NOVEL APPROACH TO OPTIMIZE THE LAYING HEN MODEL OF OVARIAN CANCER

A Dissertation

by

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ABSTRACT

No appreciable improvements in incidences and mortality rates of women with ovarian cancer have been made over the last 40 years; this fact alone indicates that scientists and clinicians lack the adequate tools to conquer this deadly disease. The American Cancer Society estimates that more than 22,000 women will be diagnosed with ovarian cancer this year, and over 14,000 deaths will be attributed to this disease; this translates to 1 out of every 75 women in the US being diagnosed with ovarian cancer, and of those diagnosed, over 60% will die from the disease. Lack of a more predictive animal model has been an obstacle to progress in ovarian cancer research. It is hypothesized that laying hens, though not fully characterized, could be an optimal animal model for the study of human ovarian cancer initiation, progression, therapy and relapse. Domestic laying hens (*Gallus gallus domesticus*), spontaneously develop ovarian cancer at a high incidence.

In an effort to better characterize ovarian cancer in laying hens, we created a surgically implantable, biocompatible port using 3D printing technology, which allows for repeated access to the ovary for laparoscopic serial sampling, observation, and imaging. With the ability to follow laying hens via easily accessible ports throughout their lifespan, our hope is to be able to detect when they develop ovarian cancer, as well as discover early diagnostic techniques for this disease.

The fact that laying hens are a spontaneous ovarian cancer model with a high incidence of disease suggests their usefulness as a preclinical animal model. Little is known about tolerability and efficacy of chemotherapeutics in the laying hen or in avian species in general. We administered a 6-week paclitaxel treatment to assess chemotherapeutic efficacy in 4.5-year-old laying hens suspect for ovarian cancer. Magnetic resonance imaging (MRI) and positron emission tomography–computed tomography (PET/CT) were used to identify cancerous laying hens as well as to assess changes in tumorigenesis throughout treatment. Results are indicative of chemotherapeutic tolerability and efficacy, as well as the value of using a non-invasive method for diagnosis of cancer within the coelomic cavity, further suggesting the potential of the laying hen as an animal model for preclinical research.

DEDICATION

I dedicate this dissertation to my father James E. Gillenwater. Daddy, you have always given the best to your family and for that, I give this to you. Mom, without your unfailing love and support, this would not be possible…THANK YOU BOTH with all my heart for everything you have done for me, I love you.

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To my children, Brooklyn and Brayden, I love you both beyond words, please know Mommy did this for you...the lesson I want you both to carry for the rest of your lives is the importance of education…and no matter the inevitable struggles you face in your life… "**NEVER GIVE UP**"!

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Contributors

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NOMENCLATURE

m^2	meter squared
^{18}F-FDG	Fluorodeoxyglucose
3 D	Three-dimensional
3T	3 Tesla
A2M	Alpha 2 macroglobulin
AUC	Area under curve
BRCA1,2	Breast cancer gene 1 and 2
Bu-1a	Bursal antigen 1a
CA 125	Cancer antigen 125
CD4, CD8	Lymphocytes
CLDN10	Claudin 10
CLDNs	Claudins
CMP	Comparative Medicine Program
COX1-2	Cyclooxygenase 1 and 2
E-cadherin	Epithelial cadherin
EOC	Epithelial ovarian cancer
F1	Follicle 1
F5	Follicle 5
GEM	Genetically engineered mouse
HGSC	High-grade serous carcinoma

IM	Intramuscular
IV	Intravenous
kg	kilogram
mg	milligram
ml	Milliliter
mMol	Millimole
MMP3	Matrix metalloproteinase 3
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
ms	milliseconds
MSLN	Mesothelin
MUC 16	Mucin 16
NaCl	Sodium chloride
p53	Tumor suppressor gene p53
PDS	Polydioxanone stuture
PET/CT	Positron emission tomography-computed tomography
PGE2	Prostaglandin E2
POSC	Poultry Science Center
SC	Subcutaneous
SERPINB11	Serpin family B member 11
SPP1	Secreted phosphoprotein 1

T1	T1 weighted imaging
T2	T2 weighted imaging
TAMU	Texas A&M University
TE	Echo time
TIPS	Texas Institute for Preclinical Studies
TR	Repetition time
TVU	Transvaginal ultrasound
VEGF	Vascular endothelial growth factor
wk	week

TABLE OF CONTENTS

Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Ovarian cancer is a disease caused by an uncontrolled division of abnormal cell growth that in 80% of patients has already metastasized by the time of diagnosis. If caught early, the prognosis for survival increases dramatically. There are 3 main types of ovarian cancer, germ cell (egg or ova), stromal cell (structural and hormone producing tissue), or epithelial cell cancer (the surface or epithelium layer). The most common type of ovarian tumor is epithelial with 90% of ovarian cancers developing from the epithelium [5]. Epithelial cell tumors are further classified into 4 histologic subtypes starting with the most common: serous (most common), mucinous, endometrioid, and clear cell. The histologic types of epithelial ovarian cancers differ in clinical behavior, descriptive epidemiology, and genetic origins [1]. In fact, some of the complexity of this disease is due to the heterogeneity of the more than 100 histopathologic subtypes of ovarian cancer [2]. Some tumors are benign (non-cancerous) and never spread beyond the ovary however if the tumor is malignant (cancerous) the tumors can spread to other organs and locations throughout the body. All women are at risk for ovarian cancer. There is a correlation of a woman's risk for developing ovarian cancer to the number of ovulations she has had in her lifetime [3]. There are some known factors for increased risk of ovarian cancer such as a family history, being post-menopausal, obesity, use of infertility drugs, women who have not had children, or those who have never used oral contraceptives [4]. If there is a family history of ovarian cancer, a woman could request

genetic testing to identify mutations in BRCA1 and BRCA2 genes (breast cancer gene 1 and 2) as it has been predicted that approximately 30% of those who have mutations in BRCA1/BRCA2 will get ovarian cancer by the age of 70 [4].

Ovarian cancer in women is so deadly because it is extremely difficult to detect at early stages. The ambiguous symptoms of this disease mimic many other common conditions. Per the American Cancer Society only 19% of ovarian cancer cases are diagnosed before there is metastatic spread outside the ovary. The most common ovarian cancer symptoms are bloating, pelvic or abdominal pain, difficulty eating or feeling full too quickly, as well as changes in urinary urgency and frequency [5]. These symptoms are largely due in part to either an ovarian mass already formed or the presence of ascites fluid in the abdomen putting pressure on other organ systems. The presence of ascites correlates with the peritoneal spread of ovarian cancer and is associated with poor disease prognosis [6]. Less common symptoms may include back pain, constipation, menstrual changes, unexplained weight loss or gain, or unusual fatigue [5]. None of these symptoms are usual causes for a woman to feel alarmed or seek immediate medical attention. At the time of diagnosis, in most cases, a woman is already at late stage cancer with metastatic spread outside of the ovaries at which point she will be faced with a 60% mortality rate.

There is no screening assay to detect early stage ovarian cancer. Routine screens such as yearly pap smears identify cervical cancer, but do not detect ovarian cancer. There

are no blood tests to detect ovarian cancer. When a woman reports any of these mild symptoms to her physician, it is at the patient and physician's discretion to investigate further. Once symptoms become problematic the usual course of action is to perform a pelvic examination which does not detect early stage cancer however, if abnormalities are found in the size of the ovary, further investigation using imaging modalities is warranted. If the pelvic exam is abnormal, follow-up would be performed with transvaginal ultrasound (TVU) and a CA 125 (cancer antigen 125) blood test. CA 125 (also known as mucin 16 or MUC 16) is one of the most commonly used blood markers associated with ovarian cancer blood markers used, however, due to both its high false positive and false negative rates, the CA 125 blood assay is not reliable for screening. Elevated CA 125 levels in the blood are not predictive nor an accurate measure of cancer so it is most commonly used as a measure of change over the course of treatment rather than for diagnostic purposes. The next treatment step would be surgical excision of the ovarian mass, followed by histological examination and grading.

There has not been any appreciable improvement in early detection, diagnosis, or treatment of ovarian cancer in decades, despite improved knowledge of etiology, surgical advances, and improvements in chemotherapy [7]. Development of a screening test for early diagnosis would help tremendously in reducing mortality rates. If ovarian cancer is diagnosed (and treated) before the cancer has spread outside the ovary (stage I), the 5-year survival rate is greater than 92% [5]. The standard model systems that exist to study ovarian cancer may also be the very obstacle preventing rapid progress.

Tumor-derived cell lines can play a critical role in facilitating *in vitro* studies of cancer biology; however, *in vivo* animal models can more accurately predict molecular characteristics of primary tumors, and provide a more pertinent preclinical testing platform [8]. Inadequate animal models that do not mimic the natural course of disease can explain, at least in part, the lack of progress toward diagnosis, early detection and improved treatments for ovarian cancer. The most commonly used animal model for ovarian cancer research is either the genetically engineered mouse (GEM) or the immunodeficient mouse used for xenograft studies with human cancer cells. Both the GEM models and xenograft models can further our understanding of key mechanisms facilitating tumorigenesis, enhanced imaging and treatment modalities [9]. GEM models most commonly (transgenic or knockout mice) have either an over-expressed or deleted gene of interest to determine the role of a particular gene in disease. Additionally, some GEM mice require the use of a drug or chemical to induce changes that lead to progression of disease. The GEM mouse has great utility; however, the unnatural nature of the model's genetic makeup is unable to mimic that of spontaneously occurring ovarian cancer within a normal human population. Xenograft studies require the use of immunodeficient mouse strains such that the human cancer cells that are implanted are not rejected. Ovarian cancer is not a disease that occurs naturally with a large quantity of cells instantly appearing under the skin or in the animal, nor is it a disease found only in women with abnormal immune systems. The xenograft model has utility for drug testing using human cancer cells that can be propagated in a live animal however, it is not without problems. In this model, the vasculature that builds around

the injected bolus of cells does not mimic that occurring during the normal progression of ovarian cancer. Drug delivery is no doubt impacted by the inherent development of a tumor from implanting a large number of tumor cells (1-10 x10^6 cells) instantly into the mouse. Tumor development is highly variable depending on cancer cell type, tissue culture conditions, and implant location. The unnatural and manipulated animal models, such as the GEM or xenograft mice certainly have their unique utility, but both also have significant drawbacks.

The chicken model, though not fully characterized, is the only animal model that gets ovarian cancer spontaneously at a high incidence. The laying hen could be the optimal animal model for the study of human ovarian cancer initiation, progression and therapy. Domestic laying hens (*Gallus gallus domesticus*), spontaneously develop ovarian cancer at a high incidence between 2-7 years of age [10]. This is thought to occur due to incessant ovulations leading to repeated DNA damage and repair of ovarian epithelial cells with ovarian cancer being directly related to the number of ovulations over time [11]. Women ovulate every 28 days whereas a laying hen ovulates almost every day (approximately every 27 hours), giving rise to higher rates of ovarian cancer in a much shorter time frame. This theory correlates to humans, where ovarian cancer primarily develops in post-menopausal women 63 years of age or older. Several studies have pointed out striking similarities in tumor types, protein and gene expression, and histologic appearance as well as symptoms in common between humans and chickens with ovarian cancers.

