Effect of body position on prostate tumor hypoxia

by

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Abstract

Introduction: Arterioles of solid tumors lack innervation and functional smooth muscle, severely limiting vasomotor control and myogenic response. Previous research has shown that increasing mean arterial pressure via an exercise bout acutely improves prostate tumor perfusion and decreases tumor hypoxia. We hypothesized that increasing prostate tumor perfusion pressure using a hydrostatic gradient introduced by 70-degree head-up tilt could also increase tumor perfusion and decrease hypoxia, which could be clinically adapted to improve radiotherapy outcomes.

Methods: 10^4 Dunning R3327 AT-1 rat prostate adenocarcinoma cells were injected directly into the ventral lobe of the prostate of male Copenhagen rats (age 6 months, n=11). Six to eight weeks following injection, rats were given an intraperitoneal injection of hypoxic marker Hypoxyprobe^{TM} -1 and placed in either level (n=4) or 70-degree head-up tilt (n=7) condition. Tumors were removed and sectioned to be examined under microscope for Hypoxyprobe^{TM} -1 binding.

Results: No significant difference was found in level of hypoxia between the level and 70-degree head-up tilt groups. Specifically, mean hypoxic cell count at supine was 1347 ± 271 and this did not change significantly in upright posture, which was 1410 ± 198 ($P>0.05$).

Discussion: Contrary to our hypothesis we found no difference in tumor hypoxia between groups. This may be due to the age of the animals and the adoption of relatively healthy prostate arterioles during tumor development. Tumor vessels originate from vessels of healthy host tissue, which likely retain vasomotor and myogenic capability in young animals. The animals in this study were relatively young and likely restricted prostate blood flow and hence tumor blood flow in the head-up tilt condition. No immunocompetent aged rat prostate cancer

model is currently available, future studies should focus on the development of this model, in order to accurately represent blood flow in host and tumor tissue.

Table of Contents

List of Figures

List of Tables

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Dedication

This thesis is dedicated to my mother Mary, sister Monica, and Grandpa Jim.

Chapter 1 - Historical Review

The first known reference to the circulatory system comes from the Edwin Smith papyrus, an Ancient Egyptian medical text dated to roughly 1600 BC (26). A description of feeling for pulse to assess the heart indicates the ancients were aware of the role of the heart to distribute to the periphery. However, knowledge of the circulatory system progressed slowly as explanations of body function, at the time, required supernatural forces. In the second century AD, Clarissimus Galen introduced a theory of the cardiovascular system in which blood was created in the intestines from absorption of food, completed within the liver, and distributed via veins and arteries to the periphery where it was consumed (26). This idea was accepted in western medicine for well over 1000 years before challenged by William Harvey in the 1600s.

Harvey, recognized with the discovery of circulation, identified the systolic phase of the heart beat as the active phase for pumping blood, and showed venous valves only permit unidirectional flow (18)(36)(46). He was able to devise that blood flows in a circle, pumped by the heart (18)(46). He showed mathematically that blood was not created or consumed and hypothesized the existence of capillaries (18)(46).

Notably in the same period as Harvey, Thomas Willis observed that arteries could change diameter, though he hypothesized the nerves themselves must constrict to elicit this arteriolar response (37). Many of these circulatory theories rely on pressure gradients, yet at this time there were no accurate measurements of blood pressure in humans or animals.

Stephen Hales became the first to qualitatively analyze blood pressure in 1733. In this experiment he restrained a mare on her back and inserted a goose wind pipe (trachea) attached to a glass tube held vertically into the crural artery, observing the height of blood in the tube and its rise and fall with each pulse (17)(32). Hales spent his time studying many fields, thus

quantitative measures of blood pressure were not made until 1828 by Jean Léonard Marie Poiseuille using a cannula attached to a mercury manometer (6). He could cannulate an artery as small as 2mm in diameter, proving that pressure is maintained throughout the arteriolar tree. He also showed that mesenteric blood flow is not affected substantially by venous pressure but responds to arterial pressure changes (6).

Thereafter, the French physician Pierre Adolphe Piorry recognized the role of blood pressure in his treating of patients with syncope, he saw how falling from loss of consciousness brought the brain to the level of the heart and brain function is restored. Further, by bleeding dogs to induce syncope then alternating between head-up and feet-up tilt, he observed the effect gravity has on circulation (19)(48). These observations were crucial in our understanding of hydrostatic gradients, within the circulation, and how body position can affect organ perfusion.

Claude Bernard and Charles-Edward Brown Sequard showed the impact of sympathetic nerves on arteries in the 1850s. Bernard discovered that sectioning nerves in rabbits lead to dilation of associated arteries and increased temperature in the region (37). Brown Sequard showed stimulation of sympathetic nerves lead to constriction of arteries (37) demonstrating the fundamental role of the sympathetic nervous system on active vasoconstriction of arteries and arterioles.

Leonard Hill and Harold Barnard recognized how the vasomotor response played a role in upright posture. In 1897, they saw that vasoconstriction in the splanchnic region offset for a fall in carotid artery pressure (20). In an experiment placing dogs in upright posture, they severed sympathetic nerves and observed substantial pressure drop as nearly the entirety of the blood gathered in the splanchnic region (20), illustrating how essential the vasomotor response is for upright posture. The relationship is explained by the following hydraulic equation:

Mean arterial pressure (MAP) = Cardiac Output (CO) x systemic vascular resistance (SVR)

Severing of the sympathetic nerve causes SVR to drop to zero, thus MAP falls to zero and lack of venous return ultimately causes CO to drop to zero as well.

