore, antigen retrieval methods are often employed (Ramos-Vara, 2005).

Antigen retrieval methods are important because they unmask antigens hidden by formalin cross-links in fixation. As such, antigen retrieval can 1) expand the range of antibodies able to be used in immunohistochemistry, 2) reduce false-negative staining results in over-fixed tissues, and 3) allow for greater diagnostic accuracy (Shi et al., 1991). Antigen retrieval methods
vary, with enzyme digestion and heat-based methods being the most popular (Ramos-Vara, 2005). Shi et al. developed the microwave-heating method in 1991. This method heated slides to up to 100°C, preferably in two separate five minute cycles in the microwave, with a one minute interval in between. New methods of heat-induced antigen retrieval came into popularity, with methods such as autoclaving, pressure cooking, steam heating, and the water bath. It has been found, however, that the method of heating is of no importance; the high temperature achieved during heating, however, is the most important factor when it comes to antigen retrieval (Shi, et al., 1997).

Once the antigens are accessible, the specific antibodies can bind to them. Antibodies, also known as immunoglobulins (Ig), are Y shaped with two identical light chains and two identical heavy chains, with both the light and heavy chains being of the same type. The light chains consist of 2 different regions. The first region is the C terminal half, which is constant (C\text{L}). The second region is the N-terminal half, which has abundant sequence variations (V\text{L}). The antigen binding portion of the Ig molecule is the Fab region which contains both types of variable regions: V\text{H} (heavy chains) and V\text{L} (light chains). The number of stable V\text{H} and V\text{L} interactions are increased by the presence of the C\text{L} domains. This contributes to the overall diversity of the antibody molecule expressed by the animal (Kindt, et al., 2007). The tail of the Y is made up of the Fc portion, which determines the biological functions of the antibody and allows it to bind to other antibodies. However, the Fc region can also allow the binding of the antibody to non-immune cells, such as inflammatory cells, creating background staining due to non-immune adherence of antibodies to tissue sections (Ramos-Vara, 2005).

The antigen binding site of the antibody is known as the paratope. The epitopes are the regions of the antigen that bind to antibodies. Epitopes can be anywhere from 5-21 amino acids
in length. The most important aspect of an epitope is its tertiary structure, or the way in which the peptide chains of the protein are folded. This is because it is the tertiary structure that dictates how a protein will interact, and hence, which antibody it will bind to. The paratope and the epitope interact by means of non covalent bonds, particularly Van Der Waals forces and electrostatic interactions. The more bonding interactions that occur, the greater the affinity between the antigen and antibody (Ramos-Vara, 2005).

In order to make specific antibodies, an animal such as a mouse, rabbit, or goat, is immunized with the particular peptide/antigen of interest (Cregger, et al., 2006; Ramos-Vara, 2005). The animal then produces antibodies that specifically recognize and bind to that antigen. There are two different types of antibodies: polyclonal and monoclonal. Polyclonal antibodies can be made in a variety of different animals. They have a higher affinity, wide reactivity, and lower specificity. Therefore, they are more likely to identify a variety of isoforms of the target antigen. Because of this, the potential for false-positive results is greater. Monoclonal antibodies, on the other hand, have a very high specificity. They are created mostly in mice, but can be generated from rabbits as well (Ramos-Vara, 2005).

For use in immunohistochemistry, antibodies are commonly made as either primary or secondary antibodies. Primary antibodies target a specific antigen and are usually unlabelled. Secondary antibodies are made to bind to the primary antibody. These secondary antibodies are labeled with a biotin carrier protein. A third reagent is then applied, streptavidin horseradish peroxidase (HRP), which binds and tags the biotinylated secondary antibody. Diamino-Benzidine (DAB) is a common HRP substrate that binds to this tag and generates a brown-colored staining product. This enables the antigens within the tissue to be viewed with the aid of light microscopy.
**Antiorthostatic Simulated Microgravity:**

The immunohistochemical study we conducted involved visualizing von Willebrand Factor within the vascular endothelial cells of hamster ovaries. We visualized vWF in two different types of tissue: cage control and antiorthostatic suspension tissue. Antiorthostatic suspension is a technique that involves orienting the animal in an approximate 30° head down tilt. It was developed in response to the necessity of studying the physiological systems that change in a microgravity environment (Chapes, et al., 1993). According to spaceflight.nasa.gov, microgravity is a state in which gravity is reduced to almost negligible levels, such as during space flight. Microgravity in space flight has been shown to have dramatic effects on the physiological systems of the body (Chapes, et al., 1999). In order to study these effects, it was necessary to create a model that parallels the changes that occur in the microgravity of space, without the drawbacks of spaceflight studies. These drawbacks include: scheduling, experimental duration, ease of experimental manipulation, the ability to repeat and extend experiments, and of course, financial drawbacks (Morey-Holton, et al., 2002).