The laying hen model use in research on ovarian cancer has not been fully characterized, though it is becoming of increasing interest due to the spontaneous nature and high incidence of the disease. Increased awareness over the last decade has brought the laying hen into the biomedical spotlight for ovarian cancer research even though it has been known for well over 80 years that laying hens have a high incidence of spontaneous ovarian cancer [12,13,14]. Fredrickson showed in 1987, within three different aged populations of laying hens that ovarian adenocarcinoma was not only the most common type of cancer found in 466 birds aged 2.5 to 7 years of age, but also that there was an increasing rate of this cancer as the birds aged, with the oldest group of birds having greater than a 34% incidence [14]. Eilati *et al*. found an increasing incidence ranging from 0% at 1 year of age to as high as 65% by 3 years of age in random samples of 20 birds per time point from a colony of 600 laying hens [15,16]. These findings further support the incessant ovulation hypothesis claiming that ovarian cancer in both humans and laying hens is correlated to the number of lifetime ovulations, and with increasing ovulatory events there is increasing inflammation which makes surface epithelial cells prone to malignancies [18]. Additionally, in a study performed by Giles and co-workers comparing normal, wild-type laying hens to a mutant strain of hens known as 'restricted ovulators' there was a direct correlation between the number of ovulatory events and the incidence of ovarian cancer in chickens [19]. It is known that factors causing a reduction in ovulatory events such as birth control pill use, pregnancy and breastfeeding also reduce the risk of females developing ovarian cancer this also holds true with the laying hen as seen in a study done by Trevino *et al.* where a significant reduction in

ovarian cancer incidence was seen when a progestin treatment was administered over age-matched controls [20]. Carver *et al*. found a 5-fold reduction in epithelial ovarian cancer of laying hens fed a reduced-calorie diet (causing a reduction in ovulation) over the control group in comparison to the full-calorie control group [21].

An increasing number of studies have pointed out remarkable similarities between humans and the laying hen regarding morphologic, molecular and epidemiologic characteristics, as well as disease progression in common between human and laying hens with ovarian cancer. CA-125, also known as mucin 16, or MUC-16, is the most commonly used ovarian cancer biomarker used, however, it is not accurate enough to be used as a stand-alone screening assay for women due to variability in both false positive and false negative results. CA-125 levels have been found to be elevated in women with ovarian cancer but also as a consequence of endometriosis, pregnancy, and different stages of their menstrual cycle [22]. Its widely used for a measure of treatment efficacy rather than as a predictive assay due to the nonspecific results. CA-125 was shown to be highly expressed in 90% of cells isolated from 15 laying hen ovarian tumors that were extracted from approximately 2-year-old hens, but not detectable in normal hen ovaries [23]. Mutations in p53, a tumor suppressor gene, have shown to have similar abnormalities in common between human and hen ovarian cancers. One group examined alterations in the *p53* tumor suppressor gene in chicken ovarian adenocarcinomas and found that 96% of 4-year-old birds versus 14 percent of 2-year-old birds had p53 mutations [24]. This shows a correlation to the reported 96% incidence of

human ovarian cancers (high grade serous tumors) in women having mutations in p53 [25]. E-cadherin, an adhesion molecule, has been found to be upregulated in human ovarian cancers and found by Ansenberger *et al.* reported significantly greater abundance of E-cadherin protein in cancerous ovarian tissue of laying hens when compared to normal ovarian tissue [26]. Overexpression of E-cadherin and VEGF (vascular endothelial growth factor) plays a role in development of ascites fluid formation and dissemination of human ovarian cancer [27]. VEGF mRNA was shown to be increased in ascites fluid in cancerous laying hens [28]. Mesothelin is a protein normally present in mesothelial cells at low level; however, in human ovarian cancer cells and some other cancers this protein is over-expressed. Yu et al. found *MSLN* mRNA in 57% (12/21) of hen ovarian tumors with none being expressed in normal ovarian tissue, and of those with *MSLN* mRNA 44% also had circulating anti-mesothelin antibodies [29]. Claudins (CLDNs) are a family of cell membrane proteins that play an important role in tight junction formation between cells and cell membranes. In human ovarian cancer, a number of claudin proteins are over-expressed [30]. This over-expression is also observed in laying hens that had more than 700-fold increase in *CLDN10* mRNA in cancerous ovaries when compared to normal ovaries [31]. Matrix metalloproteinases (MMPs) are involved in protein degradation and associated with promoting invasion, angiogenesis, and metastasis of cancer cells, with several the known MMP sub-types showing increased expression in reproductive cancers [32]. Choi *et al.* found that in cancerous ovaries of laying hens there was a 16-fold higher expression of *MMP3* mRNA than in normal ovaries and suggested there may be applications for its

use as a marker for ovarian cancer in chickens [33]. There are several novel biomarkers for ovarian cancer in the laying hen however, many show specificity to both stage of cancer and its histologic sub-type, so their broad application for ovarian cancer detection may be limited. However, their use for monitoring progression of disease with treatment should be investigated further. Alpha 2 macroglobulin (A2M), secreted phosphoprotein 1 (SPP1), and SERPINB11 may be useful as potential biomarkers in monitoring efficacy of treatment in chicken endometrioid cancer as its expression is up-regulated in cancerous but not normal ovaries [34,35]. Up-regulation of cyclooxygenase 2 (COX-2) has been reported to occur in human ovarian cancer and to be associated with poor prognosis [36]. Eilati *et al.* showed an up-regulation of both COX-1 and COX-2 enzymes as a function of laying hens age and an increase in a pro-inflammatory prostaglandin E2 (PGE_2), the downstream product of the COX enzymes. In addition, they noted further increases in COX-1 and PGE_2 in cancerous ovaries [16].

There have been many studies showing histological commonalities between the laying hen and human ovarian tumors. Rodriguez-Buford *et al.* showed that several antibodies against antigens that are frequently expressed in human ovarian tumors were cross-reactive in the laying hen [37]. Bradaric *et al.* compared normal and cancerous sections of laying hen ovaries to assess tumor type and stage as well as CD4, CD8 and Bu-1a counts of immunostained cells and determined that there were significantly more immune cells in cancerous *versus* normal ovaries [38]. The four histologic subtypes of

ovarian cancer in humans (serous, mucinous, endometrioid, and clear cell) occur in ovarian tumors of laying hens [10].

The literature points to the fact that laying hens, though not commonly used, show great potential for advancing progress in ovarian cancer. Our research aims to further validate this model and show how its application, benefits the study of ovarian cancer.

CHAPTER II

DEVELOPMENT OF 3D PRINTED PORTS FOR REPEAT OVARIAN IMAGING AND BIOPSY IN DOMESTIC LAYING HENS (*Gallus gallus domesticus*): A SPONTANEOUS MODEL OF OVARIAN CANCER

Synopsis

No appreciable improvements in mortality rates of women with ovarian cancer have been made over the last 40 years [39]. Lack of a predictive animal model has been attributed as an obstacle for progress in ovarian cancer research. It is hypothesized that laying hens, though not fully characterized, could be the optimal animal model for the study of human ovarian cancer initiation, progression and therapy. Domestic laying hens (*Gallus gallus domesticus*) are the only animal model that spontaneously, and with high incidence, develop ovarian cancer [10]. In effort to improve its practical usefulness and to further validate this model, using 3D printing technology, we fabricated a surgically implantable port that allows for repeat access to the ovary for serial sampling, observation and imaging. With the ability to easily follow laying hens via accessible ports, our goal is to be able to detect onset of ovarian cancer, as well as discover early diagnostic techniques. This port also allows repeat access to the coelomic cavity of the hen for other applications (such as chemotherapeutic delivery). Finally, it holds the potential to benefit other areas of poultry research (renal, intestinal or oviductal etc.). Our device has been implanted in multiple birds for over one year without complications

or rejection showing the potential to offer easy ovarian access over an extended period of time and perhaps even the life of the animal.

Introduction

Ovarian cancer ranks fifth in cancer deaths among women; the high mortality associated with this disease is due to late diagnosis as well as ineffective treatment and preventative therapies. The American Cancer Society estimates that 1 out of every 75 women in the US will be diagnosed with ovarian cancer, and that of those diagnosed over 60% will die from the disease. These statistics are alarming, and a growing body of evidence is pointing to the model scientists commonly use, the mouse, as being one of the biggest obstacles to progress.

Ovarian cancer is a disease caused by an uncontrolled division of abnormal cell growth in the ovaries of woman. The most common type of ovarian tumor is epithelial with 90% of ovarian cancers developing from the epithelium [5]. Epithelial cell tumors are further classified into 4 histologic subtypes starting with the most common: serous, mucinous, endometrioid, clear cell. The histologic types of epithelial ovarian cancer differ in clinical behavior, descriptive epidemiology, and genetic origins [1]. In fact, some of the complexity of this disease is due to the heterogeneity of the more than 100 histopathologic subtypes of ovarian cancer [2]. Some epithelial cell tumors are benign and never spread beyond the ovary, however if the tumor is malignant the tumors can spread to other organs and locations throughout the body. Determining the cell of origin

in ovarian cancer is difficult, there is debate whether the epithelial cells originate from the oviduct or the ovary because epithelial cells can undergo epithelial to mesenchymal transition where the epithelial cells from either tissue can migrate to the other [40]. All women are at risk for ovarian cancer. There is a correlation of a woman's risk for developing ovarian cancer with the number of ovulations she has had in her lifetime [3]. Ovarian cancer in women is so deadly because it is extremely difficult to detect at early stages. The ambiguous symptoms of this disease mimic many other common conditions. Per the American Cancer Society only 19% of ovarian cancer cases are diagnosed before there is metastatic spread outside the ovary.

There is no screening assay to detect early stage ovarian cancer. Routine screens such yearly pap smears identify cervical cancer but do not detect ovarian cancer. There are no blood tests to detect ovarian cancer. There has not been any appreciable improvement in early detection, diagnosis, or treatment of ovarian cancer in decades despite improved knowledge of etiology, surgical advances, and improvements in chemotherapy [7]. Development of a screening test for early diagnosis would help tremendously with reduction in mortality rates. If ovarian cancer is diagnosed (and treated) before the cancer has spread outside the ovary (stage I), the 5-year survival rate is greater than 92% [5].

The standard model systems that exist to study ovarian cancer may also be the very obstacles preventing rapid progress. Tumor-derived cell lines can play a critical role in

facilitating cancer biology *in vitro* studies; however, *in vivo* animal models can more accurately predict molecular characteristics of primary tumors, and represent a more pertinent pre-clinical testing platform [8]. Inadequate animal models that do not mimic the natural course of disease may be partly to blame for the lack of progress toward disease diagnosis, early detection and improved treatments for ovarian cancer. The most commonly used animal model for ovarian cancer research is either the genetically engineered mouse (GEM) or the immunodeficient mouse used for xenograft studies with human cancer cells. However, there is another option. The laying hen model, though not fully characterized, is the only animal model that gets ovarian cancer spontaneously at a high incidence. The laying hen could be the optimal animal model for the study of human ovarian cancer initiation, progression and therapy.