In 1902, William Bayless published his discovery of myogenic autoregulation, which he stumbled onto while experimenting with the vasodilator response. He saw arteries which he had denervated responded to increases and decreases in pressure by increasing and decreasing resistance, respectively. He hypothesized the response must be myogenic (5). This relationship is illustrated in the perfusion equation:

$$\text{Blood flow } (Q) = \text{Arterial pressure } (P_a) / \text{Resistance } (R)$$

Q must be constant, thus an increase or decrease in P_a must be met by an increase or decrease in R respectively. It is widely accepted that change in arterial wall tension stimulates the myogenic response; stretch activated cation channels and mechano-sensitive enzymes have been proposed as sensors but experimental evidence is so far unclear (49).

Understanding vasomotor and myogenic response to orthostatic stress has allowed researchers to explore how the mechanisms are impacted by factors such as environment (e.g., zero gravity) and aging. As Piorry used body position to study effects of gravity on partially drained dogs, altering body position has allowed researchers to use rats to simulate effects of increased cranial blood flow in zero gravity via head down tilt (55)(57), and deficiencies that arise in the upright posture with age (45). Notably, it has been identified that aged rats are

lacking in the ability to increase systemic vascular resistance when placed in head-up tilt. Young rats in the same condition were capable of increasing resistance, with decreased blood flow to splanchnic regions as compared to level (45). Diminished myogenic response was shown in the arterioles of multiple tissues from aged rats (39).

Compared to regulated tissue growth through development, cancer introduces typically uncontrolled tissue growth requiring co-opted blood vessels as well as vascular neogenesis. However, this new vasculature is structurally and functionally dissimilar to that of healthy tissue. Vessels in the tumor are primitive, lacking in basement membranes (4), innervation (2), and functioning smooth muscle (3)(4). The vessel network of the tumor is disorganized and tortuous. This network is significantly responsible for tumor hypoxia, which leads to treatment resistance (41), aggressive cancer phenotype (21)(22), and worsened clinical outcomes. Given the bulk of the tumor vasculature is deficient in smooth muscle, which is required for active myogenic constriction to counter gravity, this project seeks to utilize orthostatic stress to acutely increase tumor blood flow and thereby decrease tumor hypoxia. If successful, this concept can be rapidly adapted for clinical use.

Chapter 2 - Introduction

Significance of Prostate Cancer

Prostate cancer is the second most diagnosed cancer and second leading cause of cancer death in men. Approximately one in nine men can expect a diagnosis in his lifetime (NCI Fact Sheet; web)(ACS Fact Sheet; web). Understanding of risk factors and advancements in early detection of the disease have made prostate cancer a manageable diagnosis. Including all stages, prostate cancer has the highest 5-year survival rate (98%) of any cancer type in men in the United States (NCI). When the disease remains localized to the prostate the survival rate approaches 100%, however, survival rate drops to 30% once the disease is metastatic (NCI). World-wide there is considerable variability in prostate cancer 5-year survival.

Cancer can be separated into solid cancers and blood cancers. More than 85% of cancer deaths occur from solid tumors, including prostate cancer (NCI). Although survival for prostate cancer is high overall, the presence of hypoxia in any solid tumor presents many complications and can be devastating. Presence of hypoxia within a solid tumor is associated with worsened overall survival, disease-free survival, and biochemical failure (38)(54). Specific to prostate cancer, patients with hypoxic tumors had the highest risk of biochemical failure within 8 years, regardless of tumor stage and other factors (56).

Hypoxia/ Microenvironment/ Tumor Blood Flow

Tumor hypoxia is a result of both diffusion and perfusion limitations. Chronic hypoxia occurs as a result of diffusion limitations to cells far from vasculature. In normal tissues, only a few cell layers separate the furthest cells from vessels (28), while in tumors, cells can be 20 cell layers away or further (51). Cycling hypoxia results from perfusion limitation, exposing cells close to vessels to cycles of hypoxia and reoxygenation.

The tumor microenvironment is key in the course of tumor development. Hypoxia and the tumor vasculature have important roles in creating and evolving the microenvironment. Angiogenesis, the formation of new blood vessels, must occur for the tumor to receive oxygen and nutrients for continued growth. Tumor vasculature develops early from that of the host tissue, it has been recorded that tumors can grow to nearly 1mm in diameter without angiogenesis (12), but new vessels can also appear with as few as 100 cancerous cells present (33). It has been shown that eliminating angiogenesis in this early period via a modified form of Vascular Endothelial Growth Factor (VEGF) can halt tumor growth (33), minimizing the potential for metastasis. As the tumor grows, the vasculature that develops is disorganized and composed primarily of primitive vessels. Structurally they lack hierarchy, functioning smooth muscle (3)(4), basement membranes (4), and innervation (2). Functionally the network is chaotic, some vessels may collapse while others have flow that is turbulent, stagnant, or retrograde (29). The network contributes to a feedforward cycle of hypoxia within tumors and becomes the primary method for metastasis.

Hypoxia is a marker for cancer progression and of itself can lead to substantially worse outcomes. Tissues become hypoxic when oxygen demand exceeds oxygen supply, this can occur quickly in tumors where diseased cells rapidly reproduce. A tumor PO_2 below 10 mmHg activates many pro-oncogenes (e.g., hypoxia-inducible factor-1 (HIF-1)), resulting in poor prognosis for the patient. Specifically, when median tumor PO_2 falls below 10 mmHg, survivability, growth, metastatic potential, and cell motility of cancer cells are improved (51). When PO_2 falls below 10 mmHg, the cancer cells begin increasingly expressing genes regulated by HIF-1. In healthy tissue, expression of these genes would lead to a return to normoxia, but continued cell proliferation within the tumor contributes to continuation of the hypoxic

environment. The genes transcribed instead lead to sustained angiogenesis and resistance to oxidative stress.

