AN ABSTRACT OF THE DISSERTATION OF

Khawla Hussin Zwida for the degree of Doctor of Philosophy in Animal Sciences presented on April 4, 2022.

Title: Luteinizing Hormone Receptor Expression in Canine Lymphoma, Mastocytoma, and Hemangiosarcoma

---

Michelle Anne Kutzler

Luteinizing hormone (LH) stimulates the secretion of gonadal steroid hormones (testosterone in males and estrogen/progesterone in females) and is under negative feedback from gonadal hormones. In gonad-intact dogs, LH is secreted in low amplitude pulses of concentrations under 1 ng/mL; except for the day of the LH surge in females when the LH concentration rises between 1-2 ng/mL. However, in gonadectomized dogs, there is a loss of hormonal negative feedback, resulting in continuous supraphysiologic LH concentrations up to 20 times higher than measured for intact dogs. Although LH is mainly considered to be a reproductive hormone, there are dozens of non-reproductive tissues that contain LH/hCG receptors (LHCGRs). In dogs, gonadectomy increases the incidence of several non-reproductive long-term disorders including cancer. The purpose of this thesis research was to determine and demonstrate LHCGR expression in three canine cancers (lymphoma, mastocytoma, hemangiosarcoma). Additional research with hemangiosarcoma was conducted to

examine the effect of LHCGR activation on cell count. It was hypothesized that LHCGR would be expressed in these tissues and that differences would exist between intact and gonadectomized dogs. In addition, it was hypothesized that LH/hCG receptor activation would induce cell proliferation. This thesis research found: 1) the increased risk of lymphoma in spayed and neutered dogs is not related to body weight, sex, immunophenotype, or tumor stage; 2) mastocytoma from gonadectomized dogs had a significantly higher percentage of LHCGR -positive mastocytoma cells compared to mastocytoma from intact dogs; and 3) there was a significant effect of LHCGR agonist (human chorionic gonadotropin (hCG) and canine luteinizing hormone (cLH)) exposure time on canine splenic hemangiosarcoma cell count. These results allow for a better understanding of the relationship between spaying or neutering and the development of cancer in dogs. The long-term goal of these experiments will be to provide support for a clinical trial to reduce circulating LH concentrations as an adjunctive treatment for these canine cancers.

Luteinizing Hormone Receptor Expression in Canine Lymphoma, Mastocytoma, and Hemangiosarcoma

by

Khawla Hussin Zwida

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented April 4, 2022

Commencement June 2023

Doctor of Philosophy dissertation of Khawla Hussin Zwida presented on April 4, 2022

APPROVED:

Major Professor, representing Animal and Rangeland Sciences

Head of the Department of Animal and Rangeland Sciences

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Khawla Hussin Zwida, Author

ACKNOWLEDGEMENTS

I would like first to express thanks to my advisor, Dr. Michelle Kutzler for her support and guidance throughout my graduate career. I have learned many things since I became Dr. Kutzler's student. She spends very much time instructing me and providing useful suggestions. The idea of how to think outside of the box has truly helped me become the researcher I hope to be in near future. She always pushes me to accomplish so much in a short amount of time. I would also like to thank different people at Oregon State University. Many thanks to my fellow graduate student Hunter Ford for always helping when I asked.

I also want to thank the Ministry of Higher Education and Scientific Research in Libya for graduate school financial assistance and the American Kennel Club Canine Health Foundation for funding the hemangiosarcoma research.

Last and highly important, I owe more than thanks to my husband, Abduallaziz. Without his support, it would be impossible for me to finish college and graduate education seamlessly. I am also grateful to my family members and I cannot thank them enough for their prayers and support during my life. And to my children Maysam, Mohamed, Mayan, my little one Khawla.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

LIST OF APPENDICES

LIST OF APPENDIX FIGURES

DEDICATION

Khawla Hussin Zwida contributed to design, data collection, working on, immunocytochemistry, MTT assay, picture taking, data analysis, manuscripts writing, and editing. Dr. Michelle Kutzler provided oversight, design, and manuscript editing. Dr. Valerio Moccia performed immunohistochemistry, data analysis, and manuscript writing for the mastocytoma research. Dr. Christiane Löhr provided access to archived and graded mastocytoma tissues. Alyssa Vedus performed immunohistochemistry and data collection for the lymphoma research.

CHAPTER 1

INTRODUCTION

1.A. Cancer

1.A.1. What is cancer?

Cancer is the uncontrolled growth of the body's cells that may spread to other parts of the body (Cooper, 2000). Cancer can be benign or malignant. Benign cancers tend to grow slowly, displace, but do not tend to invade the surrounding tissues, and do not spread throughout the body (metastasize). Malignant cancers invade surrounding tissues and metastasize to other parts of the body (Cooper, 2000).

Lymphoma is a malignant cancer of lymphocyte (a type of white blood cell) in circulation or in lymph nodes. Canine lymphoma has been proposed as an animal model to study lymphoma in humans (Teske, 1994; MacEwen, 1990; Zandvliet, 2016). There is a strong association between the geographic distribution in cases of canine lymphoma and the location of waste incinerators, crematoria, polluted sites, and radioactive waste (Pastor et al., 2009; Craun et al., 2020). There appears to be a genetic susceptibility in certain breeds of dogs but any breed or mixed breed of dog can develop lymphoma (Teske et al., 1994). Tumor stage, immunophenotype, tumor grade, and response to chemotherapy are important prognostic factors with respect to total life expectance as well as remission times (Zandvliet, 2016).

Mastocytoma (MCT) is a malignant cancer of mast cells. Mast cells are derived from myeloid stem cells and located in connective tissues, predominantly in the skin and mucosal linings. The proliferation of neoplastic mast cells can lead to both localized and systemic manifestations, termed "mastocytosis" (Ahmed and Jan, 2022).

In dogs, MCT is the most common malignant cutaneous tumors, representing 10-21% of tumors (Villamil et al., 2011). Although the etiopathophysiology of MCT is not completely clear, the microenvironment of neoplastic tissue reveals an increased concentration of interleukins and growth factors (such as luteinizing hormone) that both induce proliferation of mast cells and inhibit mast cell apoptosis (Ahmed and Jan, 2022). Also, mutations in c-Kit may predispose to the occurrence of MCT (Weishaar et al., 2018). In addition, there appears to be a genetic susceptibility in certain breeds of dogs but any breed or mixed breed of dog can develop MCT (Mochizuki et al., 2017)

Hemangiosarcoma (HSA) is a common malignant cancer of dogs affecting endothelial and vascular smooth muscle cells (Moore et al., 2017). In humans, HSA is known as angiosarcoma (Juin and Shelat, 2021). In dogs, splenic HSA is highly aggressive tumor that grows rapidly and metastasizes quickly (Shiu et al., 2011). The etiopathophysiology of canine hemangiosarcomas is unknown, but age breed, and gonad removal are all predisposing factors.

1.A.2. Immunological Response to Cancer.

The canine immune system and immunological response to cancer in general are similar across all mammalian species. The main mechanism for eliminating cancer cells as they develop is with immune effector cells. There are two main types of effector cells: (1) CD8+ cytotoxic T-lymphocytes and (2) natural killer cells. Cytotoxic T-lymphocytes recognize antigenic peptide/class I MHC complexes on the surface of cancer cells via a specific T-cell receptor (Dahan and Reiter, 2012). Natural killer cells exert antibody-dependent cell-mediated cytotoxicity, secrete cytokines, and generate memory natural killer cells (Wu et al., 2020). The tumor-targeting antibodies are

directed against numerous genetic mutations present in cancer cells. The fragment crystallizable region (FcR) on tumor-targeting antibodies specifically engage with the receptors on the NK cells (Dow, 2020; Stadler, 2013).

Despite these immunological responses to cancer cells, some cancers can overcome immune–mediated destruction, especially highly aggressive cancers (Gajewski, 2013). Cancer cells can also co-opt immature immune cells. Myeloid-derived suppressive cells (MDSCs) are used by cancer cells to metastasize, resulting in a loss homeostatic control within the immune system (Ouzounova et al., 2017).

1.B. Cancer in dogs.

Similar to humans, dogs are at risk for developing cancer and some canine cancers are more common than others. In the USA, the most common malignant canine cancers are lymphoma (up to 24%) (Vail et al., 2001), osteosarcoma (up to 85% of all bone tumors in dogs (Poon et al., 2020), mastocytoma, (accounting for 10–21% of all skin tumors) (Villamil et al., 2011), and hemangiosarcoma (0.3 to 2%) (Brown et al., 1985). According to the National Cancer Institute, approximately six million new cancer diagnoses are made in dogs in USA each year (National Cancer Institute, n.d). Even with early diagnosis, half of all canine cancers are not curable (Biller et al., 2016).

The frequency of developing cancer in dogs varies depending upon world location. In the USA where 85% of dogs are spayed and neutered (Cosgrove, 2022), almost 50% of dogs over age ten will develop cancer (Adams et al., 2010; Animal Cancer Foundation, 2014; Bronson, 1982; Dobson, 2013; Vail and MacEwen, 2000).