Materials and Methods

Healthy single comb white leghorn 1 to 3-year-old laying hens were used in this experiment. All work followed Texas A&M University (TAMU) institutional guidelines and was covered under an animal use protocol approved by TAMU Animal Care and Use Committee (IACUC). The laying hens were single housed in laying cages at the TAMU poultry research center and provided feed and water *ad libitum* with a lighting schedule permissive of normal egg laying (approx. 16-18 h of light/day). On the day of procedures, hens were transported in dog crates to the surgical suite in a climate controlled vehicle and group housed until their procedure. Hens were anesthetized using a mask with isoflurane delivered at 5% until fully anesthetized and maintained at 2.5-

3.5% thereafter. Hens were kept on a circulating warming pad for the duration of the procedure. Feathers were removed from the left side of the chest and the surgical area was scrubbed with betadine followed by alcohol (repeated 3x). Using a sterile field and sterile surgical instruments a 1.5 - 2.5 cm incision was made in the skin and through the left side body wall just caudal to the last rib as this is the location of the active ovary in a hen. The pre-sterilized port was placed into the incision site and sutured into the body wall and the skin using 2-0 polydioxanone suture (PDS). The birds were recovered by removing isoflurane and administering 1 mg/kg buprenorphine IM. Post-operatively 3 mg/kg carprofen was administered SC to provide pain relief for 12 hours. After that time, birds were evaluated daily for evidence of a need for pain medication based on limping, not bearing weight, not rousing, holding wing down, holding leg up, lack of defecation, lack of activity/lethargy. The incision was closed using PDS suture. Anesthesia for the laparoscopy via the port was the same as for the port implant surgery. A laparoscope (Portascope.com, Inc) with a rigid head 4mm head was lubricated with sterile ointment, inserted through the self-sealing port and the ovary was visually observed or biopsied. Birds readily recovered after removal of isoflurane and were returned to their home cage after completion of the procedures.

Results

Throughout the course of this study surgical techniques and port design were continually improved. Early stage designs allowed for only 2-4 weeks before the port popped out of the body wall due to healing and formation of granulated tissue. When properly

anchored, all late design silicone, self-sealing ports remained in place for over a year of placement with no detrimental effects on laying, normal activity, or mobility of the hens. Hens actively laying at the time of surgery continued laying after port placement. It was common to see hens lay an egg on the day of surgery (or during) and continue laying daily post-operatively indicating minimal impact on the hen from the stress of the surgical procedure, anesthesia and port. Recovery from surgery was very rapid and generally within 1 h from the procedure the hen was back in her home cage with the ability to eat, drink and be fully weight bearing. Necropsies performed by a board certified veterinary pathologist at one-year post port placement revealed only mild tissue tacking from the caudal thoracic air sac to the port. Tissue surrounding the port looked healthy and the port was fully intact and the seal patent. The current device allows for routine access for evaluation and biopsy of organs in the coelomic cavity, specifically the ovarian tissue.

Discussion

Port design was modified over 1.5 years with changes to shape, material, texture, ability to hold suture and plug removability. The port concept was originally thought of as an animal procedural refinement with the overall number of surgical events being reduced to simple anesthetic events for the hen. This refinement also reduced the overall numbers of hens utilized in our research. The IACUC set a limit on the number of surgical events a research animal could undergo in its lifetime. With the laying hen having a wide range in years to onset of ovarian cancer, it seemed impractical to limit

the number of laparoscopic procedures over the range of 2-7 years. After brainstorming with veterinarians, the concept was developed to use a permanent "port" that would mirror a rumen cannula used in cattle (Fig. 1). Commercial devices for use in poultry were not available. Producers of rumen cannulae do not make a product small enough for a 1.5kg animal.

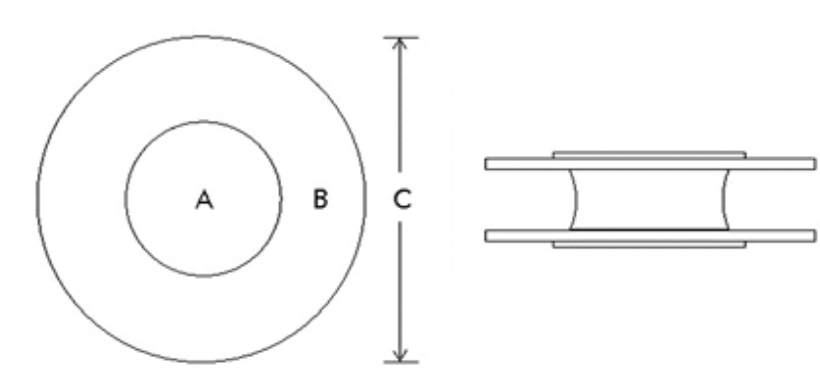

Fig. 1. Diagram of a typical commercially available flexible rumen cannula produced for cows. Typical dimensions in a cow; A = center hole diameter (100mm), B = flange width (80mm), C= total diameter (250mm).

Human pediatric devices were considered; however, no product fit the size required for the hen, or the accessibility of the laparoscope. Polycarbonate bottle tops and rubber grommets sealed with an internal plug were investigated, but adequate functionality and size parameters of both grommets and plugs were not found (Fig. 2).

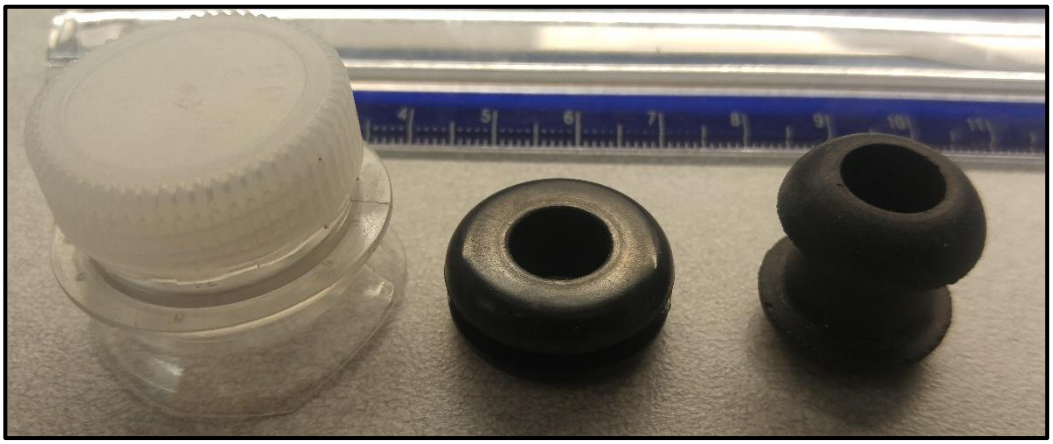

Fig. 2. Evolution of the port design. The port idea was conceived from a device that would mimic a rumen cannula scaled to a 1.5 kg hen.

Due to lack of a commercially available product that met our specifications, production of a device was discussed with Texas A&M University's Mechanical Engineering and biomedical device specialist Dr. Michael Moreno. Dr. Moreno's team included doctoral candidate Andrew Robbins who fabricated our device utilizing 3D printing technology. Working closely with Dr. Moreno's team and the laboratory animal veterinarians at Texas A&M University's Comparative Medicine Program, the port design was modified over 1.5 years with changes to shape, material, texture, ability to hold suture, and plug removability. The first prototype mimicked a rumen cannula reduced to the size of a hen, and this design and plug were completely 3D printed out of biocompatible material (Fig. 3).

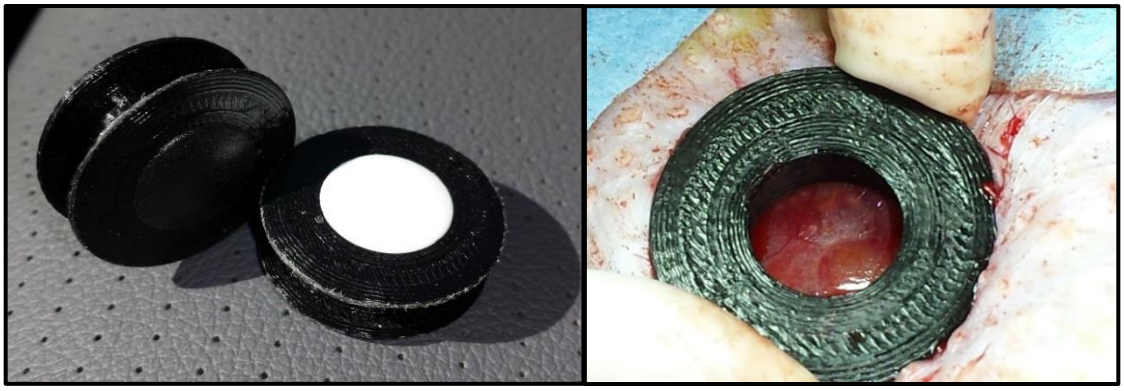

Fig. 3. First 3D printed prototype. Prototype produced by Texas A&M University's Department of Mechanical Engineering.

The prototype was circular, rigid, and spool shaped, with a central cavity containing a removable plug. The prototype was surgically implanted into a hen and it was well tolerated (Fig. 4); however, after 2-4 weeks the port would work its way out of the bird due to changes in the surrounding tissues during the normal healing process.

Fig. 4. First implanted prototype. Hens tolerated the surgical implant, and within 1h of anesthesia they were eating, drinking, moving normally and resuming egg laying.

Granulation tissue formation is a normal process in healing, however, with the shape and location of the port the tissue would build up around the port and up through the plug opening causing the port to be slowly pushed out. Further device modifications included different materials, shapes and plugs for the device. Silicone coating over the plug and port did not improve the rejection time. Suture holes were added to improve device anchoring however, rejection time was still unchanged. The shape of the next device was modified to be oval to better accommodate the location and limited space between the hens last rib and the back leg (Fig. 5).

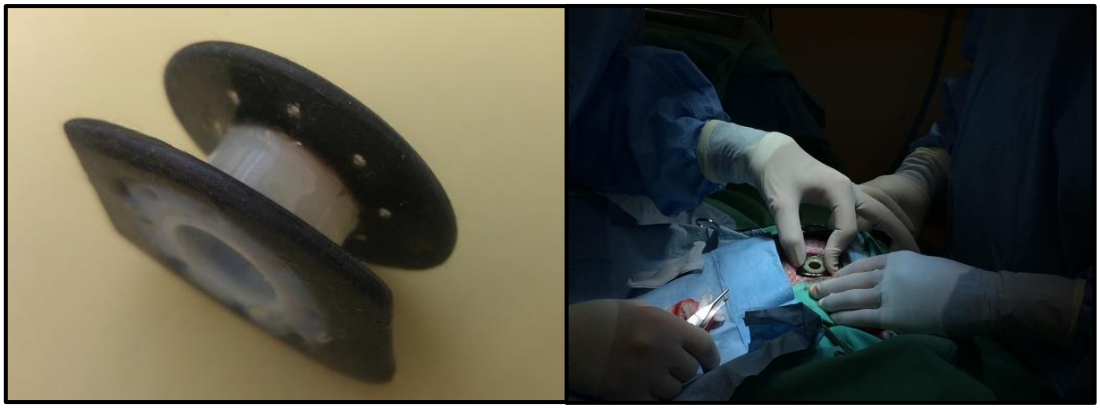

Fig. 5. Suture holes for anchoring during surgery. Suture holes were added to improve the oval-shaped port which was more flexible and designed to improve the fit in the hen.