Hypoxia Impact on Radiation/ Therapies

Radiation therapy can be used for nearly every type of cancer; over half of all cancer patients receive radiation either exclusively or as part of a treatment regimen. For patients with prostate cancer, it can be used at every stage of the disease. Its mechanism relies on the presence of oxygen to create reactive oxygen species (ROS) within the cells. ROS cause DNA damage, when the damage becomes irreparable the cell can no longer reproduce and dies. Therefore, radiation therapy must be precisely administered to target cancerous cells and eliminate their replicative capacity. For over 50 years (16), it has been known that radiation is substantially affected by tissue oxygenation. Therefore, to maximize the effect of radiation therapy, other cancer therapies depend on their deliverance into the tumor via the blood, or their mechanisms require oxygen and have limited effect due to poor tumor perfusion and hypoxia (50)(53).

Within a tumor, cells close to vessels are often normoxic, while those further away experience chronic hypoxia. Other areas of the tumor, up to 20% (51), experience cycling hypoxia. These periods of hypoxia and reoxygenation relate to reduced local blood flow (29) and greatly increase ROS production and cellular instability (51). Cyclical hypoxia is of particular importance as it increases metastasis more so than chronic hypoxia (47). It also has greater impact on HIF-1 expression than chronic hypoxia (34), which is likely to lead to a more treatment resistant phenotype. Timing the hypoxic cycle or creating a way to ensure perfusion to these cells is paramount to maximize treatment efficacy and improve treatment outcomes.

Targeting Hypoxia

Reduction or elimination of hypoxia has been the focus of much cancer research and a variety of strategies have been used including altering blood flow and oxygenation. Breathing hyperoxide gas, research has focused specifically either pure oxygen or carbogen (95% O_2, 5% CO_2), have been used to combat hypoxia since it was first identified that tumors contained radioresistant cells (7). This hyperoxide/hypercapnic inspirate increases arterial PO_2 as a means to increase the driving pressure of oxygen from the tumor microcirculation to the parenchyma to reduce tumor hypoxia prior to radiation. This intervention is commonly used for several cancer types, but often results in variable effects on tumor PO_2 (1)(30)(52).

Research has also focused on use of vasoactive drugs (e.g., vasodilators) to alter tumor blood flow. However, the intra-tumor variability is such that there is limited predicative value in vasodilators. Given dilators require a functional smooth muscle, often the dilators work well in healthy tissue and can actually shunt blood away from a tumor, worsening tumor hypoxia (8)(43). Pharmacological intervention to increase blood pressure has also been unable to increase tumor blood flow (42)(43). Specifically, as explained by the hydraulic resistance equation, increases in MAP will be combated with either a reduced cardiac output and/or SVR, both of which may limit tumor perfusion. Further, endogenous molecules known to be vasodilators are present within the tumor, though they often take on new roles. Adenosine is a potent vasodilator, but in high pharmacological concentrations within the tumor it acts as an immune system suppressant (31).

Nitric oxide (NO) is another potent vasodilator and has been used to the benefit of individuals with chronic diseases such as chronic heart failure. It has varied impact on tumors, as it contributes to HIF-1 regulation and VEGF regulation (13) which enables angiogenesis and vascular permeability (11). NO is also a radiosensitizer though less effective than oxygen (9).

Like healthy tissue, tumors produce NO via nitric oxide synthases, inhibition of endogenous nitric oxide synthase leads to decreased tumor blood flow; however, NO supplementation fails to increase tumor blood flow (14).

With interventions that impact and alter vascular resistance, change to whole body hemodynamics must be considered. Decreasing vascular resistance forces heart rate to increase to maintain mean arterial pressure, if possible, depending on venous return. This increases cardiac output, which must be met by an equal venous return, which is unlikely to happen while an individual is in a resting state without muscle pump and vasoconstriction, when much of the blood volume is directed towards compliant regions. This can cause potentially fatal drops in mean arterial pressure. The small range in which these interventions can be used limits their potential for clinical application.

Exercise/Blood Pressure and Tumor Blood Flow

Exercise can increase cardiac output by 3-6 fold, but this increase requires a tight coordination of the sympathetic nervous system (SNS) and local tissue requirements. The SNS increases resistance in splanchnic organs to direct blood where oxygen and nutrients are needed, via pressure gradients, ultimately to the active skeletal muscle where resistance is low. This paradigm also allows for substantial increases in central venous pressure to sustain high cardiac outputs. This cardiac output redistribution requires an active vasoconstriction in these compliant (e.g., mesentery) tissues. However, tumors are not innervated and lack myogenic response and thus cannot regulate their blood flow, so when other organs increase their resistance, or even compared to healthy tissue within an organ, the tumor can become a relatively low resistance destination for increased perfusion pressures (e.g., increased MAP). This exercise pressor

response is enough to overcome tumor interstitial pressure and results in an enhanced tumor perfusion and decreased tumor hypoxia during exercise (35).

Conflicting results on the effects of exercise on tumor perfusion arise from studies which utilize ectopic tumor models. In these models the tumor is grown in a skinfold window, which has been shown to be a poor representation of blood flow as compared to an orthotopic model (15), in which the tumor is grown in the cancer cells' tissue of origin. Exercise has been shown to acutely increase blood flow in an orthotopic tumor model by nearly 200% compared to resting values (35). This increase in blood flow provides a significant acute decrease in tumor hypoxia (35) which would provide tremendous clinical value. However, exercise cannot be utilized simultaneously with radiation. Radiation requires extreme precision and movement from exercise could damage or destroy healthy tissue. Therefore, a mechanism to increase arerial pressure other than exercise must be utilized to reduce hypoxia during radiotherapy.