In China, the incidence of canine cancer is much lower, only 0.3 to 1.7% dogs will develop cancer (Wang et al., 2021). Although pet ownership and routine care are a more recent societal norm in China, less than 5% of dogs in urban regions are spayed/neutered and essentially no dogs in rural regions are spayed/neutered (ICVS, 2021). In Sweden where only 7% of dogs are spayed and 1% of dogs are neutered, the mortality rate from cancer in dogs is only 16% (Bonnett et al., 1997). Similarly in Norway where there are essentially no spayed or neutered dogs, the incidence of mastocytoma and lymphoma were the most common malignancies, while hemangiosarcoma was less than 3% and osteosarcoma was less than 1% (Gamlem et al., 2008).

1.C. Causes of Cancer in Dogs.

There are many predisposing factors for the development of cancer (Wu et al., 2018; Cooper, 2000). These include genetic (breed) factors, environmental carcinogens, age, and gonadal status. Golden Retrievers, Boxers, Bernese Mountain Dogs, and Rottweilers are generally more likely to develop cancer than other breeds (Merck Manuals Staff, 2020). Exposure to herbicides, insecticides, pesticides, and second-hand tobacco smoke can predispose to cancer development (; Moore and Frimberger, 2009; Merck Manuals Staff, 2020). The lawn care herbicide 2,4 - D, paints, asbestos or solvents, as well as radiation and electromagnetic field exposure have been associated with increased risk for canine lymphoma (Moore and Frimberger, 2009). Application of insecticides increased the risk of bladder cancer in Scottish terriers (Moore and Frimberger, 2009). Dogs have been shown to have an increased risk of

developing cancer of the respiratory tract, especially of the lung and nasal cavity, when exposed to coal and kerosene heaters and second-hand tobacco smoke, especially dogs of long - nosed breeds (Moore and Frimberger, 2009).

Although young dogs can develop cancer, cancer occurs more frequently in older dogs due to a weakening immune system. In addition, the rate of cellular mutations increases with age, which increases the risk for cancer formation (Wu et al., 2018). Gonad removal is commonly performed in some parts of the world (including USA) to prevent overpopulation. In female dogs, gonad removal prior to their first estrous cycle can also significantly reduce the incidence of mammary cancer (Urfer and Kaeberlein, 2019). However, early gonad removal can increase the incidence of many other cancers including lymphoma, osteosarcoma, hemangiosarcoma, mastocytoma, and transitional cell carcinoma (Zwida and Kutzler, 2016).

1.D. Luteinizing hormone and LH/chorionic gonadotropin receptor

Luteinizing hormone (LH) is a heterodimeric gonadotrophic hormone produced and released by cells in the anterior pituitary gland. During the canine estrous cycle, the acute rise of LH ("LH surge") triggers ovulation, which leads to the development of the corpus luteum. Daily tonic release of LH stimulates luteal cells to produce progesterone in females and interstitial cells to produce testosterone in males. (Belanger et al., 2019; Ezcurra and Humaidan, 2014).

Luteinizing hormone exerts its action by binding the transmembrane LH/chorionic gonadotropin receptor (LHCGR). The LHCGR belongs to a family of rhodopsin-like seven transmembrane G-protein-coupled receptors (GPCRs). The LHCGR binds to two homologous gonadotropins: pituitary-derived LH

and placentally-derived human chorionic gonadotropin (hCG). During early embryonic development, LHCGR activation is essential for fetal interstitial cell maturation and testosterone secretion to support male sexual differentiation. Postnatally, LHCGR activation is required for male and female reproductive steroid hormone synthesis and gamete physiology (Puett and Narayan, 2003).

Ligand binding to LHCGR activates membrane-bound adenylyl cyclase, which increases intracellular cyclic adenosine monophosphate (cAMP) levels. Then cAMP acts as a second messenger to activate protein kinase A, which can activate via phosphorylation numerous transcription factors (e.g., cAMP response element binding protein (CREB), co-regulatory CREB-binding protein, steroidogenic acute regulatory protein (StAR). The latter of these transcription factors (StAR) mediates delivery of cholesterol to mitochondria as well as activates cytochrome P450 side chain cleavage, 7α-hydroxylase/17,20-lyase and 17β-hydroxysteroid dehydrogenase 3 (Puett and Narayan, 2003) (**Figure 1.1A**).

At high ligand concentrations and high receptor densities, LHCGR can also activate the phospholipase C beta (PLC-β) /inositol 1,4,5-triphosphate (IP3) signaling pathway. Phospholipase C beta cleaves IP3 from phosphatidylinositol 4,5-bisphosphate (PIP2) with the retention of 1,2-diacylg1ycero1 (DAG) in the membrane. Inositol 1,4,5-triphosphate also serves as a second messenger to promote calcium mobilization. Another second messenger, DAG works with phosphatidylserine to activate protein kinase C. Activation of IP3 and PIP2 also initiate the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) or protein kinase B (PKB) signaling pathway, which mediates cell proliferation, anti-apoptosis, differentiation,

tumorigenesis and angiogenesis (Puett and Narayan, 2003; Choi and Smitz, 2014) (**Figure 1.1B).**

1.E. Post-menopausal Women

In post-menopausal women, circulating LH concentrations are 5-10 times higher than during the follicular or luteal phase of the reproductive cycle (Brodowska et al., 2012; Schalch et al., 1968). It is of interest to note that hypothyroidism affects 10-15% of women after menopause (Giri et al, 2014). Because of the endocrine similarities between post-menopausal women gonadectomized dogs, spayed and neutered dogs could serve as a good biological model for studying disorders related to high LH concentrations.

1.F. Gonadectomized Dogs.

In the normal adult mammal, the hypothalamus secretes gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary gland to release luteinizing hormone (Vadakkadath and Atwood, 2005). Luteinizing hormone (LH) stimulates the secretion of gonadal steroid hormones (testosterone in males and estrogen/progesterone in females). These gonadal steroid hormones then provide negative feedback to the hypothalamus and anterior pituitary to decrease the secretion of GnRH and LH, respectively. However, in the gonadectomized mammal, there is no negative feedback, which results in supraphysiologic circulating concentrations of LH (**Figure 1.2**). In gonadectomized dogs, LH concentrations are more than thirty times the concentrations found in normal adult dogs (Beijerink et al., 2007). During homeostasis, responses to

high LH concentrations are limited by desensitization or down-regulation of LHCGR. However, in gonadectomized dogs, sustained supraphysiologic concentrations LH stimulates LHCGR expression (Ettinger et al., 2018; Li and Kutzler, 2020).

Although LHCGR activation is mainly discussed relative to its reproductive functions, LHCGR is present in numerous non-reproductive tissues in dogs including the skin (Welle et al., 2006), bladder and urethra (Ponglowhaphan et al., 2007), musculoskeletal tissues (ligaments, synovia, subchondral bone) (Kiefel and Kutzler, 2016), thyroid gland (Zwida and Kutzler, 2019), and adrenal cortex (Galac et al., 2010). Activation of LHCGR in the canine skin significantly alters the anagen-to-telogen ratio within the first year following gonad removal, inhibiting shedding by interfering with the hair growth cycle (Reichler et al, 2008). In incontinent spayed female dogs, LHCGR activation stimulates the release of nitric oxide within the bladder smooth muscle cells, resulting in urethral sphincter mechanism incompetence (Reichler, 2010). In addition, LHCGR expression has been reported in several neoplastic canine tissues including lymphoma (Ettinger et al., 2019), hemangiosarcoma (Zwida and Kutzler, 2016), and adrenocortical tumor (Galac et al., 2010). In other species, high LHCGR expression has been linked to cancer in the lung (Abdelbaset et al., 2017), endometrium (Noci et al., 2008), breast (Sanchez et al., 2016), and lymphoid systems (Abdelbaset et al., 2016). The effect of continuous and supraphysiologic LH concentrations had not previously been considered a predisposing factor for canine cancer. However, work in our laboratory has demonstrated that LHCGR activation results in dose-dependent cell proliferation in isolated T-lymphoma cells (Flint et al., 2019).

The purpose of the current research was to evaluate three cancers (lymphoma, mastocytoma, hemangiosarcoma) that occur more commonly in spayed and neutered dogs. It was hypothesized that LHCGR would be expressed in the canine neoplastic tissue and at a higher level of expression in spayed and neutered dogs. Additionally, it was hypothesized that activation of LHCGR in isolated canine hemangiosarcoma cells would increase cell proliferation in vitro. This research will allow for a better understanding of the relationship between spaying or neutering and the development of long-term health risks of gonadectomy in dogs.