The material the port was printed from was simultaneously changed to rigid central material that merged into softer more flexible lips surrounding the port. These changes improved surgery implant time; however, the port was still rejected. It was hypothesized that rejection was likely be from a primary design failure. Rumen cannulas were

intended to go into a sealed organ (rumen) and the port for this application only needed access to the coelomic cavity. Further brainstorming with CMP veterinarians led to the hypothesis that a fully flexible, soft port, with a flat narrow center would encourage tissue to heal around the device and allow the laparoscope to pass though the central slit. Dr. Moreno's team made a 3D printed mold to our specifications which enabled a flexible, light port to be made from pouring silicone into the mold. The new design made for much more rapid surgery and improved anchoring. The new design did away with the central hole of the previous designs and allowed for a self-sealing opening in the center where a laparoscope could easily pass through (Fig. 6).

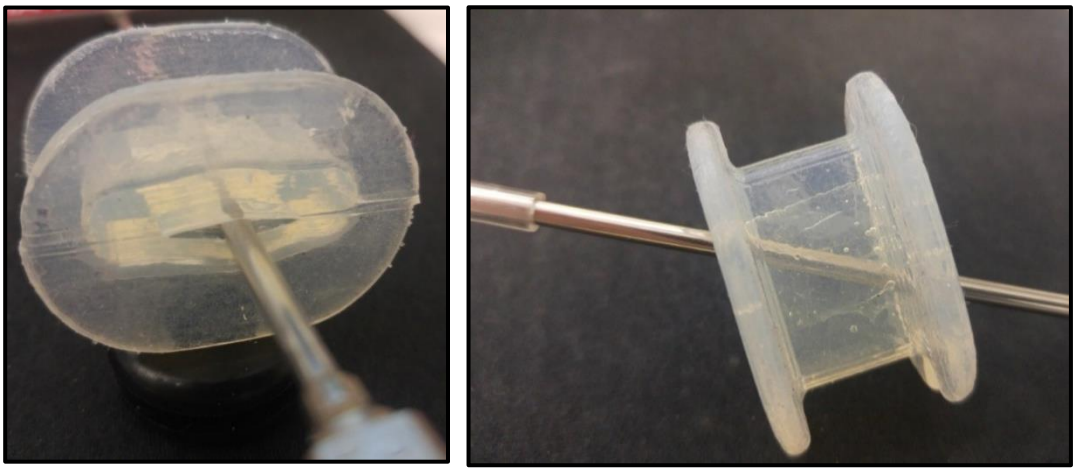

Fig. 6. The silicone self-sealing port. The flexible port was made by pouring silicone into a 3D printed mold.

The silicone port was biocompatible and able to withstand being in the hen for over 1 year without complications (Fig. 7). The current design allows for repeat laparoscopic

procedures for visualization and biopsy. Laparoscopic images of healthy versus cancerous tissue are shown (Fig. 8).

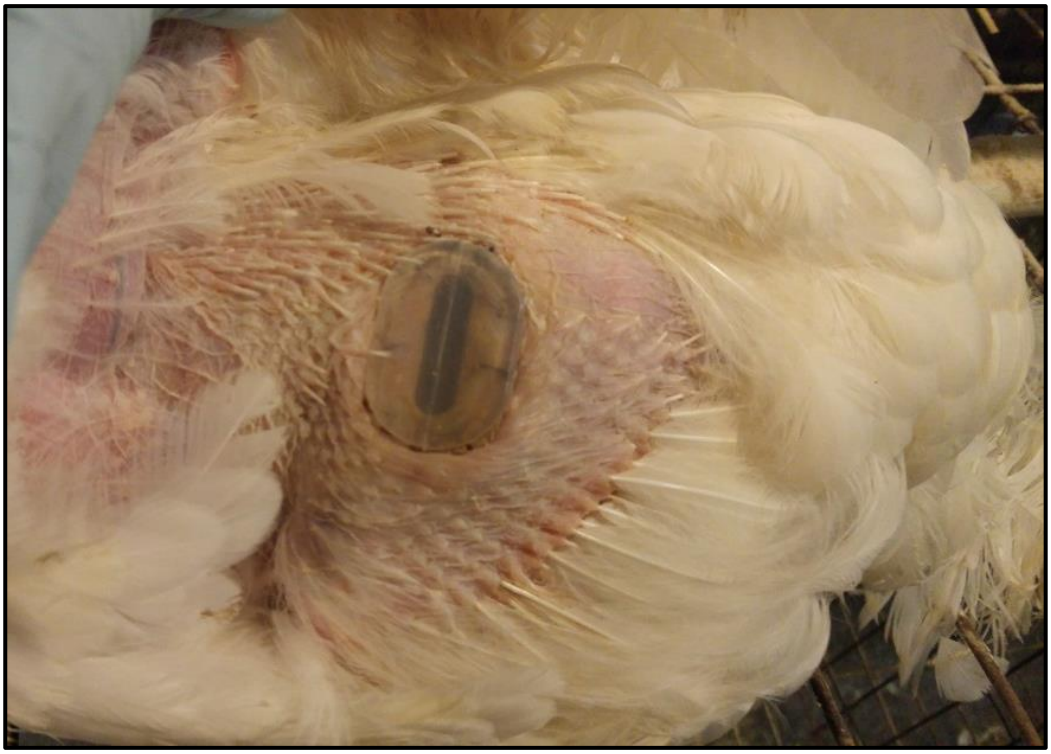

Fig. 7. The final port version. The all-silicone designed port implanted in the hen.

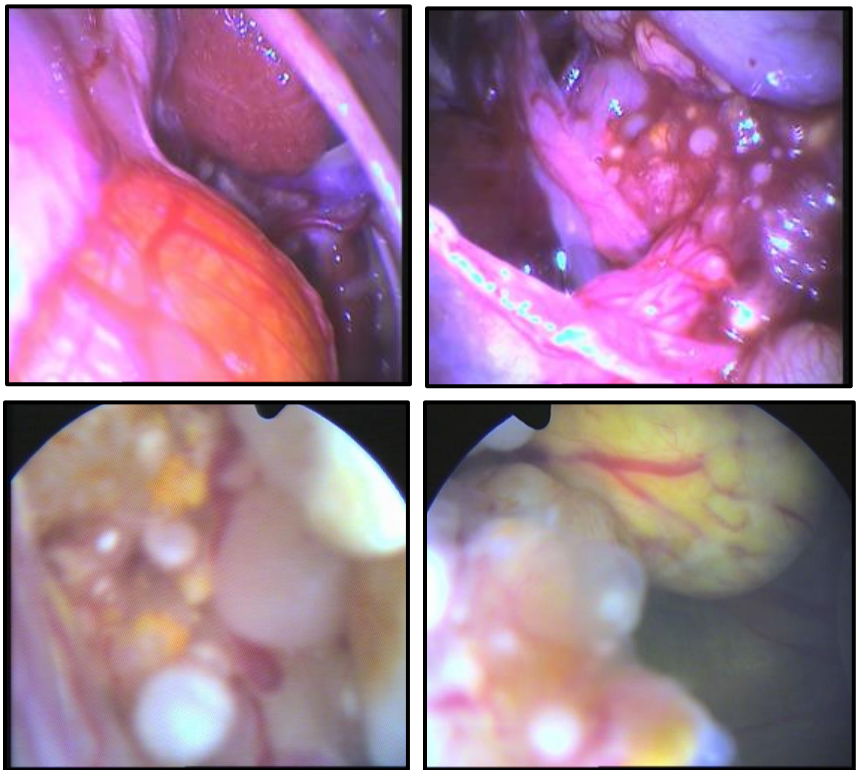

Fig. 8. Laparoscope photos. Top left and top right images show healthy ovarian tissue, and bottom left and bottom right images are cystic and cancerous with ascites present.

Hens with implanted ports were imaged using a combination of modalities including computed tomography (CT) and positron emitted topography (PET) and placement within the coelomic cavity can be well observed (Fig. 9).

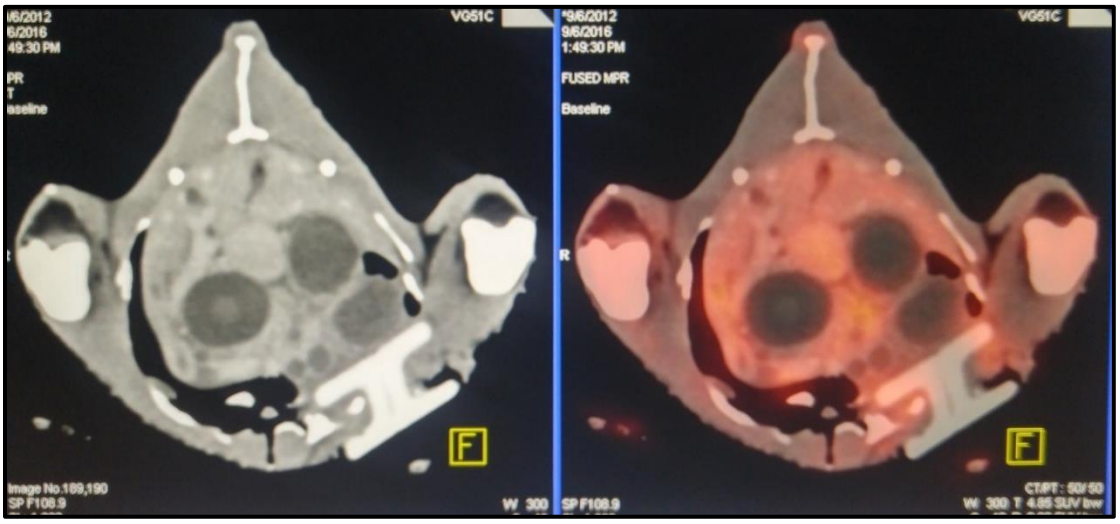

Fig. 9. Non-invasive imaging of port. Left shows computed tomography (CT) image of the implanted port, right is with positron emitted tomography (PET) image overlay.

Conclusion

A silicone device was produced that allows for repeated visualizations of the ovarian tissue as well as a means to accurately biopsy tissue of interest. This device has minimal impact to the hen and remains functional for extended periods of time. This novel approach to study a spontaneously occurring ovarian cancer animal model allows for further research and discoveries to be made in ovarian biology and ovarian cancer.

CHAPTER III

PRECLINICAL POTENTIAL OF THE LAYING HEN MODEL OF OVARIAN CANCER: INVESTIGATION OF NON-INVASIVE IMAGING MODALITIES FOR DIAGNOSIS AND EVALUATION OF TREATMENT EFFICACY

Synopsis

Laying hens spontaneously, and at high incidence, develop ovarian cancer which remarkably mimics the development of human ovarian cancer which suggests their utility as an animal model for preclinical testing of therapeutics for treatment of ovarian cancer. To further validate this model, and test this hypothesis, we identified a cohort of ten 4.5-year-old single comb, white laying hens suspected to be developing ovarian cancer. The cohort of laying hens was treated intravenously with paclitaxel chemotherapy for six weeks, and changes in the ovaries assessed using serial imaging at 0, 3, and 6 weeks of treatment. After completion of the study, hens were euthanized, necropsied, and tissues submitted for histopathology. Results are indicative of accurate diagnosis of cancer via non-invasive imaging and chemotherapeutic efficacy, further suggesting the laying hen's potential for use in preclinical research on ovarian cancer.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer [41]; the high mortality associated with this disease is attributed to late diagnosis, lack of screening methods, as well as ineffective treatment and preventative therapies. In most cases, at

the time of diagnosis, a woman has already progressed to late stage cancer with metastatic spread outside of the ovaries lending to poor prognosis. Early detection methods of EOC are greatly needed; per the American Cancer Society, only 19% of ovarian cancer cases are diagnosed before there is metastatic spread outside the ovary, and when diagnosed early a patient's 5-year survival rate exceeds 90%.