Hydrostatic Pressure Gradients

Head-up tilt has been utilized to study orthostatic stress in both humans (24)(25) and animals (45). In a supine position, there is little or no difference in arterial pressure in tissues throughout the body. Head-up tilt creates a pressure gradient in which arterial pressure decreases in tissues above heart level and increases in tissues below. For correct distribution of cardiac output, and to prevent overperfusion and a drop in MAP and possible syncope, there must be sympathetically mediated and myogenic constriction of arterioles in tissues below heart level, limiting tissue blood flow. It has been shown that aged rats have a decreased ability to constrict arterioles in response to head-up tilt (45). This decreased ability results in increased flow to tissues below heart level, including the prostate, in the head-up tilt position.

Solid tumor arterioles lack innervation and functional smooth muscle, rendering them unable to have any response to orthostatic stress. Therefore, solid tumor below the level of the hydrostatic indifference point (HIP) may demonstrate an enhanced perfusion it an increased hydrostatic gradient induced by the upright posture. However, radiation currently is administered to cancer patients in the supine position, regardless of tumor location, as this simplifies administration for patients and physicians. Given the location of the prostate is below the HIP, it is likely that the adoption of the upright posture, in combination with tumor blood vessels with no appreciable vasomotor regulation, will enhance prostate tumor perfusion and/or oxygenation due to the increased hydrostatic gradient.

Purpose

Solid tumors lack the ability to regulate their blood flow, limiting tumor blood supply and creating a hypoxic environment that promotes aggressive tumor phenotype and treatment resistance. Previous research shows head-up tilt increases blood flow to tissues below the hydrostatic point, including the prostate. We hypothesize that using head-up tilt in a prostate cancer model would reduce tumor hypoxia compared to a level (zero-degree tilt) position.

Chapter 3 - Methods

Animals

Adult male Copenhagen rats (n=11) were obtained from Charles River Laboratories, Inc. Rats were housed at 23˚C, kept on a 12:12 hour light-dark cycle, and had access to rat chow and water *ad libitum.* All procedures were approved by Institutional Animal Care and Use Committees at Kansas State University and University of Florida.

Prostate Cancer Model

This study used the Dunning R3327 AT-1 rat prostate adenocarcinoma cell line. This cell line is characterized by rapid growth rate, low metastatic potential, and comparable growth characteristics to human prostate cancer (23). Adenocarcinoma cells were cultured in RPMI-1640 media (GE Healthcare Life Sciences, Marlborough, MA) containing 10% fetal bovine serum (RMBIO, Missoula, MT), 2mM L-glutamine (Fisher Scientific), 100 mM sodium pyruvate (Thermo Fisher Scientific), 1% penicillin/streptomycin (Thermo Fisher Scientific), and 0.025 mM dexamethasone (Cayman Chemical) and kept in an incubator in 5% CO_2 and 37˚C. When 80-90% confluence was reached, a sample of cells were counted using hemocytometer in order to calculate proper dilution (100,000 cells/ml) of viable cells for tumor cell stock solution in physiological salt solution (PSS). This solution was aliquoted into 0.1 mL increments containing approximately 10^4 AT-1 cells. This method has been previously used to successfully induce prostate tumors (23)(27).

To induce orthotopic tumors, rats were anesthetized (2-5% isoflurane, oxygen balance) and a small incision of 1 cm or less was made in the abdomen, lateral to the midline, to expose the bladder and prostate complex. The ventral lobe of the prostate was isolated, and 10^4 AT-1 cells were injected using a sterile 26G insulin syringe. A sterile tipped cotton applicator was

placed alongside the needle during removal in order to prevent cells leaking into surrounding tissue. Immediately after injection the abdominal wall was closed using sterile 3-0 polyglycolic acid coated suture (DemeTECH. Miami Lakes, FL) and the overlying skin was closed using sterile 3-0 nylon filament (DemeTECH) and sealed with a skin adhesive (3M, Vet-Bond). Rats were then injected with 0.05 ml/kg buprenorphine (S.C.) to control post-operative pain as well as 0.5 mg/kg acepromazine (S.C.) as a sedative. This combination of analgesic and sedative eliminated any signs of pain or discomfort and prevented dehiscence by the rats. All surgical procedures were performed under aseptic conditions and included daily postoperative monitoring. Tumors were allowed to grow for 8-10 weeks.

Pimonidazole hydrochloride

Hypoxyprobe^{TM} -1 (Hypoxyprobe, Burlington, Massachusetts) (HP-1), chemical name pimonidazole hydrochloride, is the compound used for hypoxia detection. It is the hydrochloride salt formed from weak base pimonidazole. It is a 2-nitroimidazole, which has been shown to be bound to peptide thiols in hypoxic cells. Like other 2-nitroimidazoles it is reductively activated in hypoxic cells, it is cleared from tissues unless the NO_2 group is reduced. Additionally, other 2-nitroimidazoles have been shown not to be perfusion limited. The reduced product forms stable bonds with thiols in peptides. The antibody binds to the product with no cross-binding of non-reduced pimonidazole so there is no interference. HP-1 binds in cells where $pO_2 \leq 10$ mmHg. When PO_2 falls below 10 mmHg, tissues begin producing proteins mediated by HIF-1 including those involving resistance to oxidative stress - the mechanism of radiation therapy.

Experimental Protocol

Prior to the experiment, rats were habituated to the plexiglas restraint canopy (Rodent ECU, Braintree Scientific) for 20 minutes per day for at least 3 days. At the time of the

experiment, the dosage of HP-1 was calculated (60 mg/kg body weight) and prepared according to kit instructions and administered via intraperitoneal injection. Immediately following injection, the animal was placed in the restraint and randomly assigned to either the 70˚ (n=7) or 0˚ (n=4) tilt condition. Animals remained in this position for 45 minutes. This time is equal to the half-life of the HP-1 compound in rat plasma, ensuring binding in hypoxic tissues and minimal interference. At the end of the 45 minutes the animal was removed and placed under isoflurane anesthesia and sacrificed via cutting the abdominal aorta. The prostate tumor was immediately removed, covered in OCT compound, snap frozen in liquid nitrogen and stored at -80˚C. Using a cryostat, the tumors were sectioned at 4 micrometers and sections mounted on slides. Five core and five periphery slides were produced from each tumor. Sections for core were sliced consecutively from the center of the tumor. Sections for the periphery were sliced consecutively from the exterior of the tumor.