The antiorthostatic suspension model that has been widely accepted is the hindlimb unloading model, which has been approved by the NASA ARC Animal Care and Use Committee. The development of this hindlimb unloading model began as a cooperation between the United States and the USSR in 1975 on the Cosmos Spaceflight Mission (Morey-Holton, et al., 2002). In 1979, Morey-Holton described the simulation of space flight with the hindlimb unloading model that involved keeping rats at a 30° head-down tilt. This was accomplished by completely unloading the hindlimbs of the rat. The angle measurement was important because it
allowed for 50% of the animal’s bodyweight to be dispersed to its forelimbs, and it only unloaded the lumbar and not the cervical vertebrae (Morey-Holton, et al., 2002).

The hindlimb unloading model simulates weightlessness, and as such, mimics the many physiological changes due to microgravity. These changes include cephalad fluid shifts, movement of the abdominal organs toward the thorax, muscle atrophy, decrease in bone muscle density, and in addition, the hydrostatic gradient is minimized within the cardiovascular system. In addition to this, changes in capillarization, oxidative capacity, and immunological responses also occur in response to unloading (Chapes, et al., 1993; Morey-Holton, et al., 2002). While the hindlimb unloading model does provided similar physiological changes to the microgravity actually experienced in space, no model is perfect as one cannot completely escape the effects of gravity on Earth’s surface. In addition to this, the reentry and readaptation to gravity that occurs during spaceflight is also not accounted for during the unloading model (Morey-Holton, et al., 2002). However, until these obstacles are overcome by a better simulated microgravity model, antiorthostatic suspension in the hindlimb unloading model continues to be used and accepted.

Spaceflight microgravity and simulated microgravity induced by the hindlimb unloading model have similar physiological changes in the muscle and bone. Other similar changes occurred in the heart, pulmonary, intestine, immune, endocrine, and reproductive functions as well (Morey-Holton and Globus, 2002). This preliminary study deals with the changes in the reproductive functions due to antiorthostatic suspension. The cephalad fluid shifts that occur in response to antiorthostatic suspension could be responsible for the changes because the fluids move out of the caudal portion of the animal and settle in the cephalic region of the body. This in turn exerts a greater amount of pressure on the vasculature of that region, thereby causing changes in that vasculature (Pattison, et al., 1991). The vasculature in the reproductive organs is
hypothesized to change in response to simulated microgravity as well. This is because the fluid and blood shifts away from the lower abdomen, and therefore, the reproductive organs themselves. If blood circulation to the abdominal organs, including the ovary, has been altered, the vasculature of the antiorthostatic suspension hamster ovaries is hypothesized to be reduced compared to the vasculature of the cage control hamsters. The expression of vWF is also hypothesized to be decreased in the antiorthostatic suspension hamster ovaries.

**Materials and Methods:**

Immunohistochemistry is a procedure that detects specific protein molecules within tissue cells. This study focused on the ovarian tissue of the Golden Hamster (*Mesocricetus auratus*). All of the procedures in the study that were related to animal use were approved by the University Committee on Animal Care at East Tennessee State University in Johnson City, Tennessee. In addition, all procedures conformed to the US National Institutes of Health’s *Guide for Care and Use of Laboratory Animals* (1996). The female hamsters were continuously kept on a 14:10 hour light:dark cycle at temperatures of 21-23°C. Each hamster used experienced 3 consecutive four day estrous cycles prior to the beginning of the experiment. Day 1 of each cycle was determined by a vaginal discharge that was observed between 0700 and 0900 hours. The hamsters were then checked daily until the discharge was again observed. Hamsters were then checked on each Day 1 of their 4-day estrous cycle that followed until the suspension or perfusion took place. Once the hamsters were suspended, they could no longer be checked for their Day 1 start of their estrous cycle as that would interfere with their suspension. Therefore, the control hamsters were not checked as well.