EOC is a very complex disease in part due to the lack of a known cell of origin, variety of tumor types, and numerous histotypes. High-grade serous carcinoma (HGSC) makes up more than 70% of the primary EOC. A complicating factor to diagnosis of this disease is these tumors are histologically identical to peritoneal and fallopian tube serous tumors. The standard treatment for HGSC is surgical debulking, followed by a platinum and taxane combinatorial chemotherapeutic regimen. While this treatment shows initial efficacy, there is a very high rate of recurrence. Because of the complexity of EOC, there is a need for preclinical models that adequately mirror the heterogeneity inherent with the disease. Mice are the primary model used to study EOC; most commonly either xenograft mice, genetically engineered mice, and patient derived xenograft mice are used for ovarian cancer research. None of these models are spontaneous, and they do not mimic the normal progression of ovarian cancer. Models must be genetically altered or be completely immunodeficient to allow them to propagate human cancer cells without rejection. Each model used to study EOC has its advantages and disadvantages, and progress of this deadly disease will likely require more than just a single model to make advances. Unlike many of the mouse models of ovarian cancer, the laying hen has all

four histologic subtypes (serous, mucinous, clear cell and endometrioid) that are present in humans, in addition to the development of spontaneous ovarian cancer.

Laying hens hold promise as a superior animal model for cancer research. The laying hen is the only animal model that spontaneously develops ovarian cancer at a high incidence, which could be key to uncovering the events essential for initiating ovarian cancer and for developing early detection and screening techniques. Laying hens have an increasing incidence of ovarian cancer after age 2, which is late in its reproductive life, coinciding with that of a post-menopausal woman, whose incidence increases after the age of 63. One of the greatest assets of the laying hen is an estimated tenfold greater incidence of ovarian cancer than humans (14, 20). There is a known link between the number of ovulatory events and the incidence of ovarian cancer, although the exact mechanism through which this occurs is unknown. Research has shown that decreases in the number of ovulations, as with oral contraceptive use, reduce the risk of ovarian cancer in women. There is a similar trend in laying hens when ovulation is inhibited with a progestin, estrogen or a combination of the two [20].

We hypothesize that laying hens, though not fully characterized, are an optimal model for the study of human ovarian cancer initiation, progression and therapy. Lack of a method for predicting EOC in laying hens is an obstacle to the utilization of laying hens in preclinical studies. Historically, to establish a study cohort of laying hens, researchers must carry out studies on a large scale in hopes of obtaining enough cancerous hens to

see statistical significance of a given treatment. The method we describe here started with 20 hens that were subjected to a combination of imaging modalities including magnetic resonance imaging (MRI), computed tomography (CT) and positron emitted topography (PET) to detect ovarian cancer. Since these technologies are labor and cost intensive, we ran an initial MRI on a 4-year-old and a 1-year-old laying hen to see if we could visualize the ovary and compare cancerous and noncancerous ovaries. The first MRI scan revealed that a laying hen with cancerous ovaries was selected for comparison to normal ovaries from a young laying hen (Fig. 10). This validated our identification approach.

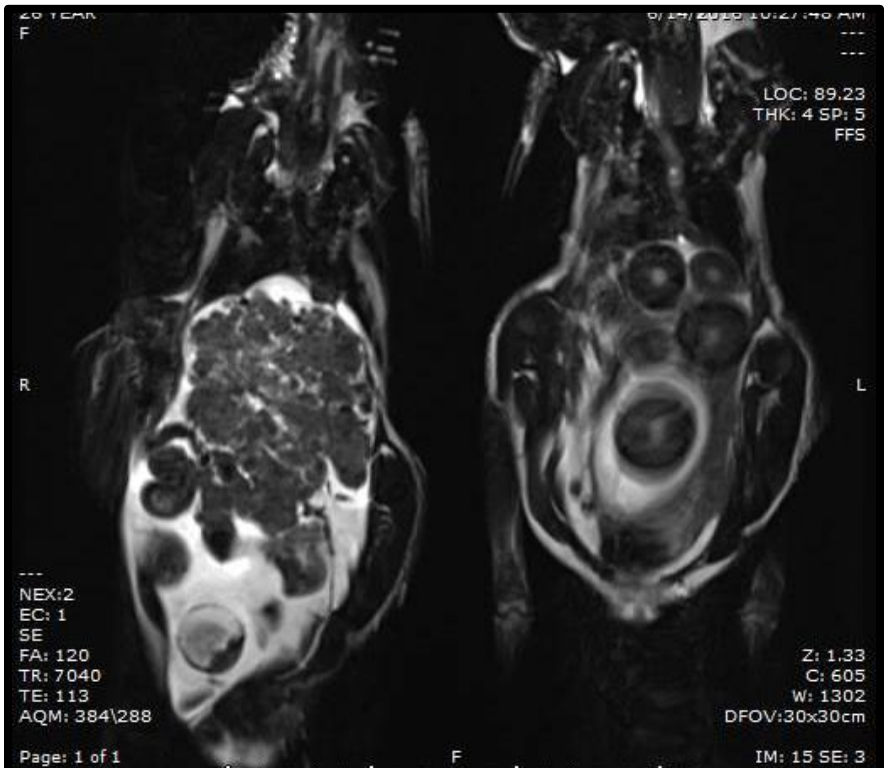

Fig. 10. Proof of concept magnetic resonance imaging (MRI). MRI T2 coronal image of a cancerous ovary in a 4-year-old laying hen (left), and a normal ovary from a 1-year-old healthy laying hen (right).

After imaging, 10 hens were selected to proceed through the chemotherapy treatment and were staged on a scale of I-IV based on visual abnormalities suggestive of coelomic cancer. Two of the 10 were included as "suspect" without a stage designation. Since a method for staging EOC via these imaging modalities in laying hens does not exist, we chose a staging system based on a histopathological approach [42]. Stage I was assigned if abnormalities appeared to be confined to the ovary (with some of the ovary having a normal phenotype); Stage II involved abnormalities that involved most of the ovary and may have oviductal involvement; Stage III involved abnormalities that included the whole ovary with other organ involvement possible, with or without ascites fluid present; and Stage IV involved extensive peritoneal metastasis and ascites fluid present.

Common standard of care in human patients is a combinatorial therapy of a taxane, such as paclitaxel, plus a platinum-based drug, such as carboplatin or cisplatin, for six cycles. Due to the limited amount of data on chemotherapeutic doses and tolerability in the laying hen or avian species in general, it was decided to use only a single drug that was predicted to show efficacy when given alone. Paclitaxel was the chemotherapeutic selected for this study as it is one of the most common chemotherapeutics used to treat human ovarian cancer, and under oncologist advisement that this drug, when given alone, would be expected to exhibit anti-cancer effects in human patients. Paclitaxel at 10mg/kg was determined in our prior studies to be well tolerated for six weekly doses. Mass spectrometry results confirmed the presence of paclitaxel in hens administered intravenous paclitaxel at 7.5mg, 10mg, and 12.5mg per kilogram (AUC =9.6, 5.7, and

3.5 ug/ml per hr respectively) (Fig. 11). The dose of 10mg/kg is slightly lower than the human equivalent dose of 175mg/m^2 when taking body surface area into account. Frequency of treatment in humans is six rounds with 3 weeks in between each round. In laboratory animals, such as mice, chemotherapy is commonly administered as a single bolus intravenous injection on a weekly regimen. Since this was an attempt to further validate the laying hen as a research model, we administered the paclitaxel IV (jugular vein) once weekly for 6 weeks.

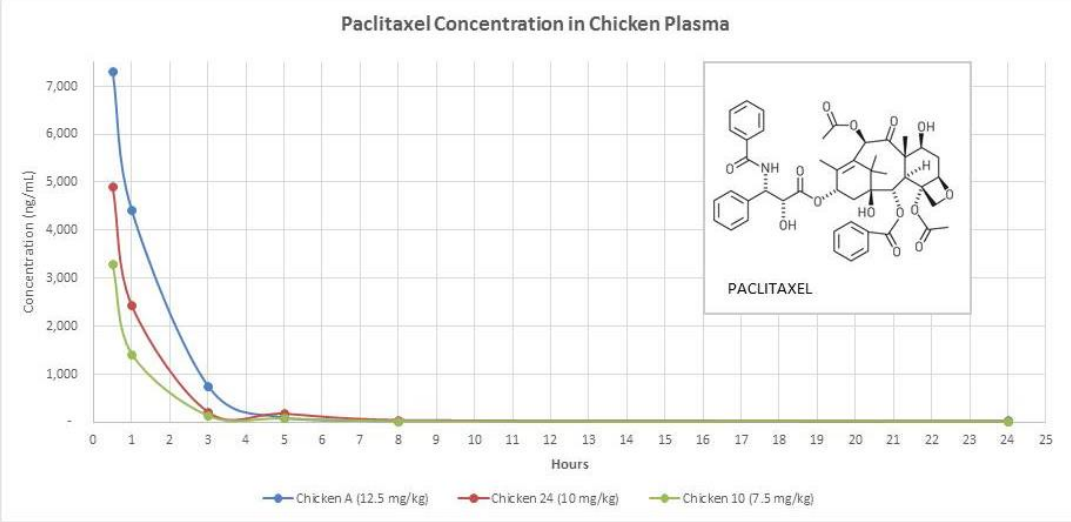

Fig. 11. Hen tolerability of paclitaxel. Tolerability was assessed by once weekly IV administration of 7.5mg, 10mg, and 12.5mg per kilogram, single bolus, AUC 3.5, 5.7, and 9.6 ug/ml per h respectively.

Materials and Methods

The Texas A&M Institute for Preclinical Studies (TIPS) performed MRI and PET/CT imaging on 20 4.5-year-old single-comb white leghorn hens to identify 10 candidates for ovarian cancer (estimated 60-70% incidence). The laying hens were identified as cancer suspects via abnormalities detected using MRI and PET/CT. A cohort of 10 laying hens was treated with paclitaxel, a common taxane chemotherapeutic (WG Critical Care, Paramus, NJ). Hens were imaged a total of three times (0, 3wk, and 6wk of treatment) to assess efficacy and improvements in tumor size and a reduction in ascites fluid. Birds were visually assessed daily and weighed weekly during the 6-week treatment period (1x weekly chemo regimen) and were monitored for any sign of decreased excrement, lethargy, anemia (pale comb/wattle) or breathing abnormalities. Blood samples were taken at 0, 3, and 6 weeks to evaluate blood chemistry, specifically, bile acids, a measure of liver function in birds, throughout the treatment period (< 0.5 ml blood drawn per time point). The hens were placed in a cage and transported in a climate controlled vehicle from the poultry science center (POSC) to the TIPS animal loading dock.