Slides were stored at -80˚C until preparation and staining according to Hypoxyprobe™ kit instructions. Specifically, slide sections are rinsed (with PBS) and incubated overnight at 4˚C with rabbit anti-pimonidazole antisera (PAb2627AP diluted 1:20 in PBS containing 0.1% bovine serum albumin and 0.1% Tween 20. Thereafter, sections were incubated for 60 min with FITC-conjugated goat anti-rabbit antibody.

Slides were examined under a Zeiss Axio Fluorescent microscope (Zeiss, Thornwood, NY) at 20X with a FITC filter (green 470-520 nm) and six images were taken from each of two periphery and two core slides from each tumor. The images were analyzed using ImageJ software to calculate an average hypoxia level for core and periphery of each tumor as detailed below.

Hypoxia Quantification

Hypoxia was quantified using ImageJ (National Institutes of Health) software. In ImageJ, images were converted to black and white to identify unstained background and stained hypoxic cells respectively. The software analyzed the black and white image and counted appropriate pixels to determine the number of hypoxic cells on each section imaged.

Statistics

A two-way repeated measures ANOVA was used to compare hypoxic cell counts between tumor core and periphery in either the supine or head-up tilt position. A two-way ANOVA was used to compare hypoxic measures between groups. A Holm-Sidak's multiple comparisons test was used to determine significance, with significance set at $P \leq 0.05$. Given the high variability in the collected data (i.e., large standard deviations) a Grubbs test for outliers was performed. Data are presented as mean $\pm$ SEM.

Chapter 4 - Results

Animals

Tumor hypoxia was successfully measured in the head-up tilt position (HUT) (n=7) and in the supine posture (n=4). Average body weight of all groups was 319±33 grams. Final age of the animals was approximately 8 months. There was no difference in body mass between animals used in the supine (300 ± 10 g) versus HUT (329 ± 14 g) experiments. Neither heart weight (supine, 0.75 ± 0.04 g; HUT, 0.77 ± 0.03 g) nor left ventricle weight (supine, 0.55 ± 0.03 g; HUT, 0.59 ± 0.02 g) was different between animals used in either position. Average tumor mass for all groups was 11.4 ± 1.3 g. Although animals were randomized into groups, tumor mass from those used in the supine posture (14.8 ± 2.9 g) was significantly greater versus those from the HUT group (9.4 ± 0.7 g; p=0.04). The animals in the supine group also had a significantly greater tumor burden as assessed as a percentage of body mass (i.e., tumor mass/body mass x 100) versus those used in in the HUT position (supine, 5.0 ± 1.2 % versus HUT, 2.9 ± 0.25 %, p=0.02)

Hypoxia

Figure 1 demonstrates representative fluorescent markers of tumor hypoxic cells, assessed via Hypoxyprobe, within the tumor core and periphery for supine and upright postures. Average hypoxic cell counts are shown in Table 1. There was no significant difference in hypoxia between the core and periphery within the same body position (Fig 2). Contrary to our hypothesis, there was no statistical difference between supine and head-up tilt groups for either the core (p=0.68) or periphery (p=0.85) (Fig. 3). Standard deviation was high in all groups indicating high variability in each tumor; however, no outliers were detected using a Grubbs test.

TABLE 1	Supine (0 degree)	HUT (70 degree)
Tumor Core (hypoxic cell counts per field)	1354 ± 229	1548 ± 312
Tumor Periphery (hypoxic cell counts per field)	1340 ± 372	1272 ± 180
Data are mean ± SEM		