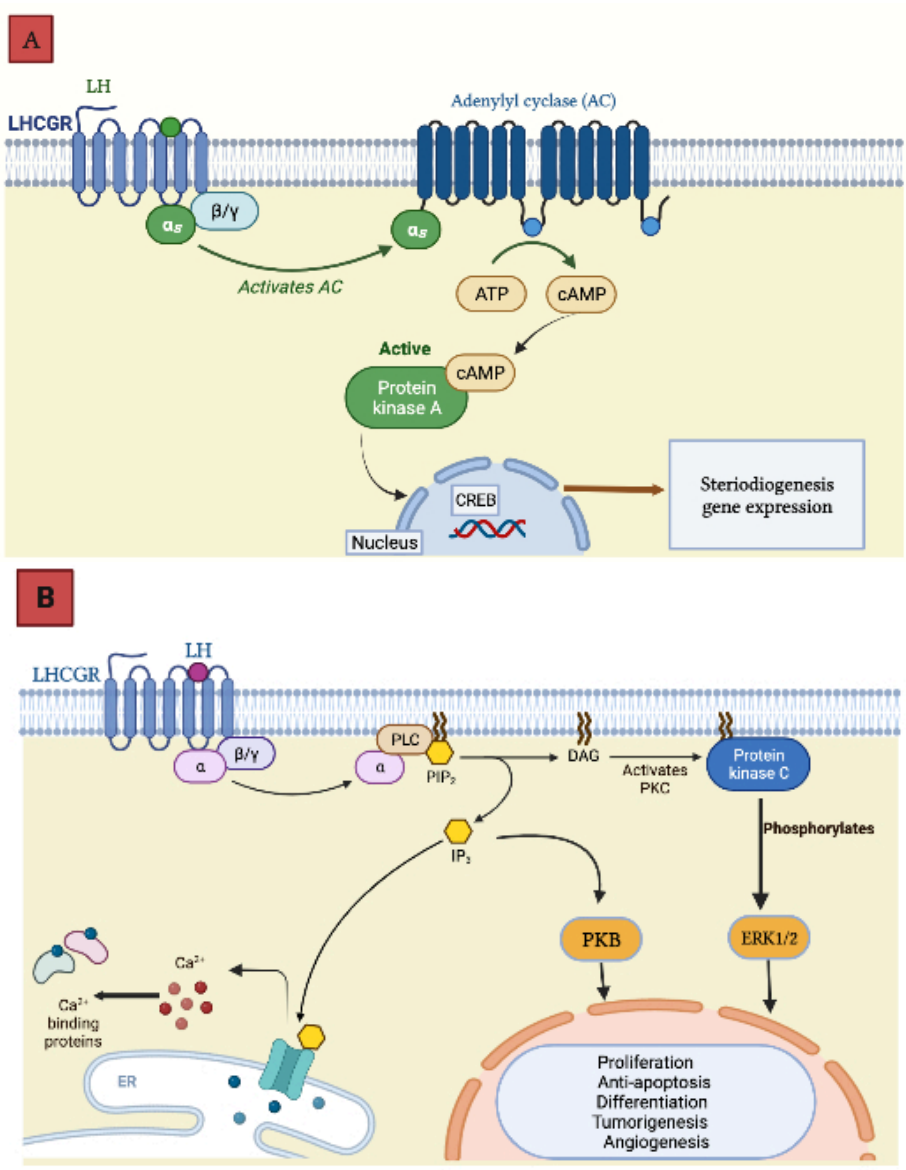


Figure 1.1. Signaling pathways mediated by the luteinizing hormone/ chorionic gonadotropin receptor (LHCGR). A. The cyclic adenosine monophosphate (cAMP) pathway. B. The phospholipase C/inositol phosphate (PLC/IP) signaling pathway. PKB: Protein kinase B, ERK: Extracellular Signal-regulated Kinases, ER: Endoplasmic Reticulum, Ca2+: Calcium, PKC: Protein kinase C, DAG: Diacylglycerol, PIP2: Phosphatidylinositol 4,5-bisphosphate, CREB: cAMP Response Element-Binding Protein, AC: Adenylale Cyclase. Figure created by Khawla Zwida.

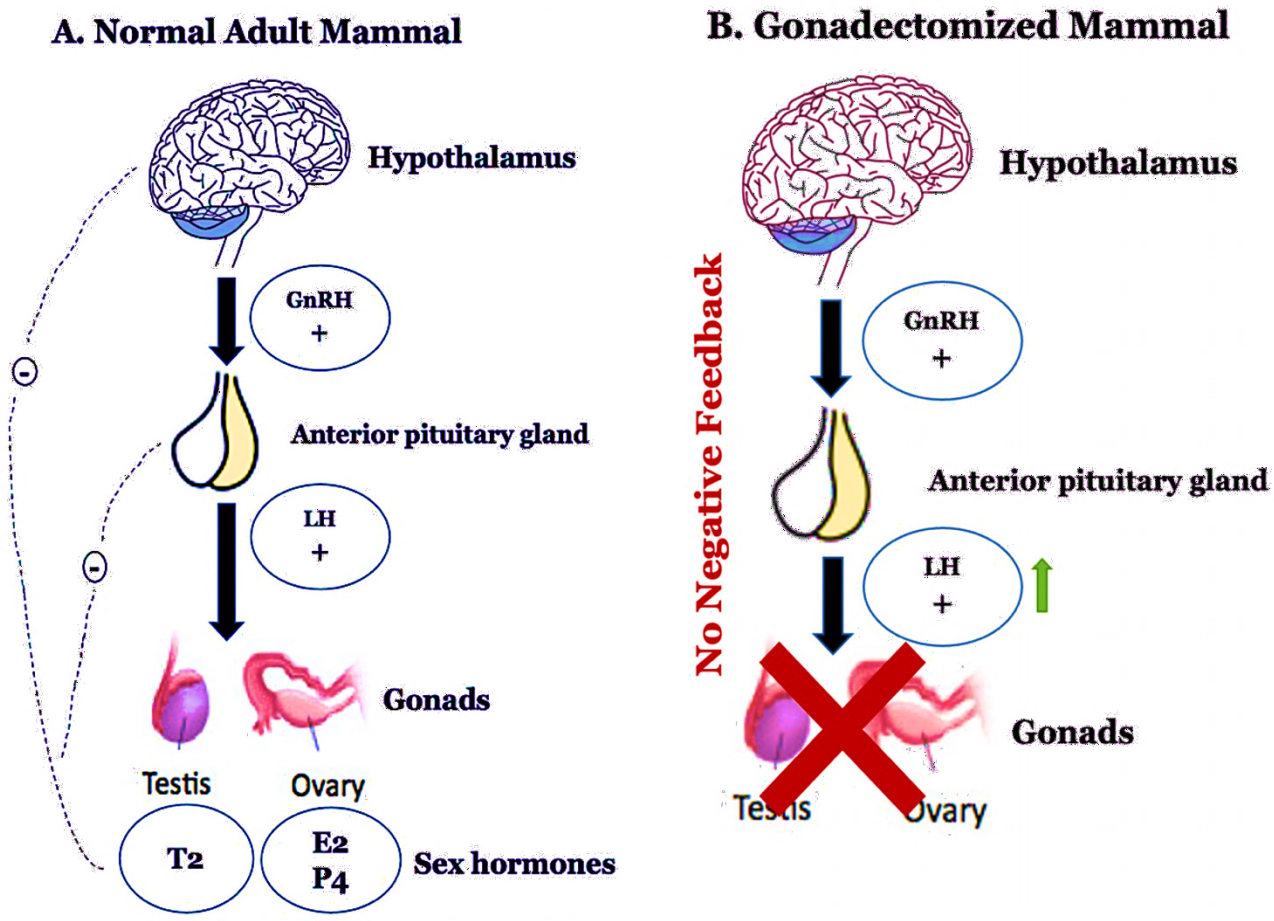


Figure 1.2. A: In the normal adult mammal, the hypothalamus secretes gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary gland to release of luteinizing hormone. Luteinizing hormone (LH) stimulates the secretion of gonadal steroid hormones (testosterone (T2) in males and estrogen (E2)/progesterone (P4) in females). These gonadal steroid hormones then negatively feedback to the hypothalamus and anterior pituitary to decrease the secretion of GnRH and LH, respectively. **B**: In the gonadectomized mammal, there is no negative feedback, which results in supraphysiologic circulating concentrations of LH.

1.2. References

Adams VJ, Evans KM, Sampson J, Wood JLN. (2010). Methods and mortality results of a health survey of purebred dogs in the UK. J Small Anim Pract, 51, 512–524.

Abdelbaset-Ismail A, Borkowska S, Janowska-Wieczorek A, et al. (2016). Novel evidence that pituitary gonadotropins directly stimulate human leukemic cells-studies of myeloid cell lines and primary patient AML and CML cells. Oncotarget, 7(3), 3033–3046.

Abdelbaset-Ismail A, Pedziwiatr D, Schneider G, et al. (2017). Sex hormones enhance the pro-metastatic potential of human lung cancer cells by downregulating the intracellular expression of heme oxygenase-1. Int J Oncol, 50(1): 317-328.

Ahmed A, Jan A. (2022). Mastocytoma. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538252/

Animal Cancer Foundation. Home page. (2014). Available from: http://www.acfoundation.org. Accessed on April 1, 2014.

Beijerink NJ, Buijtels JJ, Okkens AC, Kooistra HS, Dieleman SJ. (2007). Basal and GnRH-induced secretion of FSH and LH in anestrous versus ovariectomized bitches. Theriogenology. 2007 Mar 15;67(5):1039-45. doi: 10.1016/j.theriogenology.2006.12.003

Belanger J, Tremblay C, Davis A, Arnocky S. (2019). Luteinizing hormone. In: Shakelford T, Weekes-Shackelford V (eds), Encyclopedia of Evolutionary

Psychological Science. Springer, Cham. Doi: 10.1007/978-3-319-16999-6_1812-1

Biller B, Berg J, Garrett L, et al. (2016). 2016 AAHA Oncology Guidelines for Dogs and Cats. JAAHA, 52(4), 181–204.