After hens arrived in the surgical preparation room, they were examined for health prior to dosing with butorphenol (1mg/kg IM) as a pre-anesthetic. Anesthesia in the hens was induced and maintained with isoflurane using a vaporizer and nose cone with a charcoal scavenging system. Once fully anesthetized, their wing vein was catheterized and flushed with heparinized saline to allow access for contrast to be injected into the blood stream prior to imaging. MRI scans were done using a Siemens 3T MRI scanner. After

localizer sequences, pre-contrast coronal T2-weighted images with and without fat saturation, as well as axial T2-weighted fat-saturated and T1-weighted images were obtained. Intravenous contrast was then given (Gadavist 1mMol/ml; 1ml in 5ml NaCl). Post-contrast sequences included T1-weighted fat-saturated axial and coronal images. The parameters for the T1-weighted sequences included TR of 779 to 973ms and TE of 11-12ms. The parameters for T2-weighted sequences included TR of 5350 to 7010ms and TE of 93-116ms. Slice thickness of 3mm was used for coronal images and 4mm for axial images. While in the MRI, under anesthesia, birds were injected with ^{18}F-FDG (5-10 mCi/kg) approximately 1h prior to CT scan to aid in PET imaging for contrast and in identification of hypermetabolic areas. Feed was withheld the evening prior to the procedures and birds were kept in a dark, quiet holding room for 1 hour prior to anesthesia to minimize excess digestion and movement. ^{18}F-FDG fluorodeoxyglucose is a radiolabeled glucose analog used to identify highly metabolic areas such as those that would occur with cancer. TIPS radiation safety procedures were followed including separation of the radioactive materials (containers, animals, feces, syringes and blood) until they were cleared by a TIPS radiation safety staff member or radiation permit holder. Hens were analyzed individually under isoflurane anesthesia and moved on a transport cart along with the vaporizer to the MRI imaging room where they were transitioned to an MRI-safe vaporizer and placed in the magnet. After MRI scans were completed the hen was moved into the CT imaging room, reconnected to a vaporizer and maintained on isoflurane until imaging was complete. For the CT and PET, scans were obtained on a 128-slice Siemens Biograph CT/PET scanner. After localizing topograms

were acquired, a whole-body pre-contrast CT scan was performed. This was followed by acquisition of a whole-body PET scan. Iodinated CT (omnipaque, 2ml 350mg/ml followed by 3ml NaCl) contrast was then given into the venous catheter and a post-contrast whole body CT scan was obtained. It took approximately 1.5 hours to image each hen using MRI, CT and PET. Supplemental heat was provided by warmed bags of rice and MRI-compatible disposable warmers. Monitoring under anesthesia was accomplished by using a respiration pillow, fiber optic temperature rectal probe, pulse oximetry and a blood pressure cuff (SA Instruments, Stony Brook, NY). The hens were recovered from anesthesia by providing oxygen only via the nose cone and administering 35ml warmed fluids (NaCl) subcutaneously. Hens were returned to their cage under a warming lamp, provided feed and water ad libitum and held until cleared by the radiation safety officer, and finally transported back to the POSC center and placed in their home cages.

Results

Necropsy of the hens after final imaging revealed that 7 of 10 (70%) hens had metastatic cancer, and 3 of 10 (30%) hens were non-cancerous. The breakdown of the metastatic cancer based on histopathology was ovarian carcinoma with metastasis in 6 out of 10 (60%) hens and 1 of 10 (10%) had oviductal carcinoma with metastasis

Analysis of MRI and CT images at 0, 3 and 6 weeks of treatment correlate well with diagnosing cancer and assessing changes post-treatment within the coelomic cavity of the laying hen. Image analysis throughout the study by board certified avian veterinarians accurately observed indications suggestive of the presence of cancer within the coelomic cavity in all but one of the hens (#104); in this case the results were confounded by the presence of fibrin and yolk masses found on necropsy and histopathology. Although identification of the presence of cancer was accurate, initial staging of the hens was performed after only one MRI and CT/PET image collection event (timepoint 0). One-half of the staging assigned at this point correlated well 5/10 (50%) were accurately assessed late stage cancer; however, we found confounding presentation of disease (massive ascites or the presence of fibrin within the coelomic cavity) or less than ideal image quality (breathing artifacts and slice size of MRI) led to an inaccurate assignment when staging from just a single set of images. When taking 3wk and 6wk images into account collectively, stage assessment would have been significantly more accurate. Additionally, ^{18}F-FDG uptake and reflux variability led to difficulties making an accurate diagnosis based on standardized uptake value (SUV) alone. Since the ^{18}F-FDG is excreted by the kidney and there can be reflux into the cloaca, colon and coecal pouches, it was not always a definitive sign of hyper-metabolism for cancer diagnostic purposes, however, in instances where a clear mass was visible, PET imaging was an effective means of following response to treatment. Our study found that MRI was most informative for diagnosis and assessment of

changes; however, CT and PET images proved to be helpful methods for clarification of MRI findings and to aid identification and progression of cancer in the hen.

Paclitaxel appeared to cause a reduction in ascites fluid, ovarian quiescence, reduction in ventriculus dilation, reduction of bowel wall thickness and a reduction in tumor ^{18}F-FDG SUV in several hens over the course of treatment (even if only an initial response). Ascites fluid was present in 8 out of 10 hens at the start of the study. Of those 8, (75%) showed a reduction in fluid volume in the coelomic cavity (Fig. 12); one additional hen (#108) showed a reduction, however, had coelomic tap (ascites removal) performed at intervals throughout the study to remove excess fluid, so the reduction cannot solely be attributed to the chemotherapeutic regimen alone. Only one hen had an increase in ascites fluid volume throughout the study (#75) and was diagnosed with oviductal cancer.

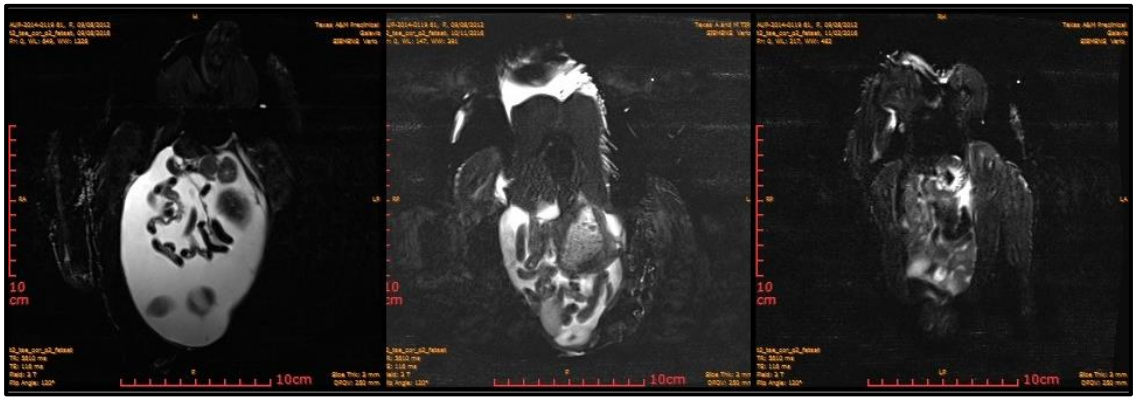

Fig. 12. Diminished ascites with paclitaxel treatment. Hen# 61 from left to right MRI images at 0, 3wk, 6wk of paclitaxel treatment showing decreased ascites fluid over each time point.

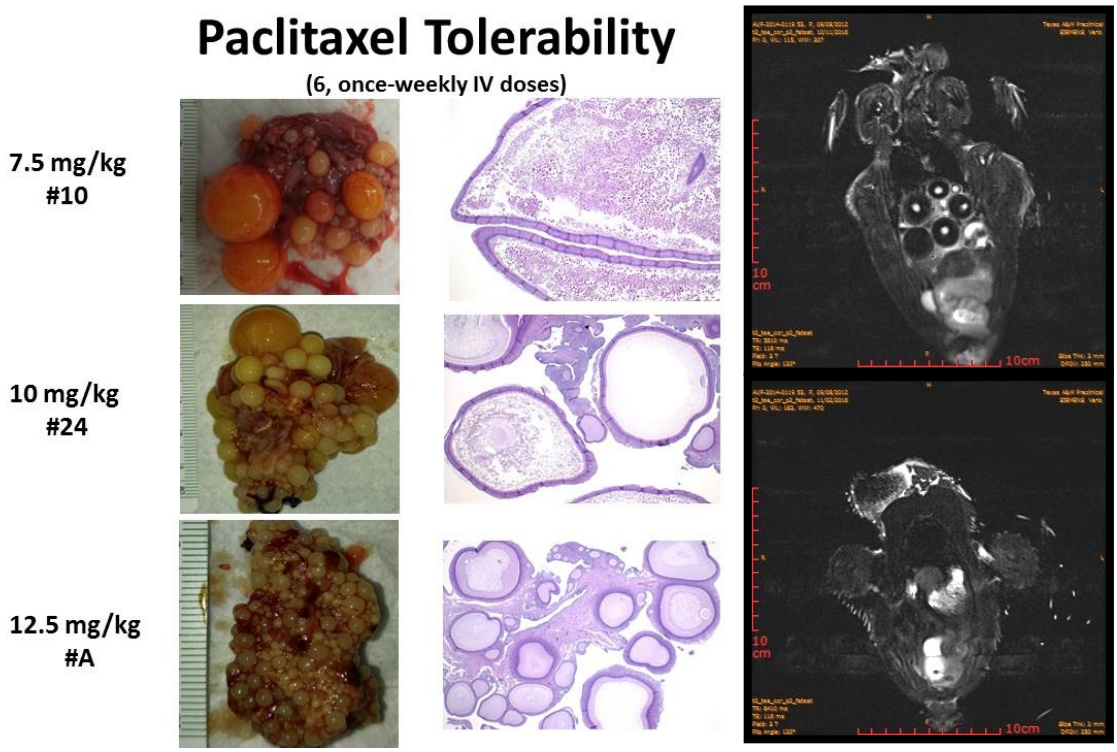

Fig. 13. Ovarian quiescence with paclitaxel. The left panel show ovaries from laying hens receiving once-weekly IV bolus injections of 7.5mg, 10mg, and 12.5mg per kilogram dose paclitaxel for 6 weeks had a dose response of ovarian quiescence, with histologic confirmation. The top right panel is an MRI of hen #53, with an active ovary with clear F1-F5 follicles, whereas the bottom right panel shows no detectable follicles after treatment.

Ovarian changes throughout the study were quite remarkable and occurred in both the cancerous and non-cancerous hens and was attributed to the chemotherapeutic regimen. Sixty percent (6 of 10) hens had a notable shift from an active ovary to a completely quiescent ovary by the end of the study. This was also noted in a prior tolerability study where 1.5-year-old, actively laying hens given paclitaxel for 6 weeks, showed a dose dependent reduction in follicular development over the course of treatment (Fig. 13).