Twenty Golden Hamsters were used for the antiorthostatic suspension study in two separate groups of ten. One group consisted of control hamsters that were kept in conventional
cages. The other group of ten hamsters was placed in suspension cages at 1700 hour on Day 4 of their estrous cycle. The suspension cages consisted of wire mesh bottom cages equipped with a metal rod running length-wise along the top of the cage. The wire mesh bottom was used as opposed to traditional cage bedding in order to reduce the possibility of the bedding being piled up, thereby reducing the suspension angle of the hamsters. The hamsters were suspended according to a modified technique used by Wronski, Morey-Holton (Armstrong et al., 1993). In the normal suspension technique, animals (typically rats or mice) are suspended by their tails. This is problematic because hamsters do not have much of a tail. Therefore, modifications of the technique described by Armstrong et al. were necessary. These modifications involved a lot of strategic taping of the hamsters. The hind feet and ankles were wrapped in medical tape in order to ensure the foot was maintained at a 90° angle to the leg. Tape was then wrapped around the lower abdomen, hips, and thighs to fix the thighs in a continual and slight forward/flexed position. Plaster casting strips were placed over the tape around the lower abdomen to prevent the hamsters from gnawing through the tape. Once the plaster hardened, the suspension mechanism was taped to the dorsal surface of the cast (Figure 1). The suspension mechanism consisted of a small metal ring around the suspension bar above the cage, a 360° swivel, and extended paperclips to adjust the length of the apparatus in order to keep the hamsters at a constant approximate head-down tilt angle of 30°. In order to reduce friction of the metal ring on the bar, scotch tape was wrapped around the surface of the suspension bar. This made it easier for the ring to slide, allowing free range of movement with the hamsters’ front limbs while their rear limbs remained elevated (New and Forsman, 2001).

All of the hamsters used in the study were in the weight range of 101.9g-136.1g. The cage antiorthostatic microgravititivity hamsters were left suspended for approximately one week
before euthenization. Euthenization of the ten cage control and the ten cage suspended hamsters occurred at the following day and times in their estrous cycle: 0400, 1000, and 2200 of Day 1, 1000 and 2200 of Day 2, 1000 and 2200 of Day 3, and 1000, 1600, and 2200 of Day 4. These days and times of their estrous cycle were approximated from the hamsters’ three consecutive estrous cycles prior to suspension. The hamsters were subjected to CO₂ euthanasia, the abdominal cavities were incised, and the ovaries were removed. The ovaries were then immediately immersed in a 1% formalin fixative.

The tissue used was fixed and paraffin embedded and prepared by Jeff Stirman, a student working in the lab of Allan Forsman. The fixation involved a 12 hour emersion of the tissue at 4°C in 1% paraformaldehyde, followed by another 12 hours at 4°C in 4% paraformaldehyde. The paraformaldehyde soaks were then followed by three different one-hour emersions in a sucrose solution comprised of 0.1M PO₄, 2% sucrose, and 50mM NH₄Cl. Two separate one-hour incubations in 0.1 M PO₄ and 50mM NH₄Cl followed. This was then followed by a series of thirty minute washes in 30% EtOH, 50% EtOH, and 70% EtOH.

Next, the tissue was dehydrated in order for it to be embedded in paraffin, starting with thirty-minute emersions each in 80%, 85%, 90%, 95%, and two in 100% EtOH. These EtOH emersions were then followed by a thirty minute bath of a 50/50 100% EtOH/Hemo-De solution and a thirty minute emersion in 100% Hemo-De. The tissue was then gradually subjected to an embedding paraffin called paraplast by submersing the tissue in a 50/50 solution of Hemo-De/paraplast for thirty minutes at 58°C. The paraplast was then further infiltrated into the tissue by immersing the tissue in two separate containers of pure paraplast at 58°C for one hour each. Finally, the paraffin embedded tissue was placed in embedding molds, covered with melted paraffin, and allowed to cool completely.
The embedded tissue was then sectioned using a Microm HM325 microtome. Due to time restraints, this study used tissue from ten of the twenty different hamster specimens: 5 cage control hamster tissues, and 5 cage suspension hamster tissues. The tissue for both cage control and cage suspension tissues were from Day 1 (PM), Day 2 (PM), Day 3 (AM), Day 3 (PM), and Day 4 (PM). The tissue blocks were cut in a manner that shaved the excess paraffin off so that only a small amount remained around the tissue in a trapezoid shape. The tissue block was then positioned in the microtome and the microtome was set to cut the tissue in 4 micrometer sections. Once the tissue was cut, the tissue was then floated out in a warm floatation water bath filled with distilled water with two drops of glycerin. The tissue was then mounted on acid-washed microscope slides and placed on a slide warmer to dry overnight.