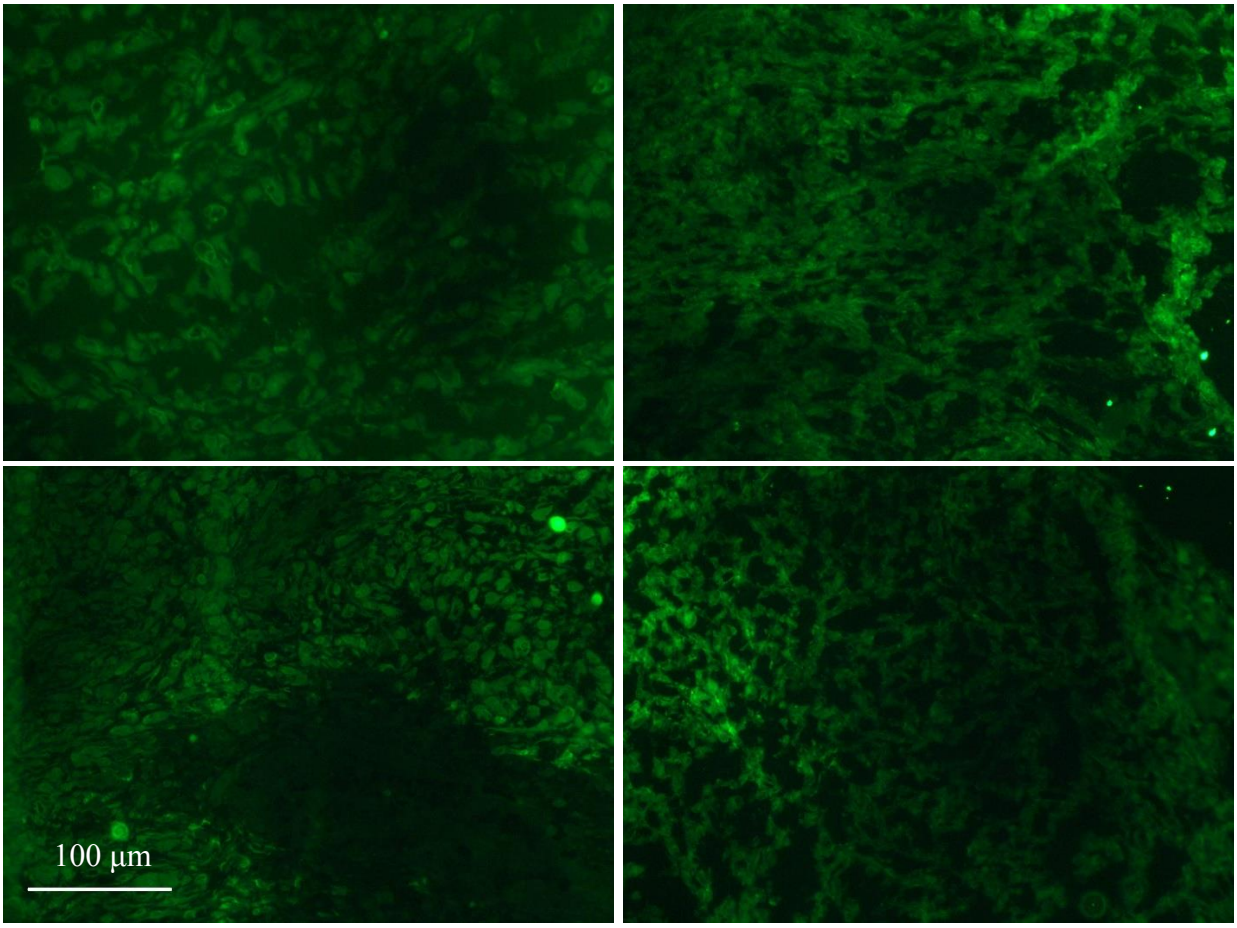

Fig. 1. Representative images of hypoxic positive cells (green) in the tumor core and periphery in the supine and HUT body positions. Top left: Tumor core from supine position (hypoxic cell count 1451). Top right: Core from 70-degree HUT (hypoxic cell count 1656). Bottom left: tumor periphery from supine position (hypoxic cell count 1221). Bottom right: periphery from 70-degree HUT (hypoxic cell count 1517).

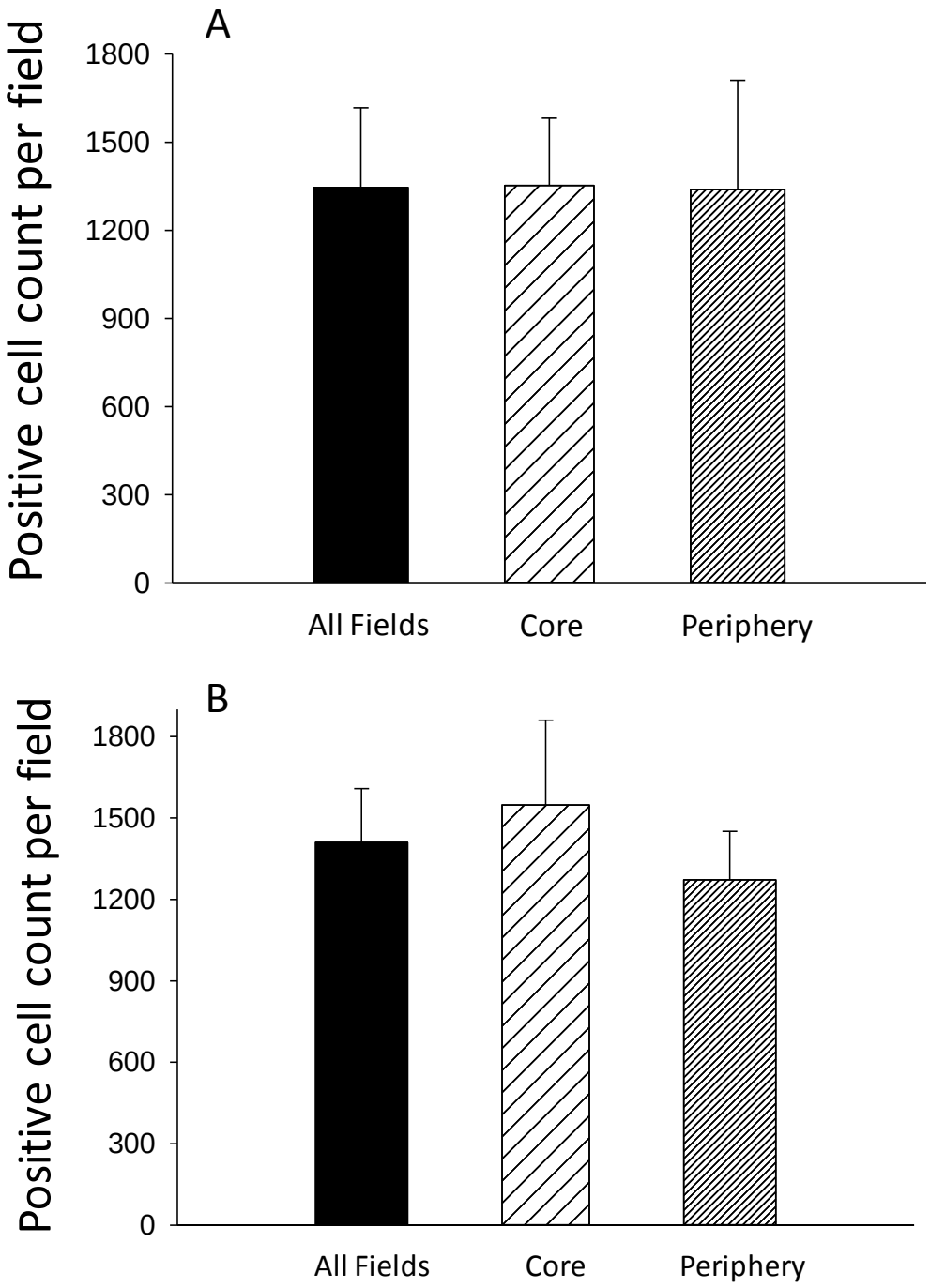

Figure 2. Hypoxic cell count in tumors from A) the supine and B) HUT body positions. No differences in positive cells were observed between the tumor core and periphery in either body position.