Significant dilation of the ventriculus (gizzard) was noted in three hens, one of which was shown to have cancer in nearby tissue of the esophagus and proventriculus (#127). This ventriculus dilation was suspected to occur from poor gastrointestinal clearance due to the presence of cancer that had metastasized to the intestines. In all three cases, the severity of the ventriculus dilation was reduced throughout the 6-week treatment. This reduction in ventriculus dilation can be clearly seen on MRI (Fig. 14).

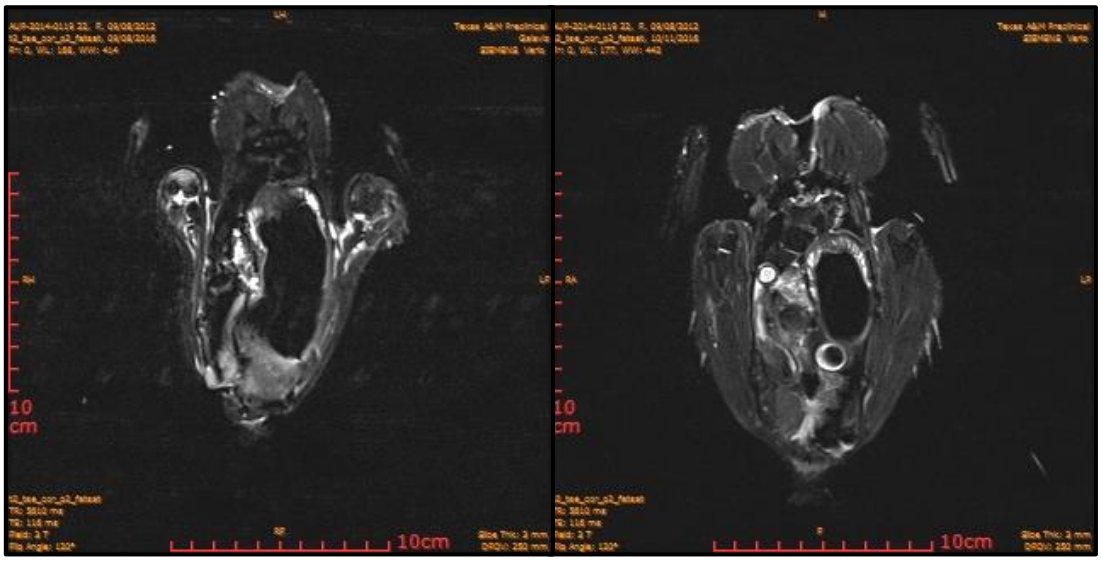

Fig. 14. Reduction in ventriculus dilation with treatment. Hen#22 shown on left prior to paclitaxel treatment, on right, after 3 weeks of treatment the ventriculus dilation is greatly reduced.

These results show proof of concept of a non-invasive measure that can be used for identification of coelomic cancer and assessment of changes due to treatment. These results provide further validation of the laying hen model for preclinical studies of ovarian cancer.

Discussion

The treatment goal of human ovarian cancer is dependent on the stage of cancer. If fortunate enough to be diagnosed at an early stage (occurs in only 20% of cases), the focus of treatment is progression free survival, whereas, in late stage diagnosis, the objective is palliative care for control of symptoms and pain relief [43]. Our study was 6 weeks in duration and ended with necropsy of the laying hens therefore, prolonging their life span could be the focus of future studies. The cohort had a 70% survival rate to the end of study; one hen (#22) died under anesthesia during the MRI at week 3, and the remaining two laying hens (#73, #75) died under anesthesia during the imaging process at week 6. All three hens who died had late stage cancer. Most treated hens appeared physically healthier, better groomed, and more active for the first 5 weeks of treatment, which could be attributed to improvements due to chemotherapy, fluid administration and/or topical mite treatment during the study. Some of the observed improvements were likely due to reduced ascites and ovarian and follicular atrophy resulting in more room within the coelomic cavity allowing for improved gastrointestinal clearance and comfort of the hens throughout treatment. In the 6^{th} and final week, all hens were given daily supportive care (30ml saline subcutaneously and 0.5ml Nutrical) during their final week due to observations of weight loss in the final week in this and previous tolerability studies. Caution should be used when administering weekly dosing of a paclitaxel regimen as the 6^{th} dose seems to be the most difficult for the birds to tolerate, however, with monitoring and care, the weight loss is manageable (Fig. 15).

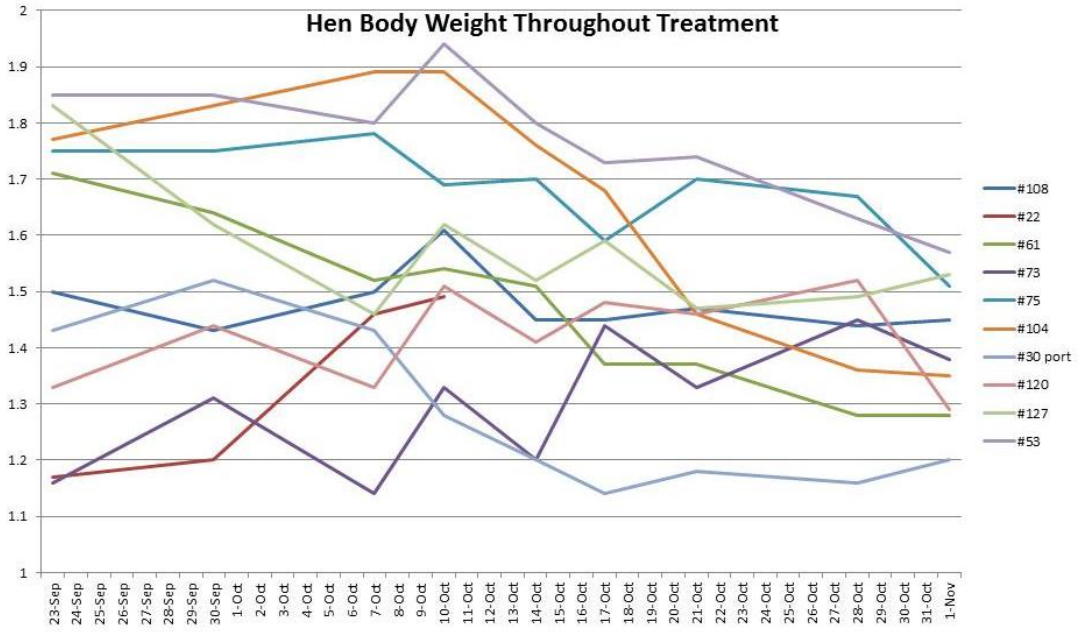

Fig. 15. Body weights of hens throughout the paclitaxel treatment. In the final week, hens were supplemented with daily subcutaneous fluids and oral gavage of 0.5ml nutrical caloric supplement to aid in weight loss.

Diagnosis of cancer within the coelomic cavity over a single image collection timepoint was not an effective method to establish accurate stage of cancer accurately, but images correlated well with the presence of cancerous versus non-cancerous hens, especially over multiple days of imaging. Staging multiple image collection sets allows for an overall reduction in false results based on breathing artifacts, movement, and fluid flux. We found that position of the bird was important for the tolerability of the imaging procedure. Initially, hens were laid on their backs for imaging, however, due to their cancerous condition and the presence of ascites fluid, we observed an upright “roosting”

position was optimal for hens. This was accomplished by using cut out baskets which allowed for monitoring equipment connection and a normal resting position in which the birds could breathe comfortably (Fig. 16).

Fig. 16. Basket for proper positioning during imaging. Hen in "roosting" position for imaging, which accommodated connection of the monitoring devices and optimal breathing for hens with ascites fluid.

The MRI provided the most informative measure of disease of all imaging modalities used, however, both CT and PET helped clarify findings from MRI. In the future, it may be important to perform dynamic imaging studies to rule out effects of fluid shifts (such as cloaca reflux) causing the appearance of areas of high ^{18}F-FDG uptake. Additionally, more selective radiolabeled markers may be useful in targeting specific areas of ovarian tissues for future studies.

The use of MRI in laying hens, and potentially other birds, was useful for visualizing F1-F5 follicles to determine normal reproductive activity compared to abnormal activity. This study showed that birds with active follicles became reproductively quiescent in response to treatment with paclitaxel, which is an important finding as these hens are hard wired to lay eggs constantly. MRI also proved useful for visualizing intestinal dilatation which improved across treatment in each of the hens affected with this abnormality. MRI further showed its utility for detecting and characterizing the pancreas which has been informally reported to be impossible. Results of this study suggest that these imaging modalities have great diagnostic and prognostic potential in birds and can be used serially and safely in future studies.

Conclusion

The results of this study show proof of concept of a non-invasive technique that can be used for identification of coelomic cancer and assessment of changes due to treatment further validating the laying hen as an animal model for preclinical studies of ovarian cancer. Additionally, the results of this study revealed an ability to correlate neoplasia and reproductive disease using non-invasive imaging modalities, as well as the tolerability and efficacy of a 6-week 10mg/kg paclitaxel regimen in laying hens. These are valuable tools for the advancement of ovarian cancer research and use by avian clinicians in the treatment of client owned birds. Our work with paclitaxel may provide a novel platform to expand knowledge of its mechanism of action, explore the potential for improving cancer treatment regimens, investigate its role in off-label uses where the

induction of ovarian quiescence may be beneficial or therapeutic in human medicine (fertility protection during chemotherapy, etc.).

CHAPTER IV

FUTURE DIRECTIONS AND CONCLUSIONS

Future Directions

Future directions of our studies may include combining serial MRI imaging and laparoscopic techniques for following the progression of ovarian cancer and metastasis in the laying hen. Utilization of the self-sealing port after diagnosis allows for drug delivery into the coelomic cavity to mimic intraperitoneal dosing in a human. Further, the port could be used to follow ovarian changes in the hen after diagnosis with MRI.

The port allows for repeated access to the ovary or coelomic cavity for visualization or biopsy, but may also be useful in the delivery of directed nanoparticles that could aid in identification and imaging of ovarian cancer and metastasis within the hen. Metastasis is a key problem in ovarian cancer and further research needs to be done to determine the early events leading up to the shedding and spread of cancer cells from the primary tumor throughout the peritoneal cavity in humans and correspondingly, ovarian cancer metastasis within the coelomic cavity of hens.

Improved methods are needed for identifying cancerous hens at an early stage, as is true for human ovarian cancer patients. The earlier the diagnosis, the greater a patient's odds are for survival, and in the laying hen, the earlier cancer can be identified the greater the ability to design studies with fewer animal numbers and greater statistical significance.

Those methods could involve identification of biomarkers in the blood, or protein expression in ovarian biopsies collected laparoscopically through the port.

The data generated from MRI and PET/CT holds significant information that could benefit many other areas of study that were not the focus of this ovarian study (bone, renal, hepatic, cardiac, pancreatic etc.). Little information exists on aged populations of laying hens because they routinely do not survive past 2 years in commercial production systems. MRI studies done in birds, much less 4.5-year-old laying hens, is sparse, and the data we collected could benefit multiple areas of radiologic training and research. Finding the best outlet for this data will be a pursued as a priority.