Bonnett BN, Egenvall A, Olson P, et al. (1997). Mortality in insured Swedish dogs: rates and causes of death in various breeds. Vet Rec, 141(2), 40–44.

Brodowska A, Laszczyńska, MJ, Masiuk M, et al. (2012). Analysis of pituitary gonadotropin concentration in blood serum and immunolocalization and immunoexpression of follicle stimulating hormone and luteinising hormone receptors in ovaries of postmenopausal women. Histo histopathol, 27(2), 241–248.

Bronson RT. (1982). Variation in age at death of dogs of different sexes and breeds. Am J Vet Res, 43,2057–2059.

Brown NO, Patnaik AK, MacEwen EG. 1985. Canine hemangiosarcoma: retrospective analysis of 104 cases. J Am Vet Med Assoc, 186(1), 56-58.

Choi J, Smitz J. (2014). Luteinizing hormone and human chorionic gonadotropin: origins of difference. Mol Cell Endocrinol, 383, 203–13.

Cooper GM. (2000). The Cell, A Molecular Approach, 2nd ed. Sunderland, MA: Sinauer Associates,. The Development and Causes of Cancer. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9963/ Accessed on: June 27, 2022.

Cosgrove N. (2022). How many dogs and cats are neutered in the US? Statistics to know in 2022. Available at: https://petkeen.com/cat-dog-neuter

statistics1In_the_US_85_of_dogs_have_been_spayed_or_neutered Accessed on: June 27, 2022.

Craun K, Ekena J, Sacco J, et al. (2020). Genetic and environmental risk for lymphoma in boxer dogs. Journal of veterinary internal medicine, 34(5), 2068–2077.

Dahan R, Reiter Y. (2012). T-cell-receptor-like antibodies - generation, function and applications. Expert Rev Mol Med,14, e6. doi:10.1017/erm.2012.2

Dobson JM. (2013). Breed-predispositions to cancer in pedigree dogs. ISRN Vet Sci, doi:10.1155/2013/941275. 2013

Dow S. (2020). A role for dogs in advancing cancer immunotherapy research. Front. Immunol. 10:2935. doi:10.3389/fimmu.2019.02935

Ettinger AM, Gust SK, Kutzler MA. (2019). Luteinizing hormone receptor expression by nonneoplastic and neoplastic canine lymphocytes. Am J Vet Res, 80(6), 572-577.

Ezcurra D, Humaidan P. (2014). A review of luteinising hormone and human chorionic gonadotropin when used in assisted reproductive technology. Reprod Biol Endocrinol, 12, 95. doi: 10.1186/1477-7827-12-95

Flint C, Gust S, Kutzler M. (2019). Luteinizing hormone receptor mediated proliferation of isolated canine T lymphoma cells. Clin Theriogenology, 11, 477- 24.

Galac S, Kars VJ, Klarenbeek S, et al. (2010). Expression of receptors for luteinizing hormone, gastric-inhibitory polypeptide, and vasopressin in normal adrenal glands and cortisol-secreting adrenocortical tumors in dogs. Domest Anim Endocrinol, 39(1), 63-75.

Gamlem N, Nordstoga K, Glattre E. (2008). Canine neoplasia - Introductory paper. APMIS Suppl 125):5-18. doi: 10.1111/j.1600-0463.2008. 125m2.x

Gajewski TF, Schreiber H, Fu YX. (2013). Innate and adaptive immune cells in the tumor microenvironment. Nat immunol, 14(10), 1014–1022.

Giri A, Edwards TL, LeGrys VA, et al. (2014). Subclinical hypothyroidism and risk for incident ischemic stroke among postmenopausal women. Thyroid, 24(8), 1210-1217.

ICVS (International Center for Veterinary Services). (2021). Humane Animal Welfare Programs: Spay/Neuter Sponsorship for Stray/Rescued/Shelter Dogs and Cats. Available at: http://www.icvsasia.com/index.php?option=com_content&view=category&layout=blog&id=62&Itemid=&lang=en; Accessed on: June 27, 2022.

Juin HBL, Shelat VG. (2021). Spleen angiosarcoma: a world review. Expert Rev Gastroenterol Hepatol, 15(10), 1115–1141.

Kiefel C, Kutzler M. (2016). Luteinizing hormone receptor expression in canine anterior cruciate and femoral head ligaments. International Symposium on Canine and Feline Reproduction VIII, Paris; p.151.

Li W, Kutzler M. (2020). Luteinizing hormone receptor gene expression in canine neoplastic lymphoma cells is upregulated by treatment with human chorionic gonadotropin. Abstract presented at the 2020 Society for the Study of Reproduction virtual meeting.

MacEwen EG. (1990). Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer metastasis rev, 9(2), 125–136.

Merck Manuals Staff. (2020). Causes of Cancer. In: Merck Veterinary Manual. Available at: https://www.merckvetmanual.com/special-pet-topics/cancer-and-tumors/causes-of-cancer; Accessed on: June 27, 2022.

Mochizuki H, Motsinger-Reif A, Bettini C, et al. (2017). Association of breed and histopathological grade in canine mast cell tumours. Vet Comp Oncol, 15(3), 829–839.

Moore AS, Frimberger AE. (2009). Causes of Cancer in Pets. In: Oncology for Veterinary Technicians and Nurses. Hoboken, NJ: John Wiley and Sons, Inc., pp. 9-12.

Moore AS, Rassnick KM, Frimberger, AE. (2017). Evaluation of clinical and histologic factors associated with survival time in dogs with stage II splenic hemangiosarcoma treated by splenectomy and adjuvant chemotherapy: 30 cases (2011-2014). Journal of the American Veterinary Medical Association, 251(5), 559–565.

Noci I, Pillozzi S, Lastraioli E, et al. (2008). hLH/hCG-receptor expression correlates with in vitro invasiveness in human primary endometrial cancer. Gynecol Oncol,111(3):496-501.

Ouzounova, M., Lee, E., Piranlioglu, R. et al. (2017). Monocytic and granulocytic myeloid derived suppressor cells differentially regulate spatiotemporal tumour plasticity during metastatic cascade. Nat Commun, 8, 14979 Doi: 10.1038/ncomms14979

Pastor M, Chalvet-Monfray K, Marchal T, et al. (2009). Genetic and environmental risk indicators in canine non-Hodgkin's lymphomas: breed associations and

geographic distribution of 608 cases diagnosed throughout France over 1 year. JVIM, 23(2), 301–310.

Puett D, Narayan P. (2003). Luteinizing Hormone Receptor Signaling. In: Henry, H (ed.), Encyclopedia of Hormones, Vol. 2. Cambridge, MA: Academic Press imprint of Elsevier, pp. 612–616.

Ponglowhapan S, Church DB, Scaramuzzi RJ, et al. (2007). Luteinizing hormone and follicle-stimulating hormone receptors and their transcribed genes (mRNA) are present in the lower urinary tract of intact male and female dogs. Theriogenology, 67(2), 353-366.

Poon AC, Matsuyama A, Mutsaers AJ. (2020). Recent and current clinical trials in canine appendicular osteosarcoma. Can Vet J, 61(3), 301-308.

Reichler IM. (2010). Urinary incontinence and puppy coat due to spaying in the bitch. An overview of pathophysiology, diagnosis and therapy. Tierarztl Prax Ausg K Kleintiere Heimtiere, 38(3), 57-62.

Reichler IM, Welle M, Eckrich C, et al. (2008). Spaying-induced coat changes: the role of gonadotropins, GnRH and GnRH treatment on the hair cycle of female dogs. Vet Dermatol, 19(2), 77–87.

Sanchez AM, Flamini MI, Russo E, et al. (2016). LH and FSH promote migration and invasion properties of a breast cancer cell line through regulatory actions on the actin cytoskeleton. Molecul Cell Endocrinol, 437, 22–34.

Schalch DS, Parlow AF, Boon RC, Reichlin S. (1968). Measurement of human luteinizing hormone in plasma by radioimmunoassay. J Clin Invest, 47(3),665-78.

Shiu KB, Flory AB, Anderson CL, Wypij J, Saba C, Wilson H, Kurzman I, Chun R. (2011). Predictors of outcome in dogs with subcutaneous or intramuscular hemangiosarcoma. J Am Vet Med Assoc;238(4):472-9. Doi: 10.2460/javma.238.4.472

Stockner PK. (1991). The economics of spaying and neutering: market forces and owners' values affecting pet population control. J Am Vet Med Assoc, 198(7), 1180-1182.

Stadler WMM. (2013). Cancer biology review: A case-based approach. Warren, MI: Springer Publishing Company. Created from OSU source on 2022-05-02 18:39:57.

Teske E. (1994). Canine malignant lymphoma: a review and comparison with human non-Hodgkin's lymphoma. Vet Quarterly, 16(4), 209-219.

Teske E, de Vos JP, Egberink HF, et al. (1994). Clustering in canine malignant lymphoma. Vet Q, 16(2), 134–136.