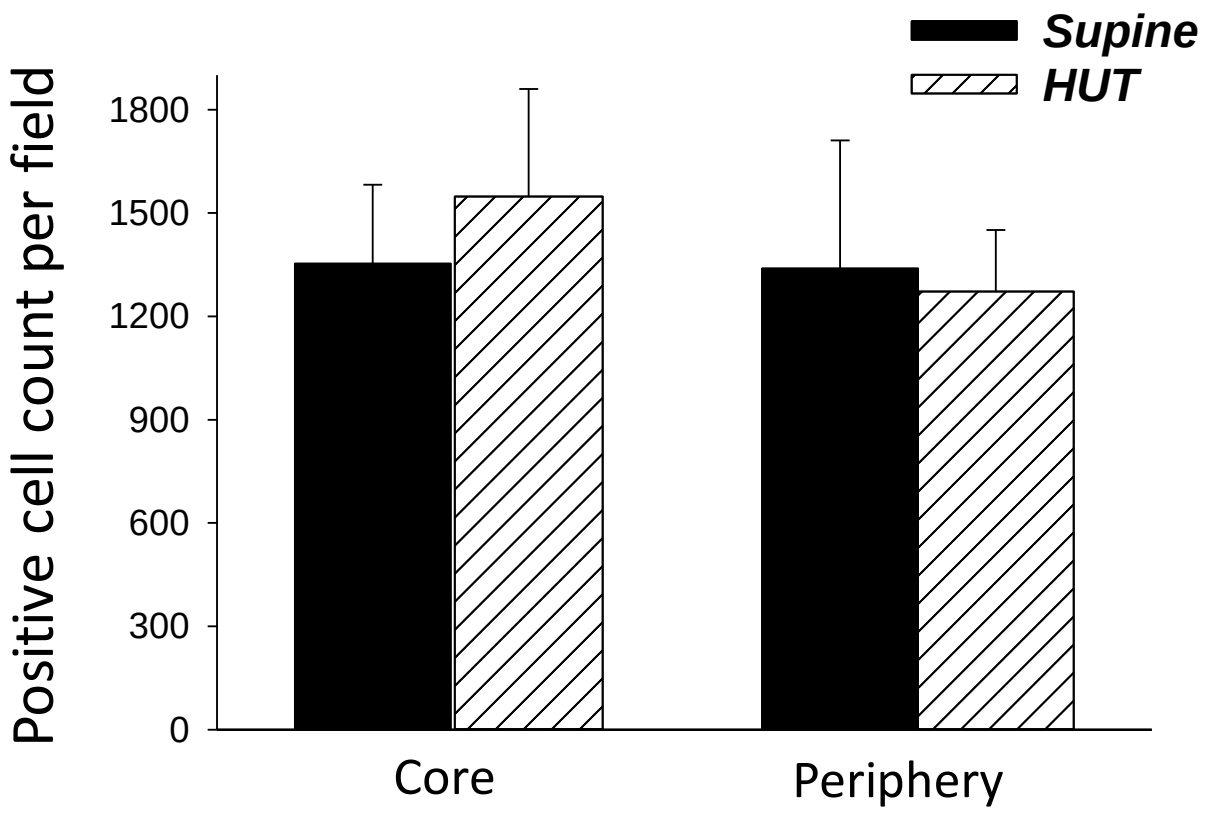

Figure 3. Hypoxic cell count in the tumor core and tumor periphery across body positions. There were no differences in hypoxic cell count between groups across body positions.

Chapter 5 - Discussion

The purpose of this study was to determine if head-up tilt could decrease the amount of hypoxia present in rat prostate tumors. The results opposed our hypothesis as there was no significant difference in hypoxic cell count between 70 degree and supine position groups. Standard deviation was high, likely indicating substantial variance in the vasculature of each individual tumor, which could be attributed to the uncontrolled and disorganized growth of the tumor vascular network.

Tumor hemodynamics

It is well known that the vasculature of solid tumors is disorganized and individual vessels are primitive, lacking functional smooth muscle (3)(4) and nervous innervation (2). This network handicaps tumor blood flow and can lead to hypoxia, an important factor in the hostile tumor microenvironment as well as unfavorable clinical outcomes. Combating hypoxia has long been a goal of cancer researchers, testing for ways to increase O_2 diffusion in the tumor and to increase tumor perfusion. Studies looking to increase diffusion in the tumor have produced heterogenous results (1)(30)(52). Those looking to increase tumor perfusion using pharmacological intervention have frequently found the opposite effect (8)(43). However, research has shown that using exercise to increase tumor blood flow has significant success (35). This is because the tumor lacks the ability to regulate its vascular resistance in response to changing mean arterial pressure. The exercise protocol used by McCullough et. al produced significant tumor hypoxia reduction (35), though this response is acute and cannot be translated to clinical cases because of the precision required to protect healthy tissue during radiation treatment. Our study sought to use a novel way to increase tumor perfusion pressure, taking advantage of primitive tumor vasculature, that could be easily adapted into clinical practice.