Our findings of induction of ovarian quiescence spurs many scientific questions about paclitaxel's use. There is a great need for optimization of the standard combinatorial therapy using paclitaxel and platinum drugs for treatment of human ovarian cancer enabling maximum benefit for the patient. Our work may provide the platform needed to further this investigation, as well as aid in the understanding of the full mechanism of action of this drug; it is suspected paclitaxel is doing more than just disrupting mitosis, which is seen by tumor regression, and not just halting growth. The potential exists that there may be off-label uses for paclitaxel in areas of human medicine where ovarian quiescence may provide therapeutic benefit such as a possible treatment to offer ovarian protection for future fertility post-cancer treatment.

Conclusion

The fact that decades of ovarian cancer research has not yielded significant improvements in incidence and mortality rates of women with ovarian cancer, suggests the need for better approaches in studies of ovarian cancer. I believe that the tools currently being employed in research on ovarian cancer are insufficient to allow scientists and clinicians to conquer this deadly disease. The goal of this research project was to highlight an animal model that is not used frequently, but shows huge potential for offering insights into spontaneously occurring human ovarian cancer. By further validating the laying hen model of ovarian cancer and showing its relevance, research-friendly nature, and ability to serve as a preclinical model, we hope to draw attention to the laying hen as a platform for many future studies that will advance the field of ovarian cancer research, and potentially many other areas of avian and human medicine.

REFERENCES

1. Kurian AW, Balise RR, McGuire V, Whittemore AS. Histologic types of epithelial ovarian cancer: have they different risk factors? *Gynecol Oncol*. 2005;96(2):520-530. doi:10.1016/j.ygyno.2004.10.037.

2. Cho KR, Shih I-M. Ovarian cancer. *Annu Rev Pathol*. 2009;4:287-313. doi:10.1146/annurev.pathol.4.110807.092246.

3. Fathalla MF. Incessant ovulation and ovarian cancer - a hypothesis re-visited. *Facts, Views Vis ObGyn*. 2013;5(4):292-297 http://www.ncbi.nlm.nih.gov/pubmed/24753957.

4. CDC - Ovarian Cancer Statistics. https://www.cdc.gov/cancer/ovarian/statistics/. Accessed January 9, 2017.

5. Ovarian Cancer | American Cancer Society. https://www.cancer.org/cancer/ovarian-cancer.html. Accessed January 9, 2017.

6. Ahmed N, Stenvers KL. Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research. *Front Oncol*. 2013;3:256. doi:10.3389/fonc.2013.00256.

7. Vanderhyden BC, Shaw TJ, Ethier J-F. Animal models of ovarian cancer. *Reprod Biol Endocrinol*. 2003;1:67. doi:10.1186/1477-7827-1-67.

8. Khaled WT, Liu P. Cancer mouse models: past, present and future. *Semin Cell Dev Biol*. 2014;27:54-60. doi:10.1016/j.semcdb.2014.04.003.

9. Hasan N, Ohman AW, Dinulescu DM. The promise and challenge of ovarian cancer models. *Transl Cancer Res*. 2015;4(1):14-28. doi:10.3978/j.issn.2218-676X.2015.01.02.

10. Barua A, Bitterman P, Abramowicz JS, Dirks AL, Bahr JM, et al. Histopathology of tumors in laying hens. *Int J Gynecol Cancer*. 2009;19(4):531-539. doi:10.1111/IGC.0b013e3181a41613.

11. Murdoch WJ, Martinchick JF. Oxidative damage to DNA of ovarian surface epithelial cells affected by ovulation: carcinogenic implication and chemoprevention. *Exp Biol Med (Maywood)*. 2004;229(6):546-552. http://www.ncbi.nlm.nih.gov/pubmed/15169974.

12. McGowan JP. The biological significance of ovarian tumors in the fowl. *J Cancer Res*. 1930;14(4).

13. Papasolomontos PA, Appleby EC, Mayor OY. Pathological findings in condemned chickens: a survey of 1,000 carcases. *Vet Rec*. 1969;84(17):459-464. http://www.ncbi.nlm.nih.gov/pubmed/5387897.

14. Fredrickson TN. Ovarian tumors of the hen. *Environ Health Perspect*. 1987;73:35-51. http://www.ncbi.nlm.nih.gov/pubmed/3665870.

15. Eilati E, Bahr JM, Hales DB. Long term consumption of flaxseed enriched diet decreased ovarian cancer incidence and prostaglandin E_2 in hens. *Gynecol Oncol*. 2013;130(3):620-628. doi:10.1016/j.ygyno.2013.05.018.

16. Eilati E, Pan L, Bahr JM, Hales DB. Age dependent increase in prostaglandin pathway coincides with onset of ovarian cancer in laying hens. *Prostaglandins Leukot Essent Fatty Acids*. 2012;87(6):177-184. doi:10.1016/j.plefa.2012.09.003.

17. Gilbert AB, Wood-Gush DG. A technique for the fistulation of the hen's oviduct through the abdominal wall, with recovery of the ovum. *J Reprod Fertil*. 1963;5:451-453. http://www.ncbi.nlm.nih.gov/pubmed/13947798.

18. Fathalla MF. Incessant ovulation--a factor in ovarian neoplasia? *Lancet (London, England)*. 1971;2(7716):163. http://www.ncbi.nlm.nih.gov/pubmed/4104488.

19. Giles JR, Elkin RG, Trevino LS, Urick ME, Ramachandran R, Johnson PA. The restricted ovulator chicken: a unique animal model for investigating the etiology of ovarian cancer. *Int J Gynecol Cancer*. 2010;20(5):738-744. http://www.ncbi.nlm.nih.gov/pubmed/20973263..

20. Treviño LS, Buckles EL, Johnson PA. Oral contraceptives decrease the prevalence of ovarian cancer in the hen. *Cancer Prev Res (Phila)*. 2012;5(2):343-349. doi:10.1158/1940-6207.CAPR-11-0344.

21. Carver DK, Barnes HJ, Anderson KE, Petitte JN, Whitaker R, et al. Reduction of ovarian and oviductal cancers in calorie-restricted laying chickens. *Cancer Prev Res*. 2011;4(4):562-567. doi:10.1158/1940-6207.CAPR-10-0294.

22. Scholler N, Urban N. CA125 in ovarian cancer. *Biomark Med*. 2007;1(4):513-523. doi:10.2217/17520363.1.4.513.

23. Jackson E, Anderson K, Ashwell C, Petitte J, Mozdziak PE. CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. *Gynecol Oncol*. 2007;104(1):192-198. doi:10.1016/j.ygyno.2006.07.024.

24. Hakim AA, Barry CP, Barnes HJ, Anderson KE, Petitte J, et al. Ovarian adenocarcinomas in the laying hen and women share similar alterations in p53, ras, and HER-2/neu. *Cancer Prev Res*. 2009;2(2).

25. Bell D, Berchuck A, Birrer M, Chien J, Cramer D, et al. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474(7353):609-615. doi:10.1038/nature10166.

26. Ansenberger K, Zhuge Y, Lagman JAJ, Richards C, Barua A, et al. E-cadherin expression in ovarian cancer in the laying hen, *Gallus domesticus*, compared to human ovarian cancer. *Gynecol Oncol*. 2009;113(3):362-369. doi:10.1016/j.ygyno.2009.02.011.

27. Akutagawa N, Nishikawa A, Iwasaki M, Fujimoto T, Teramoto M, et al. Expression of vascular endothelial growth factor and E-cadherin in human ovarian cancer: association with ascites fluid accumulation and peritoneal dissemination in mouse ascites model. *Jpn J Cancer Res*. 2002;93(6):644-651. doi:10.1111/J.1349-7006.2002.TB01302.X.

28. Urick ME, Giles JR, Johnson PA. VEGF expression and the effect of NSAIDs on ascites cell proliferation in the hen model of ovarian cancer. *Gynecol Oncol*. 2008;110(3):418-424. doi:10.1016/j.ygyno.2008.05.018.

29. Yu Y, Edassery SL, Barua A, Abramowicz JS, Bahr JM, et al. The hen model of human ovarian cancer develops anti-mesothelin autoantibodies in response to mesothelin expressing tumors. doi:10.1186/1757-2215-4-12.

30. Bose CK, Mukhopadhyay A. Claudin and ovarian cancer. *J Turkish Ger Gynecol Assoc*. 2010;11(1):48-54. http://www.ncbi.nlm.nih.gov/pubmed/24591894.

31. Seo HW, Rengaraj D, Choi JW, Ahn SE, Song YS, et al. Claudin 10 is a glandular epithelial marker in the chicken model as human epithelial ovarian cancer. *Int J Gynecol Cancer*. 2010;20(9):1465-1473. http://www.ncbi.nlm.nih.gov/pubmed/21370593.

32. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer*. 2002;2(3):161-174. doi:10.1038/nrc745.

33. Choi JW, Ahn SE, Rengaraj D, Seo HW, Lim W, et al. Matrix metalloproteinase 3 is a stromal marker for chicken ovarian cancer. *Oncol Lett*. 2011;2(6):1047-1051. doi:10.3892/ol.2011.391.

34. Lim W, Song G. Pivotal roles for hormonally regulated expression of the HEP21 gene in the reproductive tract of chickens for oviduct development and in ovarian

carcinogenesis. *Domest Anim Endocrinol*. 2014;48:136-144. doi:10.1016/j.domaniend.2014.03.003.

35. Lim W, Kim J-H, Ahn SE, Jeong W, Kim J, et al. Avian SERPINB11 gene: a marker for ovarian endometrioid cancer in chickens. *Exp Biol Med*. 2012;237(2):150-159. doi:10.1258/ebm.2011.011250.

36. Qiu X, Cheng J-C, Chang H-M, Leung PCK. COX2 and PGE2 mediate EGF-induced E-cadherin-independent human ovarian cancer cell invasion. *Endocr Relat Cancer*. 2014;21(4):533-543. doi:10.1530/ERC-13-0450.

37. Rodríguez-Burford C, Barnes MN, Berry W, Partridge EE, Grizzle WE. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. doi:10.1006/gyno.2001.6191.

38. Bradaric MJ, Penumatsa K, Barua A, Edassery SL, Yu Y, et al. Immune cells in the normal ovary and spontaneous ovarian tumors in the laying hen (*Gallus domesticus*) model of human ovarian cancer. Samant R, ed. *PLoS One*. 2013;8(9):e74147. doi:10.1371/journal.pone.0074147.

39. Cancer of the ovary - cancer stat facts. https://seer.cancer.gov/statfacts/html/ovary.html. Accessed January 9, 2017.

40. Vergara D, Merlot B, Lucot J-P, et al. Epithelial–mesenchymal transition in ovarian cancer. *Cancer Lett*. 2010;291(1):59-66. doi:10.1016/j.canlet.2009.09.017.

41. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7-30. doi:10.3322/caac.21387.

42. Ansenberger K, Richards C, Zhuge Y, et al. Decreased severity of ovarian cancer and increased survival in hens fed a flaxseed-enriched diet for 1 year. *Gynecol Oncol*. 2010;117(2):341-347. doi:10.1016/j.ygyno.2010.01.021.

43. Ozols RF. Treatment goals in ovarian cancer. *Int J Gynecol Cancer*. 2005;15(s1):3-11. doi:10.1111/j.1525-1438.2005.15351.x.