Urfer SR, Kaeberlein M. (2019). Desexing Dogs: A Review of the Current Literature. Animals (Basel), 9(12),1086.

Vadakkadath MS, Atwood CS. (2005). The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain. Cellular and molecular life sciences. CMLS, 62(3), 257–270.

Vail DM, MacEwen, EG, Young KM. (2001). Canine lymphoma and lymphoid leukemia. In: Withrow SJ, MacEwen EG: editors. Small Animal Clinical Oncology, 3 nd edition, Philadelphia; WB Saunders: p. 558-590.

Vail D, MacEwen E. (2000). Spontaneously occurring tumors of companion animals as models for human cancer. Cancer Invest, 18, 781–792.

Villamil JA, Henry CJ, Bryan JN, et al. (2011). Identification of the most common cutaneous neoplasms in dogs and evaluation of breed and age distributions for selected neoplasms. J Am Vet Med Assoc, 239(7), 960-965.

Wang W, Li W, Chu D, et al. (2021). Long-term assessment of risk factors for canine tumors registered in Xi'an, China. Anim Diseases, 1(30), 1-8.

Weishaar KM, Ehrhart EJ, Avery AC, et al. (2018). c-Kit Mutation and Localization Status as Response Predictors in Mast Cell Tumors in Dogs Treated with Prednisone and Toceranib or Vinblastine. JVIM, 32(1), 394–405.

Welle MM, Reichler IM, Barth A, et al. (2006). Immunohistochemical localization and quantitative assessment of GnRH-, FSH-, and LH-receptor mRNA expression in canine skin: a powerful tool to study the pathogenesis of side effects after spaying. Histochem Cell Bio, 126(5), 527–535.

Wu S, Zhu W, Thompson P, et al. (2018). Evaluating intrinsic and non-intrinsic cancer risk factors. Nat commun, 9(1), 3490.

Zandvliet. (2016). Canine lymphoma: a review. Vet Quar, 36(2), 76–104.

Zwida K, Kutzler M. (2019). Luteinizing hormone receptor is immunoexpressed within the canine thyroid. Clin Theriogenology, 11(1), 23-29.

Zwida K, Kutzler M. (2016). Canine hemangiosarcoma expresses luteinizing hormone (LH) receptors. J Anim Sci, 94 (Suppl 5), 205-206.

CHAPTER 2

OVERVIEW OF IMMUNOCHEMISTRY METHODOLOGY

2.1. IMMUNOHISTOCHEMISTRY

2.1.1 Summary

Immunohistochemistry is a highly sensitive method that exploits the specific binding between an antibody and antigen to allows localization of specific antigens within cells and tissue (Magaki et al., 2019). Immunohistochemistry connects three major disciplines: immunology, histology, and chemistry as its names indicated. Immunohistochemical staining is used widely in the diagnosis of abnormal cells such as cancerous tumors including the breast, lung, gastrointestinal tract hematolymphoid and central nervous systems and basic research to understand the localization and distribution of biomarkers and expressed proteins (Yong et al., 2014). The sequential steps in IHC can be discussed (**Figure 2.1**).

2.1.2. Tissue Section Types

Tissue samples are taken from specimens of various sources including biopsy, surgery, animal model and autopsy. The biopsy, surgery, and animal model types of specimens give fresh tissues while the last one is taken after an animal has died for two hours which is postmortem autolysis. As antigens may denature, disappear and diffuse, autopsy specimen should be fixated as soon as possible so as not to influence its label (Anonymous, n.d[b]).

The frozen sections are the most important feature for of this section is to keep antigen's immune competence completely especially for the antigen cell surface and fresh and fixed tissues both they can be processed as frozen tissues. However, the tissues must be primary fixed and stored at low temperature (Anonymous, n.d[1]). Frozen tissues are potentially problematic in that research freezers are not carefully monitored and it can be accepted but should be flagged as a returned specimen in case of degradation (Yong et al., 2014). These sections are cut by using a pre-cooled cryostat and mounted to adhesive-coated glass slides and dried overnight at room temperature and are usually post-fixed by immersion in pre-cooled (-20°C) acetone, fresh paraformaldehyde, or formaldehyde/formalin at ambient temperature (Anonymous, n.d[2]).

Paraffin-embedded tissue section is normally sliced by a microtome to give a thickness of 2-7 μm. With proper treatment, the section reveals clear tissue structure and exact antigen location. This section type can be stored at 4°C for long term use. These sections are then mounted onto glass slides that are coated with a tissue adhesive. This adhesive is commonly added by surface-treating glass slides with 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine. These two leave amino groups on the surface of the glass so, the tissue can adhere. slides can be coated too with actual adhesives including egg albumin, gelatin, or Elmer's glue. After mounting, the sections are dried in an oven or microwaved in preparation for de-paraffinization (Anonymous, n.d[2]).

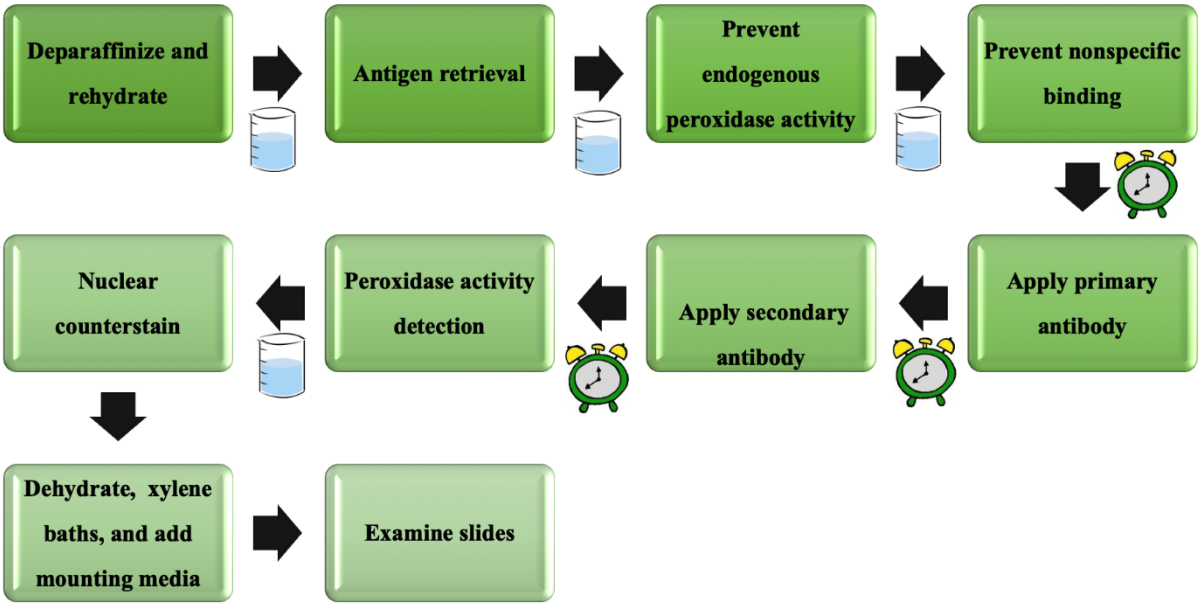


Figure 2.1. Flowchart of immunohistochemistry protocol

In order to be able to use antibodies, the samples should be processed or fixed in a very specific manner. For example, many antibodies only recognize proteins that have been reduced and denatured, while others detect protein epitopes only when the proteins are in their native/folded conformation. Some antibodies should only be used with unfixed, frozen tissue. Many antibodies cannot bind to their target epitopes in formalin-fixed, paraffin-embedded tissues unless an antigen retrieval step is included to reverse the cross-links that result from fixation (Anonymous, n.d[2]).

There is possible cause for no or weak staining that the tissues slides might lose signal over time during storage and to solve this problem, always prepare slides with freshly-slice tissues or store slides at 4°C (Anonymous, n.d[1]). Make sure to cover the tissues in solutions at all time during the IHC experiment to avoid tissues from dry out because that also will cause no or weak staining (Anonymous, n.d[1]).

2.1.3. Tissue Fixation

Tissue fixation which depends on the type of antigen that need to be detected. Some antigens can be destroyed during fixation in formaldehyde and tissues have to be frozen or fixed in a different fixative (Ramos, 2011). Formaldehyde is standard or gold standard of fixative for routine histology and immunohistochemistry (**Table 2.1**). The water-soluble, colorless, toxic, and pungent gas reacts with primary amines on proteins and nucleic acids to form partially-reversible methylene bridge crosslinks.

Formaldehyde in solution is capable of binding many amino acids including lysine, tyrosine, asparagines, histidine, arginine, cysteine, and glutamine. The basic

mechanism of fixation with formaldehyde is the formation of addition products between the formalin and uncharged reactive amino groups (–NH or NH2), forming cross-links. Once the addition product (reactive hydroxy methyl compound) is formed, additional cross-linking will happen (**Figure 2.2**). Therefore, the hydroxymethyl group will form a methylene bridge in the presence of a second reactive hydrogen (Ramos, 2005).