The acid-washed slides were prepared using an acid wash of 10% HCl concentration. This was prepared by adding 60mL of 100% HCl to 540mL 70% EtOH. This acid wash was done in order to ensure the slides were completely clean. This is important for use in immunohistochemistry because the cleaner the slides, the better the adhesion of the tissue sections. This helps prevent the tissue sections floating off of the slides during the immunohistochemical staining procedure. The slides were dipped in the acid wash basin for 5 minutes. This was followed by two subsequent emersions in distilled water for a few minutes each. The slides were then put into an oven at 58°C overnight to ensure the slides were completely dry before storage or use.

A Blood Vessel Staining Kit Peroxidase System was purchased from Millipore (Catalog No. ECM590) to be used for immunohistochemical analysis. First, the reagents provided within the kit needed to be prepared for use. The 20x Rinse Buffer was diluted to a 1x Rinse Buffer using distilled water. The chromagen reagent that was provided in the kit was 3,3′
diaminobenzidine tetrachloride (DAB). The chromagen reagent was prepared so that there was a 1:25 ratio of the supplied DAB Chromagen A and DAB Chromagen B. Also, a citrate buffer was prepared using 2.1 citrate monohydrate and 1000mL distilled water, and adjusted until the solution was at a pH of 6.0.

Once all of the reagents were prepared, immunohistochemistry could begin. First, it was important to properly label each slide so that slides do not get mixed up as to which antibodies are being applied and which slides are controls. The procedure then began with the deparaffinizing and rehydration of the tissue. The tissue-mounted slides were submersed in Coplin jars filled with various solutions. To deparaffinize the tissue, the slides were submersed in two separate jars of pure xylene each for five minutes. This was followed by two submersions in 100% EtOH for two minutes each. The slides were then submersed in jars of 95%, 80%, and 70% EtOH for two minutes each. Finally, the slides were immersed in jars of distilled water and 1x Rinse Buffer for five minutes each.

A microwave antigen retrieval method was used according to histochem.net in order to retrieve any antigens which were masked by the tissue fixation. A Coplin jar containing the deparaffinized slides was filled with prepared citrate buffer and microwaved at medium-low power for 5 minutes. Additional citrate buffer was added to replace any evaporated losses and the jar/slides microwaved for an additional 5 minutes at medium-low power. Citrate Buffer was again added and the slides cooled in solution to room temperature. At this time, the slides were blotted dry and a Secure-Seal hybridization chamber gasket (Molecular Probes. Lot # 38006A) was applied to each slide and 3% hydrogen peroxide was added until the tissue specimen was covered. The hydrogen peroxide aides in opening the binding sites. The hydrogen peroxide was
left on for 10-30 minutes. The tissue sections were then rinsed with 1x Rinse Buffer two times for five minutes each.

The staining procedure follows the tissue pretreatment, and it is critical to note that at absolutely no time during the staining procedure should the tissue sections be allowed to dry out. First, a blocking step is performed in order to avoid any non-specific binding of the primary antibody. The kit provided a ready-to-use blocking reagent, and this was applied to each tissue specimen until it was completely covered and was left on for 15-30 minutes. The slides were then rinsed with 1x Rinse Buffer for at least fifteen seconds. The rabbit anti-vWF polyclonal primary antibody provided in the kit was diluted to a 1:400 dilution. To prepare this solution, two drops of the blocking reagent were added to 5 mL of 1x Phosphate Buffer Saline (PBS). Then, 997.5 µL of this blocking reagent/PBS solution was transferred to a small vial and 2.5 µL of the rabbit anti-von Willebrand Factor polyclonal primary antibody was added to achieve the 1:400 dilution. The 1:400 diluted antibody (or a negative control of PBS) was then applied to each slide for 2 hours, making sure that at no time did the antibody solution dry out within the hybridization chamber. Following the incubation time, the slides were rinsed three times for five minutes each in 1x Rinse Buffer.

The next step was to apply the provided goat anti-rabbit secondary antibody specific for the primary antibody or a PBS negative control. The antibody solution was applied so that it completely covered the tissue specimen on the slide. The slides were monitored so that they would not dry out during the fifteen minute incubation time. The tissue was subsequently rinsed three times with 1x Rinse Buffer for five minutes each. Following treatment with secondary antibody (or PBS control) the tissues were treated with Streptavidin-Horseradish Peroxidase. Streptavidin-Horseradish Peroxidase amplifies a weak signal by allowing 2 labeled and tagged
secondary antibodies to bind to each primary antibody, and then 2 Streptavidin-Horseradish Peroxidase molecules can bind to each labeled secondary antibody, for a total of 4 Streptavidin-Horseradish Peroxidases. This is done with high sensitivity and low background staining, which allows a target molecule, in this case vWF, to be detected. The Streptavidin-HRP was applied for fifteen minutes in an enclosed container, followed by three consecutive washes of 1xRinse Buffer for five minutes each.