At rest the tumor has relatively high resistance due to its interstitial pressure, however, its inability to vasoconstrict means it becomes an area of relatively low resistance during exercise with a large exercise pressor response. The animals in our study remained in a resting state as is currently necessary for radiation treatment. Based on previous research (35)(45), it was hypothesized that introduction of head-up tilt could provide enough of an increase in pressure at the level of the tumor to overcome tumor interstitial pressure and capitalize on the inability of the tumor to vasoconstrict. Failure to do so in our experiment could indicate that head-up tilt does not provide enough of a pressure increase. It is possible this could be overcome in a larger subject, e.g. human, with a larger hydrostatic column between the heart and prostate tumor. It could be the case that at rest, there is no way to increase regional pressure enough to overcome tumor interstitial pressure.

Aging Hemodynamics

Tumor vasculature originates from that of its host tissue. Thus, it is necessary to understand hemodynamics of the normal vasculature for a full understanding of tumor hemodynamics. This is especially important in cancers, like prostate cancer, where the average age of diagnosis is 66 years (ACS). With age comes declines in cardiovascular performance and regulation. Maximal heart rate and potentially (if no aerobic training) stroke volume decline, contributing to decreased aerobic capacity. Blood vessels are impacted by aging. Proctor et al demonstrated that older men experience less leg blood flow and decreased vascular conductance than young men in dynamic exercise at the same power output (44). Donato et. al showed decreased quad blood flow and increased vascular resistance in older men (10). The mechanism for these decreased skeletal muscle blood flow levels was indicated by Muller-Delp et. al - decreased endothelium dependent vasodilation (39). Aging impacts blood flow distribution

within a tissue in addition to whole tissue flow. A study by Musch et al comparing whole hindlimb flow during submaximal exercise between young and old rats showed that despite the same whole limb flow, aged rats sent significantly more blood to glycolytic fibers instead of oxidative fibers like younger counterparts (40). Though these studies all use exercise as a stress and examine skeletal muscle, they illustrate the alterations to the cardiovascular system with age. Using head-up tilt as a stress, Ramsey et. al demonstrated that young animals decrease blood flow to the prostate and other splanchnic organs during head-up tilt compared to rest, and decrease vascular conductance in the same manner, aged animals had no significant differences in either measure for the prostate (45). This indicates a loss of vascular control at the prostate level with aging. In young animals it indicates a vasoconstriction leading to decreased prostate perfusion. Inability of the prostate vessels in aged animals to respond to orthostatic stress from head-up tilt is important in that these are the vessels from which prostate tumor vasculature emerges. Since the prostate vascular is unable to respond to an orthostatic stress introduced by head-up tilt, it is reasonable to hypothesize that the tumor will experience the same blood flow alteration as the host tissue when introduced to the stress. Since no aged immunocompetent rat prostate cancer model exists, we had to use younger rats which would have a more intact vasoconstrictive response to the stress in prostate vasculature, which could have prevented an increased blood volume from reaching the prostate tumor. In rats of appropriate age (24 months) it is possible that due to decreased myogenic response, head-up tilt could increase tumor perfusion.

Postural position

Postural position plays a significant role in tissue perfusion pressure. In a supine position, there is little difference in pressure in major arteries throughout the body (48).

however, upright posture creates a hydrostatic gradient, through a hydrostatic column, where pressure decreases in tissues above heart level and increases in tissues below heart level. The increase in pressure must be great enough to overcome the high interstitial pressure within the tumor. The increased pressure in upright posture compared to supine is dependent on the height of the hydrostatic column. In rats the increase may not be enough to overcome tumor interstitial pressure, but in humans or another relatively large organism with an increased heart to prostate distance and larger hydrostatic column could possibly see a great enough pressure increase. However, it is well known that rats experience significant changes in blood flow from tilt, as they have been used to study orthostatic stress for years. Head-up tilt has been used to mimic upright posture in humans and study effects of aging on hemodynamics (45). Head down tilt has been used to study increased cranial blood flow as seen in zero gravity space flight and from prolonged bedrest (55)(57).

Limitations

There are several limitations within this study. The younger relative age of the rats in this study means they are likely to have significantly greater ability to respond to orthostatic stress, specifically that introduced by head-up tilt, compared to aged counterparts. This limits the translation to aged humans. Blood flow was not measured in this experiment. Our laboratory has done similar experiments using head-up tilt and measured blood flow using microspheres, however microspheres could not be used in this study as they could block vessels preventing Hypoxyprobe™ from reaching hypoxic tumor locations. Because each animal was only able to be placed in one condition, it is possible that rats experiencing the head-up tilt condition did see a reduction in hypoxia, and the animals that remained in supine position could have had significantly reduced hypoxia if placed in the head-up tilt condition. This is unlikely,

but it must be noted that the chaotic and unregulated nature of tumors means that individual tumors and the associated vasculature will progress at different rates. Plasma flow could have increased and been undetected in this study due to its low O_2 carrying capacity. This could indicate head-up tilt is not effective for improving radiotherapy outcomes but could contribute to increased delivery of other cancer combative modalities, e.g. pharmacological agent delivery.

Conclusions and Future directions

This study shows head-up tilt is unsuccessful at reducing tumor hypoxia in a preclinical rat prostate cancer model. It must be noted that the animals in this study were relatively young compared to the average age of human prostate cancer patients at diagnosis (ACS), and to the animals that demonstrated a decreased response to orthostatic stress presented by head-up tilt (45). The benefit of increased tumor blood flow is clear (35) and developing a method to maximize blood flow while being able to administer radiotherapy should remain paramount. Head-up tilt remains as one viable option. Currently there is no aged, immunocompetent preclinical rat cancer model, which should be developed in future studies to reduce limitations. A larger preclinical animal model, with greater distance between heart and prostate, could also be developed, though the subjects must be appropriately aged.

Chapter 6 - References

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