The terms "formalin" and "formaldehyde" are often used interchangeably, although the chemical composition of each fixative is different. Formalin is made with formaldehyde but the percentage denotes a different formaldehyde concentration than true formaldehyde solutions. To be clarify, the formalin is commercial concentrated (37–40%) solution of formaldehyde. Formaldehyde fixative is the simplest aldehyde, its chemical formula is H_2CO, and made up of formalin diluted to a 10% solution (3.74% formaldehyde). Formaldehyde dissolved in phosphate buffered saline can be recommended as a universal fixative for the routine use (Igor and Werner, 2010). When using neutral buffered formalin, the pH is shifted to neutrality causing dissociation of hydrogen ions from the charged amino groups (–NH3) of the side chains of proteins, resulting in uncharged amino groups (–NH2) (Ramos, 2005). The use of 10% buffered formalin will produce more cross-links than non-buffered formalin and it will have more deleterious effects for immunohistochemistry (Ramos, 2005; Hayat, 2002).

Using formalin and paraformaldehyde fixatives can masked the epitope which can cause possibility for low or no stating. This can be solved by using sufficient

different antigen retrieval methods to unmask the epitope (HIER or PIER) or fix the sections in a shorter time (Anonymous, n.d[1]).

2.1.4. Deparaffinization and Rehydration

By using standard methods, slides immerse in three washes of xylene, 10 min for each. xylene acts as a fusing agent for paraffin wax. Slides then need to be hydrated it by dipped in graded alcohols sequentially from 100%, 100%, 80%, to 70%. Alcohols are the most commonly used dehydrating agent and it has a strong water separation. However, since ethanol has strong penetration and contractility, so its concentration should be increased progressively to avoid tissue excessive shrinking. After then, immerse in two changes of deionized water and let it sit in deionized water for 5 min to adequately remove alcohol.

There is possible cause for high background due to insufficient deparaffinization and that's can be solve by increase the deparaffinization time or use fresh dimethylbenzene.

2.1.5. Antigen Retrieval (AR)

This method intended to retrieve the loss of antigenicity or returning proteins to their pre-fixation conformation. Approximately 84% of antigens fixed in formalin require some type of antigen retrieval to optimize the immunoreactions (Ramos et al., 2000). During the fixation, there is methylene bridges formed cross-link proteins and mask the sites of antigen.

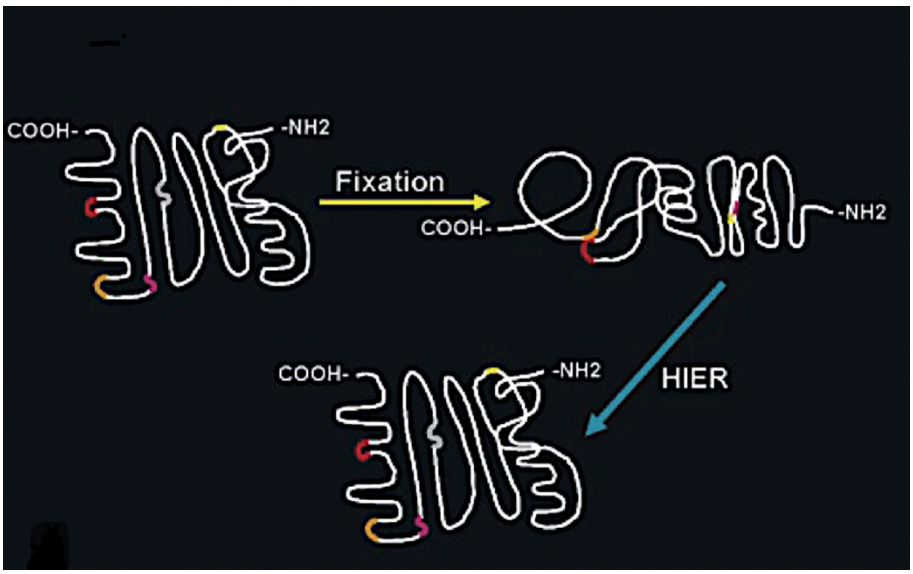


Figure 2.2. Conformational changes in the protein structure after fixation with cross-linking fixatives. Different epitopes (small segments in different colors) is modified, which has access to specific antibodies. Heat-induced epitope retrieval (HIER) methods reverse conformational changes produced by the fixative. Source: Ramos, 2011.

Fixative	**How does work?**	**Does antigen retrieval step need?**	**Advantage**	**Disadvantage**
Formalin	When introduced to sample, formalin diffuses and reacts with amino acids in sample to form reactive groups. These reactive groups combine with one another to form methylene bridges (crosslinks).	Formalin fixation can mask epitope and limit antibody-epitope binding and to unmask epitope and restore antigenicity an antigen retrieval step prior to IHC staining is recommended.	Better preservation of tissue morphology. Long term storage if paraffin embedded.	Can mask epitope and reduce antigenicity. Can affect expression of post translational modifications
Alcohol	Ethanol and methanol replace water in the tissue, exposing the internal hydrophobic proteins and breaking hydrophobic bonds to alter tertiary structure. Alcohol is considered a precipitating fixative.	Not recommended on alcohol fixed tissues since it is often too harsh and can impact tissue integrity.	Better preservation of antigenicity. Better suited for the study of DNA, RNA, and post translational modifications.	Can distort nuclear and cytoplasmic detail.

Table 2.1. Comparison between two fixatives.

Antigen retrieval methods break these bridges and expose antigenic sites to allow antibodies to bind. The two methods for antigen retrieval are heat induced epitope retrieval (HIER) and proteolitic-induced epitope retrieval (PIER). PIER is the most commonly method used antigen retrieval before the advent of HIER. Many enzymes have been used including trypsin, proteinase K, pronase, and pepsin. The mechanism behind the PIER is likely digest the protein cross-linkages that introduced during formalin fixation. The effect of PIER depends on the concentration and type of enzyme, incubation time, temperature, and PH as well as on the duration of fixation (Ramos, 2011). Although these enzymes digest bonds in the native proteins, but may cause a reduce in staining intensity or loss of immunoreactivity if the appropriate epitope is cleaved (Mcnicol and Richmond, 1993)

HIER is a common approach as it typically has a higher success rate for restoring antigenicity than an enzymatic method (Magaki et al., 2019). The mechanism involved in this method is unknown, but its final effect is to revert conformational changes that produced during the fixation. It is often performed using a pressure cooker, a microwave oven, or a vegetable steamer. slides need to be placed in boiling sodium citrate in a cooker and boiling continued in a microwave for a total of 10 minutes.

Antigenicity also may be recovered without heating by prolonged immersion of fixed tissues in water (Puchtler and Meloan, 1985) or aqueous solutions (Elias, 1990). Sodium hydroxide–methanol has also been successfully applied (Linthicum et al., 1995), as well as urea (Hausen and Dreyer, 1982) and formic acid (Kitamoto et al., 1987). Enhancement has been also achieved by refixation in zinc sulphate–formalin (Abbondanzo at el., 1991). After that, those fixed slides should allow to be cool at room

temperature for about 20 minutes, then should be rinse in distilled water to be ready for next step.

2.1.6. Blocking Endogenous Peroxidase

Endogenous peroxidase is naturally present in many cells such as red blood cells (pseudoperoxidase), granulocytes (myeloperoxidase), and neurons that can react with the chromogen, thus producing staining identical to specific immunoperoxidase (Elias, 2003). However, the endogenous peroxidase activity is destroyed almost completely during formalin fixation, but pretreatment of tissue sections with a diluted solution 0.003 to 3% of H_2O_2 will reduce or completely abolish pseudoperoxidase activity of red blood cells and peroxidase activity in myeloid cells (Igor and Werner, 2010; Ramos, 2005). Some tissue sections that abundant hemorrhages or with acid hematin, a stronger solution of 10% H_2O_2 (Abbondanzo, 1991) might be needed to remove this endogenous activity or a longer incubation in less concentrated solutions could work (Elias, 2003).

When the endogenous peroxide or phosphatase is active, it will cause possibility of high background. To solve this, troubleshoot, quench the endogenous peroxidase or phosphatase activity by enzyme inhibitors like peroxidase: H_2O_2 and methanol (v/v: 0.3%:99.7%) or phosphatase (2 mM levamisole).

2.1.7. Blocking Non-Specific Binding

This step is important because the antibody applications rely on the specific binding of an antibody to the target epitope and there are specific interactions that can

also contribute to non-specific binding including ionic interactions, hydrophobic interactions, and hydrogen bonding. Apply several drops of diluted normal blocking serum (1% horse serum) to tissue until completely covered, then incubates at room temperature for 20 minutes.

Make sure to block according to the provided protocol to avoid the possible cause for high background. If the blocking is insufficient can cause high background. In this situation, it should increase blocking incubation period or change blocking reagent. For example, for sections, use 10% normal serum for a hour and for cell culture, use 1-5% BSA for half hour (Anonymous, n.d[1]).

2.1.8. Antibody Application

The antibody concentration that provides the strongest staining of the target antigen and lowest background staining should be determined by serial dilutions of a concentrated antibody so, it is easiest to start with the dilution recommended by the manufacturer (Magaki et al., 2019). Apply diluted primary antibody at 1:100 or 1:50. Incubate slide for 60 min at room temperature. Negative controls from each tissue should be similarly treated, but without primary antibody. Sections then reacted for 30 minutes with species specific secondary antibody, which has specificity against primary antibody and is usually applied to provide a contrast that helps the primary stain more distinct.