In order to actually see the targeted protein molecules (von Willebrand Factor), a stain must be applied. To accomplish this, a chromagen reagent was used. The previously prepared chromagen reagent was applied to the slides for ten minutes. Immediately following incubation with the chromagen reagent, the Secure-Seal hybridization chamber gaskets were removed and the slides were placed in a Coplin jar filled with tap water. The jar was then placed under a running tap faucet for 5-10 minutes. The last step of the immunohistochemistry staining technique involved doing a dehydration process. The dehydration involves a graduated series of alcohol baths in order to drive out any residual water from the tissue, in addition to removing excess DAB reagent. This involved putting the slides in 70%, 80%, 95%, and two 100% EtOH Coplin jars for three minutes each. This was followed by clearing the tissue in xylene with two consecutive submersions in 100% xylene, each for five minutes. Clearing in xylene makes the sections hydrophobic so that a coverslip can be applied. Also, the xylene has a similar refractive index as the proteins in the tissue which minimizes the light diffraction and promotes nearly optically perfect images (Gill, 2010). Finally, 1-2 drops of Permount, a xylene-based mounting medium, was applied to the tissue-mounted slides and a coverslip was slowly lowered so that the Permount completely spread. The slides were then allowed to dry overnight before storing in slide boxes.
Once the immunohistochemistry procedure was complete, it was now possible to analyze the slides under a microscope. This study used a Fisher Scientific Micromaster Microscope in order to view the slides and photograph them with the aid of Westover Scientific Micron Imaging Software. Blood vessel and Follicular counts were done manually with the use of this technology.

**Results:**

**Mouse vs. Hamster:**

The Blood Vessel Staining Kit Peroxidase System (Millipore #ECM590) was designed for use with mouse and human tissue. Dr. Allan Forsman’s laboratory had available both actual spaceflight mouse tissue and simulated microgravity hamster tissue. Initially, we started with practice ground control mouse tissue from the spaceflight project. Several mouse tissues were subjected to the immunohistochemistry technique including ovary, uterus, kidney, brain, muscle, heart, and spinal cord. No staining resulted in any of the various tissue types. Figure 2 shows no positive staining within the mouse ovary. Figure 3 shows no positive staining within mouse muscle tissue. We then decided to test if the kit showed cross reactivity with hamster tissue. Hamster ovarian tissue was subjected to immunohistochemistry alongside mouse ovarian tissue. Figure 4 shows that positive staining did occur within the hamster tissue. Therefore, hamster ovarian tissue was used throughout the rest of the study.

**Cage Control Hamsters:**

The section through the ovary from the hamster euthanized at 2200 on Day 1 of the estrous cycle (H14-LO-B) had 19 follicles of varying size. Three follicles were unilaminar (one
layer of granulosa cells surrounding the oocyte). Three follicles were bilaminar, three follicles were trilaminar, and ten follicles were multilaminar. Within the tissue sections, there were 13 blood vessels (Table 1). Figure 5 shows that most of the positive staining for vWF occurs in the blood vessels in between several multilaminar follicles. In addition to this, there is a lot of small excess staining in endothelial cells that are potential new blood vessels.

The section through the ovary from the hamster euthanized at 2200 on Day 2 of the estrous cycle (H17-RO-B) had 1 corpus luteum and multiple follicles. The follicles consisted of 23 multilaminar follicles, 2 trilaminar follicles, 1 bilaminar follicle, and 3 unilaminar follicles. There were approximately 94 blood vessels within the tissue section (Table 2). Most of the vasculature was surrounding the multilaminar follicles, especially in regions where the follicles appear to share neighboring vessels. Staining for vWF occurred heavily in the vasculature of the corpus luteum, in addition to many small and flat vessels surrounding the multilaminar follicles. Figure 6 shows the staining that occurred within the small vessels in between multiple multilaminar follicles.

The section through the ovary from the hamster euthanized at 1000 on Day 3 of the estrous cycle (H11-RO-B) contained 11 follicles. Of these follicles, 5 were unilaminar, 1 was bilaminar and 5 were multilaminar. There were also 2 corpora lutea within the ovarian tissue section. There were 22 blood vessels counted within the ovary, not including the numerous amounts of tiny vessels seen within the corpora lutea (Table 3). The vessels that stained positive for vWF were mostly oval shaped, with some being flat and skinny vessels. These vessels were in the regions surrounding the multilaminar follicles. There was also staining within the vasculature of the corpora lutea.
The section through the ovary from the hamster euthanized at 2200 on Day 3 of the estrous cycle (H13-RO-B) had 2 corpora lutea that contained high levels of vasculature and high levels of positive staining for vWF in the vascular endothelial cells. There were numerous follicles, including 2 unilaminar follicles, 4 bilaminar follicles, 1 trilaminar follicle, and 5 multilaminar follicles. There were 31 blood vessels within the ovarian tissue section (Table 4). The majority of the vessels that stained for vWF were small and oval and located in regions of neighboring follicles.

The section through the ovary from the hamster euthanized at 2200 on Day 4 of the estrous cycle (H19-LO-B) contained 14 follicles. Of these, there were 3 unilaminar follicles, 4 trilaminar follicles, and 7 multilaminar follicles. There were also 2 large corpora lutea present. When it came to the blood vessel counts, there were 92 blood vessels, in addition to the numerous vessels in the corpora lutea (Table 5). The vast majority of the positive staining for vWF occurred in the corpora lutea and in regions surrounding the follicles, particularly the multilaminar follicles.

**Antiorthostatic Suspension Hamsters:**

The section through the ovary from the antiorthostatic cage suspended hamster euthanized at 2200 on Day 1 of the estrous cycle (H9-LO-B) contained 5 follicles. Of these follicles, 2 were bilaminar, 2 were trilaminar, and 3 were multilaminar. No corpus luteum was present. There were 15 blood vessels that were minute in size with minimal staining for vWF in the vascular endothelial cells surrounding them (Table 6). The vessels that did have positive staining were surrounding one of the larger multilaminar follicles.

The section through the ovary from the antiorthostatic cage suspended hamster euthanized at 2200 on Day 2 of the estrous cycle (H6-LO-B) contained 25 follicles. These
follicles consisted of 7 unilaminar, 5 bilaminar, 3 trilaminar, and 10 multilaminar. In addition to the numerous follicles present within the ovarian tissue section, there were also 2 corpora lutea (not shown in Figure 7). The corpora lutea contained extensive vasculature, and within this vasculature, there was extensive staining of vWF. There were 63 blood vessels throughout the tissue section, not including the vasculature in the corpora lutea (Table 7). The vasculature was predominately located around the larger follicles, especially the multilaminar follicles. There were not a lot of blood vessels in the regions of unilameter follicles (Figure 7).

The section through the ovary from the antiorthostatic cage suspended hamster euthanized at 1000 on Day 3 of the estrous cycle (H1-RO-B) contained the most vasculature of the various tissue sections. It contained 114 vessels among 10 multilaminar follicles. In addition, there were 3 corpora lutea present that also contained extensive vasculature not included in the previous blood vessel count (Table 8). Most of the vessels resided in the middle of the tissue section where they were surrounded by several large follicles. The staining of the vascular endothelial cells occurred extensively in the corpora lutea (Figure 8). There was also a great deal of positive staining in small oval vessels surrounding multilaminar follicles (Figure 9).

The section through the ovary from the antiorthostatic cage suspended hamster euthanized at 2200 on Day 3 of the estrous cycle (H2-LO-A) contained 3 unilaminar follicles, 1 bilaminar follicle, 3 trilaminar follicles, and 9 multilaminar follicles. There were 24 vessels within the tissue section (Table 9). The blood vessels that stained positively for vWF were small and oval in shape (Figure 10).

The section through the ovary from the antiorthostatic cage suspended hamster euthanized at 2200 on Day 4 of the estrous cycle (H3-LO-A) contained 2 corpora lutea and 18 follicles. The makeup of these follicles is as follows: 5 unilaminar, 2 bilaminar, 3 trilaminar, and
8 multilaminar. There were 91 blood vessels within the tissue section, in addition to the elaborate vasculature in the corpora lutea (Table 10). The blood vessels that stained positively were mostly located between 4 multilaminar follicles and a neighboring corpus luteum, which also had extensive staining in its vasculature. The positively stained blood vessels in this tissue section were unique in that they were much larger in size than any of the previous tissue sections.

**Discussion:**

One of the problems with immunohistochemical staining is that it lacks standardization when it comes to tissue fixation conditions, specimen pretreatment, reagents used, detection methods employed, and the interpretation of results. The interpretation of results is difficult because statistics used for immunohistochemistry are varied and currently require expensive commercially available automated analysis systems specifically designed for use with immunohistochemistry (Cregger et al., 2006). We did not have such analysis systems available to us; therefore this study was forced to take a qualitative approach to data analysis as opposed to a quantitative one.