If the antibody used is not suitable for IHC procedures might cause possibility for low or no stating. This can be solved by checking datasheet of the antibody to make sure that it has been validated for IHC assays or checking the antibody is applicable to

the right IHC samples (paraffin sections vs. frozen samples). Also, if the primary and/or secondary antibody have lost its activity due to dilution or excessive freezing and thawing or improper storage can cause possibility for low or no stating so, storing the antibodies according to manufacturer instructions or use positive controls to ensure that the antibodies are working well can solve this problem. Other troubleshoot that cause weak or no stating is that the insufficient antibody to detect protein of interest so, using a higher concentration or incubate for a longer time (e.g. overnight) at 4°C can solve this (Anonymous, n.d[1]). High background is also problem due to the high concentration of primary antibody concentration. That's why it should titrate the antibody to determine the optimal concentration (Anonymous, n.d[1]).

If the primary antibody was raised in the same species as source of tissue (therefore, secondary antibody recognizes and binds to everywhere on the entire tissue because it was raised against that species) will cause highbackground too. The solution for that by using primary antibody raised against a species which is different from the source of tissue and use biotinylated primary antibody, and conjugated streptavidin for the detection system (Anonymous, n.d[1]).

2.1.9. The Avidin-Biotin-Peroxidase Complex (ABC) Method

In this method, the second antibody is biotinylated and the third reagent is a complex of avidin mixed with biotin linked with label appropriately. The labeled biotin and avidin are allowed to react together for 30 minutes before being applied, which results in the formation of a large complex with many molecules (e.g., enzyme) (Polak and Noorden., 2003). Avidin is a large glycoprotein that is extracted from egg white

and it has 4 binding sites per molecule and high affinity for a low–molecular mass vitamin called biotin. Biotin has one binding site for avidin and can be attached through other sites to an antibody (biotinylated antibody) or any other macromolecule, such as an enzyme, fluorochromes, or other label (Polak and Noorden., 2003).

Another commonly used avidin–biotin method is the labeled avidin–biotin (LAB) or labeled streptavidin–biotin (LSAB) method, which uses a biotinylated secondary antibody and a third reagent of peroxidase or alkaline phosphatase labeled avidin. The sensitivity of this method is higher than standard ABC. The avidin can produce background by binding to lectins that in the tissue through its carbohydrate groups and through electrostatic binding because its isoelectric point (pI) and this background can be greatly reduced by substituting avidin with streptavidin. (Ramos, 2005; Boenisch, 1999).

The main disadvantages of any avidin–biotin system is the possibility of producing high background. High levels of endogenous biotin in biotin-based detection systems for samples (e.g., liver and kidney tissues) can cause this problem to solve this it should perform biotin block after normal blocking procedure (before primary antibody incubation) (Anonymous, n.d[1]).

2.1.10 Chromogen Application

Chromogens are chemical compounds that produce a colored product that can be visualized by bright-field microscope. There are two commonly used chromogens: 3,3'-diaminobenzidine (DAB), which is brown or alkaline phosphatase (AP) that is red. DAB typically used as a signal enhancer in conjunction with the HRP-based

immunostaining systems and it provides strong and permanent stains. Since DAB may cause skin and bladder cancers, it is advised that personal protective equipment should be used and skin/mucosa should be avoided. AP Red (or another red chromogen) is used mainly for skin sections while the brown DAB masked by brown melanin pigment. Red sometimes used in the same tissue section to allow the pathologist to visualize two antigens in the one slide (Anderson et al., 2021). The one that we use in our laboratory is Vector® NovaRED® Substrate Kit, Peroxidase (HRP). This is red chromogen and should be used with non-aqueous (permanent) mounting.

The only problem of using chromogens is that the possibility of producing high background. Reaction between chromogens and PBS buffer in tissue or cell samples can cause this high background. The solution of this is that use Tris buffer to wash the samples before incubating with the substrate (Anonymous, n.d[1]).

2.1.11. Counterstaining

A counterstain is a dye or stain that has a color contrasting to the first stain (primary antibody). After staining a target of interest with a primary antibody (first stain), the counterstain is then applied as a second chemical stain (Conrad, 2020). Counterstains are used to differentiate the various cell types or subcellular structures seen in cell staining (Rodig, 2019). When selecting a counterstain, it is important to ensure the counterstains are different color with sufficiently different absorption/emission characteristics so that they are distinguishable easily (Conrad, 2020).

There are four types of methods that are used in cell staining to label the detecting reagent. These are fluorochromes, enzymes, gold, and iodine. Fluorochrome and enzyme labels are the most common (Rodig, 2019). To choose an appropriate counterstain, determine whether the cell-staining detection reagent first, which is usually a chromogenic substrate and is soluble in alcohol (Rodig, 2019). There are common chromogenic and fluorescent counterstains that are used in immunohistochemistry staining will be summarized below.

2.1.12. Counterstains for Enzyme/Chromogen Detection Systems

Hematoxylin or haematoxylin is the most commonly nuclear counterstains that used for enzyme/chromogen detection systems (Aziz and Rekha, 2016; Conrad, 2020). Hematoxylin is combined with aluminum ions to form an active metal–dye complex that stains the nuclei of cells blue by binding to lysine residues on nuclear histones as opposed to other nuclear dyes that target the nucleic acids. There are two modes of hematoxylin staining which are regressive and progressive. When a regressive mode is used, the tissue will be overstained with excess regressive hematoxylin (Harris), then decolorized by immersion in an acid solution (e.g.,1% acid alcohol). This step of decolorizing removes the nonspecific background staining (Conrad, 2020).

Progressive stains are used for optimizing development easily to allows the compatibilist with alcohol soluble enzyme/substrate such as those in HRP (horseradish peroxidase) enzyme/AEC (3-Amino-9-ethylcarbazole) substrate systems. Once the desired staining is achieved, the stain is then “blued” with bluing reagent to convert

reddish purple to a deep blue or purple. At acid PH, hematoxylins stain the nuclei red. At alkaline pH, hematoxylins stain the nuclei blue (Conrad, 2020).

Counterstaining the cytoplasm may be necessary to add more staining properties to nuclear stains with hematoxylin. Hematoxylin is often paired with eosin, an orange/pink xanthene dye that is acidic and binds to proteins in the cytoplasm and connective tissue. This helps to clarify both nuclear and cytoplasmic structures in tissue (Conrad, 2020). Other common nuclear counterstains are methylene blue and methyl green and both are dyes that target nucleic acids.

2.1.13. Counterstains for Fluorescent Immunostaining Systems

Fluorescent immunostaining involves the use of fluorescent chemicals such as DAPI or Hoechst. DAPI (4', 6-diamidino-2-phenylindole) and Hoechst 33342 are common nuclear dyes that work by intercalating into the DNA and producing a strong blue color under UV excitation. DAPI binds selectively to dsDNA without background staining in cytoplasm and it has semi-permeability to living cells and can be used to stain fixed cells and/or tissue sections. Hoechst 33342 is a primary counterstain which is used against yellow fluorescence. Propidium iodide is another common primary counterstain which is used for nucleus and chromosome staining against yellow/red fluorescence (Anonymous, n.d[1]; Conrad, 2020).

2.1.14. Dehydration, Clearing, and Mounting Media

The mounting media for immunohistochemical technique must be compatible with the detection method used. A mounting medium used to attach a coverslip or may

itself used to replace the coverslip (Rodig, 2019). The medium selection depends on some factors including the chemical compatibility with chromogen, counterstain, and the preservation period. There are two types of mounting medium. Neutral mounting medium, which usually refers to an oily substance with pH 7.0 such as neutral gum (resin) but the sample should be treated with dimethyl benzene, transparent and dehydrated for long-term storage sections. The second one is water-soluble mounting medium. Popularly used in immunofluorescent staining for short-term storage sections, this mounting medium usually consists of 50% glycerol (Anonymous, n.d[1]). The choice of mounting medium among different enzymes, chromogens and counterstains summarized in **Table 2.2**.

After mounting the sections, the slides should be left at room temperature overnight to allow water that may be trapped under the section to dry out. In the event that tissue sections adhere to the slide, it may be incubated at 60ºC for a few hours (Anonymous, n.d[1]). Slides with paraffin-embedded tissue sections can be kept for up to 3 years at 2-8°C, depending on the antigen in question. Frozen tissue sections from snap-frozen tissue blocks should be carefully wrapped in aluminum foil and stored at -20°C or lower for up to six months (Anonymous, n.d[1]).