Twenty sections of the cage control hamster ovarian tissue were viewed, and there were an average of 17 follicles per section. The average composition of follicles is as follows: 3.2 unilaminar, 1.8 bilaminar, 2 trilaminar, and 10 multilaminar follicles per section. There were an average of 1.4 corpora lutea per section and 50.4 blood vessels, not including the extensive vasculature within each corpus luteum. Most of the vessels were surrounding multilaminar follicles, with an average of 3-6 vessels per multilaminar follicle. The vascular endothelial cells that stained positively for vWF were typically small, oval vessels in regions neighboring multilaminar follicles.
The antiorthostatic cage suspended hamster ovarian tissue sections had a slightly lower average follicle number than the cage control tissue with an average of 15.2 follicles per section, with 32 sections viewed. These were comprised of an average of 3 unilaminar, 2 bilaminar, 2.2 trilaminar, and 8 multilaminar follicles per section. There was an average of 1.4 corpora lutea per section, the same average as the cage control tissue. The average number of blood vessels was higher in the anitorthostatic cage suspended tissue with 61.4 vessels per section, not including the vasculature of the corpora lutea. This is the opposite of what was initially hypothesized that the vasculature would decrease in the antiorthostatic cage suspended hamsters. The staining of vascular endothelial cells for vWF also appeared to be increased in the antiorthostatic cage suspended hamsters. This is to be expected since there was an increase in the vasculature in the antiorthostatic cage suspended tissue. However, this goes against the findings of Pattison, et al., 1991 that showed that the blood and fluid flow away from the abdominal organs to the cephalic region of the body. This shift in blood flow presumably causes lower blood pressure in the abdominal vessels, such as the vasculature within the ovary.

The type and location of vessels that stained seemed to be consistent with the cage control hamsters in that most were small, oval vessels near multilaminar follicles. Because of their small oval shape, it would be nice to be able to say that they are flat veins, as described by Forsman and McCormack, 1993. This would coincide with the research that vWF expression is higher on the venous side of capillary circulation (Pusztaszeri, et al., 2006; Zanetta, et al., 2000). Unfortunately, this cannot be stated definitively as it is difficult to distinguish between the different types of vessels within the tissue sections in this study. The vessels within the ovary are present in every direction, and we are unable to determine which angle the blade of the microtome sliced through each vessel. Therefore, we are unable to determine whether the
vessels seen in our tissue sections were cross sections, longitudinal sections, or oblique sections through the vessels. Therefore, further studies would need to be done to determine the types of vessels that stain for vWF with the highest percentage.

If more time and money were available for this study, more tissue-mounted slides would be subjected to the immunohistochemistry techniques. This would allow for more data to eliminate any statistical outliers and significant differences due to small sample size. Also, with more data, time, and money, statistics could be run with the proper software in order to have a quantitative study. Therefore, future studies need to be done in order to obtain statistically relevant data.

This study did determine significant information that will be useful for future studies on this subject. The Blood Vessel Staining Kit Peroxidase System purchased from Millipore was created for use on mouse and human tissue; other tissue types were not specified. Our study shows that the antibodies and staining kit did not work on mouse tissue, but instead worked on hamster tissue. Therefore, we will be notifying the Millipore company with this information to benefit other researchers.

Xylene is used extensively in the immunohistochemical process. Xylene has numerous hazardous effects as it is flammable, a strong skin irritant, and should not be inhaled or ingested. CitriSolv is a xylene substitute that is much safer to handle and work with. The final immunohistochemistry run of this study added three additional slides, 2 standard and 1 control, in order to test if the use of CitriSolv had any effect on the staining of the tissue sections. The results show that CitriSolv does not have any impact on the staining technique. Therefore, it is advised that future experimentation on this subject use the safer CitriSolv over xylene.
The use of hematoxylin, a counterstain that stains the nuclei of cells blue while retaining the brown color of positive stains, is optional in the immunohistochemistry technique. When this counterstain was applied in this study, differentiation between the blue staining and brown staining was difficult. It could be observed with difficulty with the use of the eyepiece on the microscope; however, the camera would not identify the color distinction as it was not of the highest quality available.

In conclusion, while this preliminary study was a qualitative one with inconclusive results, much has been learned that can aid researchers in future experimentation on the expression of von Willebrand Factor within the vasculature in hamster ovaries. It has been observed that vWF has been expressed greatly in small, oval vessels in regions of multilaminar follicles. It has been observed in this study that vasculature and thereby vWF expression increased in antiorthostatic cage suspension hamster ovarian tissue sections. This goes against the hypothesis that vasculature would decrease in antiorthostatic cage suspension hamsters due to research that has shown that blood and fluid flow away from the abdominal organs into the cephalic region of the body. Angiogenesis normally occurs as a physiological process within the ovary; however, angiogenesis is also involved in pathological conditions such as wound healing and inflammatory processes (Augustin, et al., 1995). Perhaps an explanation for our reverse findings is that antiorthostatic cage suspension could have caused a pathological condition, therefore stimulating angiogenesis and leading to an increase in vasculature compared to cage control hamster ovarian tissue. Another probable explanation for these reverse findings is that whether the hamster is subjected to antiorthostatic simulated microgravity or not, it still requires the same amount of blood since the mature corpus luteum has the greatest blood supply in the body (Kaczmarek, et al., 2005). Because it is presumable that the cephalic fluid shifts causes
decreased blood pressure in the ovarian and other abdominal vasculature, perhaps more
angiogenesis occurs to compensate for the decreased blood flow, since the organ still requires the
copious amount of blood. Further experimentation is necessary to gather more data with
statistics to further test these explanations and these findings.
References:


Gill, G.W. “H&E Staining.” *Connection* 2010;14:137-143.
Histochem. *Antigen Retrieval*. Histochem.net. 2006.  
http://www.histochem.net/histochemistry%20protocol%20antigen%20retrieval.htm


http://spaceflight.nasa.gov/history/shuttle-mir/references/glossaries/science/sc-gloss-g_m.htm.  


Figures & Tables
Figure 1: Hamster with suspension apparatus. Photo taken by New and Forsman, 2001.
Figure 2: Mouse Muscle Tissue. 11/9/2010. In 100X resolution.

Figure 3: Mouse ovarian tissue. STS118P. 11/12/2010. In 100X resolution.

Figure 4: Hamster Ovarian Tissue. H16-RO-B. 11/12/2010. In 100X resoloution. Arrows show blood vessels with positive staining.
Figure 5: H14-LO-B. 2200 Day 1. In 100X resolution. 11/19/2010. 1) Positive staining in the vascular endothelial cells. 2) A neighboring multilaminar follicle. 3&4) Excess staining in endothelial cells; potential new blood vessels.

Figure 6: H17-RO-B. Slide 1. 2200 Day 2. 11/15/2010. In 100X resolution. Arrows show multilaminar follicles.
Figure 7: H6-LO-B. 2200 Day 2. 11/21/10. In 100X resolution. 1) Region of unilaminar follicles with no vasculature surrounding them. 2) a multilaminar follicle with surrounding vasculature. This section was counterstained with hematoxylin. The camera could not distinguish the color differentiation between hematoxylin and positive staining.

Figure 8: H1-RO-B. slide 2. 1000 of Day 3. 11/23/2010. In 100X resolution. One of the 3 Corpora Lutea. Arrows show staining in the extensive vasculature within the CL’s.
Figure 9: H1-RO-B. slide 1. 1000 Day 3. 11/23/2010. In 100X Resolution. Small oval vessels stained positive for vWF surrounding a multilaminar follicle.

Figure 10: H2-LO-A. 2200 Day 3. 11/23/2010. In 100X Resolution. Small oval vessels stained positive for vWF.
Table 1: Data for Cage Control Hamster H14-LO-B. 2200 on Day 1.

Table 2: Data for Cage Control Hamster H17-RO-B. 2100 on Day 2.
Table 3: Data for Cage Control Hamster H11-RO-B. 1000 on Day 3.

Table 4: Data for Cage Control Hamster H13-RO-B. 2200 on Day 3.
Table 5: Data for Cage Control Hamster H19-RO-B. 2200 on Day 4.

Table 6: Data for Antiorthostatic Suspension Hamster H9-LO-B. 2200 on Day 1.
Table 7: Data for Antiorthostatic Suspension Hamster H6-LO-B. 2100 on Day 2.

Table 8: Data for Antiorthostatic Suspension Hamster H1-RO-B. 1000 on Day 3.
Table 9: Data for Antiorthostatic Suspension for Hamster H2-LO-A. 2200 on Day 3.

Table 10: Data for Antiorthostatic Suspension Hamster H3-LO-A. 2200 on Day 4.
Acknowledgments

Dr. Allan Forsman, Thesis Advisor

Dr. Bert Lampson, Committee Member

Dr. Michael Stone, Committee Member

Dr. Rebecca Pyles, University Honors Director

Dr. Foster Levy, Student-Faculty Collaborative Grant Provider

Dr. Ranjan Chakraborty

Dr. Michelle Duffourc

Thomas Barber

East Tennessee State University Honors Program