2.2. Immunocytochemistry

2.2.1. Summary

The microscopy widely remains valuable and important technique because it responsible for visualization of cells, organelles, and tissues, which determine the presence of a disease or the distribution of cells of interest. immunocytochemistry is

Enzyme	Chromogen	Counterstain	Mounting media
HRP	DAB	Hematoxylin, Methyl Green, Methyl Blue	Neutral
HRP	AEC	Hematoxylin, Methyl Blue	Water soluble
AP	BCIP/NBT	Nuclear Fast Red, Brilliant Green	Neutral
AP	Fast Red	Hematoxylin, Methyl Green, Brilliant Green	Water soluble

Table 2.2. Selection of some of appropriate enzyme substrate for chromogenic detection. Horseradish Peroxidase (HRP), Alkaline phosphatase (AP); 3,3'-diaminobenzidine (DAB), 3-Amino-9-ethylcarbazole (AEC), (5-Bromo-4-Chloro-3-Indolyl-Phosphate (BCIP), Nitro Blue Tetrazolium (NBT).

still one of valuable ancillary technique (Beesley, 2001). Immunocytochemistry is method to detect and visualize of proteins or other antigens in cells using specific antibodies that recognize the target of interest. This antibody linked to fluorophore or enzyme directly or indirectly, then these enzymes give rise to the signal such as fluorescence or color from an enzymatic reaction, which can be detected by microscope (Burry, 2011). Immunocytochemistry is usually performed in four sequential steps (**Figure 2.3**).

2.2.2. Cell Culture for Immunocytochemistry

2.2.2.1. Cell Seeding and Cell Culture Technique

The cells are seeded on a solid support to allow easy handling in subsequent procedures. The solid support usually is glass slide or a glass-bottom plate. The incubation time is so important before proceeding with immunostaining and that's depending on the type of cells. In case of seeding adherent cells, the cells attach to the solid support surface during the incubation, which varies depending on cell types. The suspension cells can be centrifuged onto glass slides and bound to solid support by using chemical linkers or handled in suspension.

When preforming the cell passaging, try to a strict schedule to ensures reproducible behavior and allows to monitor their health status and the seeding density of cultures until achieve consistent growth. Fresh media will be added every 2-3 days until cells reach >80% confluence. Cells are treated with trypsin and incubated at 37°C for 3-5 minutes, checked under a microscope to confirm that cells are no longer adhered and then suitable media should be added to stop the trypsin digestion. The cells then be

transferred into a sterile conical tube and centrifuged at 200Xg for 5 minutes. The supernatant will be removed, media are added, and repeat this step. Then the cells should be counted on a hemocytometer. Add a volume of cell suspension (1M cells/ml) over each coverslip in the dish. Grow the cells at 37°C in a humidified 5% CO2 incubator and wait one day and next day, remove media and add fresh media and 2 days until reaching 70-80% confluence. Looking through all coverslips (all replicates) and make selection of what should be can use because some of them have too many cells and others not.

2.2.2.2. Cell Fixation and Immunostaining

Fixation retains the proteins at their location in the cell and preserves their chemical and structural state at the time of fixation. It can be done by crosslinking or by precipitating the proteins using organic solvents. Upon permeabilization, membranes are punctured with the use of solvents or detergents, allowing the relatively large antibodies to cross the cellular membranes. The permeabilization requires fixation, and hence limits the technique to studying dead cells. During antibody incubation, the antibodies are allowed to bind to target antigens within the cells, after which unbound antibodies are removed by washing. (Anonymous, n.d[3]).

There are different types of reporters used to identify proteins in the cells; First, the enzyme-coupled antibodies, which create a color reaction when bound to the antigen. Second one is fluorophores, which absorb a wavelength of light and then emit additional wavelengths of various lengths using a fluorescence microscope. Multiple fluorophores can be combined in one sample if they react to different wavelengths and

also emit different colors. The direct method of immunocytochemistry only uses one antibody for immunostaining, which is done quickly and is accurate. However, it may not be sensitive enough because it requires many proteins to be present to determine a reaction. Indirect immunocytochemistry uses a primary and secondary reporter. The primary antibody is applied to the sample and then a secondary reporter-paired antibody is applied to the primary antibody. This process takes longer but increases the sensitivity of the reaction.

2.2.2.3. Imaging and Image Analysis

The cells and antibodies' locations that bound to target antigens are visualized by using microscopy. Images are acquired using a camera, and the images are analyzed and cellular structures annotated in the final step.

2.3. Comparison between Immunocytochemistry and Immunohistochemistry

2.3.1. Sample Source

Immunohistochemistry (IHC) stains tissue sections, whereas immunocytochemistry (ICC) stains single layers of cells grown in culture or from a patient sample. However, the tissues are indeed made of cells, immunohistochemistry preserves the original architecture of the tissue sample. The cells are arranged how they are in the body tissues including the extracellular components (proteins and carbohydrates). In immunocytochemistry, cells are grown in a single layer and lack the properties of an entire tissue. This allows researchers to zoom in on the properties of single cells instead looking at the entire tissue.

2.3.2. Sample Procedure

The samples in both techniques need to be fixed with a fixative agent, which preserves the structure of the cell or tissue. Tissues with IHC need additional steps and must be embedded in paraffin wax to preserve their structure prior to being sectioned and mounted. However, the paraffin interferes with the staining procedure and must be removed before the staining starts. This processing can cause the intended targets for the staining to become obscured, so IHC samples must undergo another process called antigen retrieval to re-expose the target proteins to restore tissue antigenicity (Anonymous, 2017 [4]). IHC often requires blocking non-specific binding epitopes during sample preparation. compared to immunohistochemistry, immunocytochemistry undergoes a shorter fixation period.

For the cells, it has different challenges for preparation prior to ICC. Individual cells must be permeabilized to open up the cell membrane and allow antibodies into the cell during staining. The permeabilization provides access to intracellular or intraorganellar antigens. There are two general types of reagents are commonly used: organic solvents, such as methanol and acetone, and detergents such as saponin, Triton X-100 and Tween-20. The organic solvents dissolve lipids from cell membranes which then make them to be permeable to antibodies. Saponin interacts with membrane cholesterol, selectively removing it and leaving holes in the membrane. The disadvantage of detergents such as Triton X-100 and Tween-20 is that they are non-selective in nature and may extract proteins along with the lipids (Jamur and Oliver, 2010). Immunohistochemistry samples may not have to undergo a separate permeabilization step.

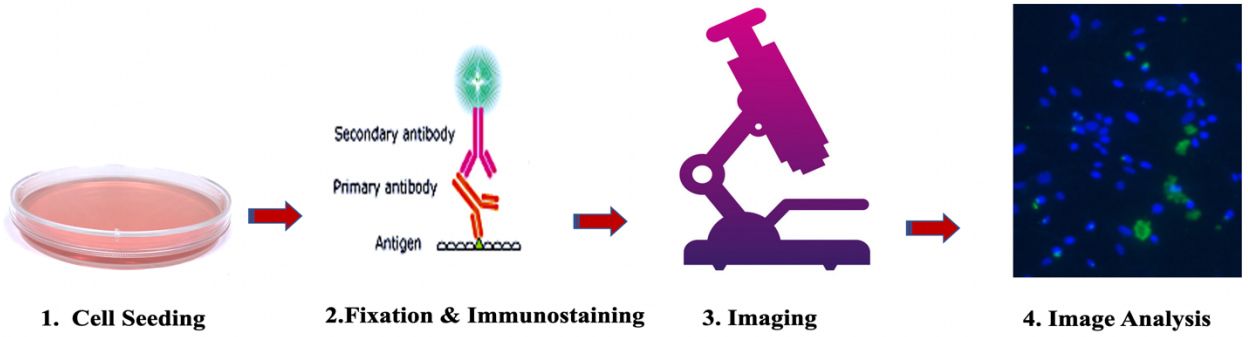


Figure 2.3. Flow chart describes the four steps of immunocytochemistry: cell seeding, immunostaining, imaging, and image analysis.

2.3.3. Stains

Immunohistochemistry uses chromogens for their staining method while immunocytochemistry uses fluorophores. Fluorescent molecules that are bound to the secondary antibody. First, a primary antibody is incubated with the sample that will bind to the target protein. Then, the fluorescent labeled secondary antibody is added, which binds to the primary antibody. When a special light source is applied, the fluorophore lights up, allowing researchers to locate the protein of interest.

Although fluorescence is traditionally used with immunocytochemistry, it is becoming more popular to use with immunohistochemistry as well. It's easier to stain for multiple proteins at once using fluorescence compared to the chromogens, and tends to have fewer steps than chromogen staining.

2.4. Animal Tissue Culture Techniques

2.4.1. Summary and Historical Background

Animal cell culture is an important tool for biological research. Cell culture involves isolation of cells from a tissue before establishing a culture in the suitable artificial environment. This isolation of cells from the tissues can be achieved by disaggregation using enzymatic or mechanical methods. The source of the isolated cells is an in vivo environment, but sometimes cells are also derived from an existing cell line or cell strain. Animal cell culture offers suitable model systems for investigating the following factors including drug screening and development, mutagenesis and carcinogenesis, normal physiology/biochemistry of cells, and potential effects of drugs and toxic compounds on the cells (Bhatia et al., 2019).

Tissue or cell culture as a technique was first devised at the beginning of the 20th century as a method to study the behavior of animal cells in vitro. Roux in 1885, was able to maintain embryonic chicken cells in warm saline and established the principles of tissue culture. In 1911, Carrel and Burrows demonstrated that the survival of cells isolated from blood and connective tissue can be made in serum and plasma. Harrison in 1907 established the methodology of tissue culture and observed cell growth in clotted lymph fluid from explants of frog embryo tissue by the "hanging drop" method (Swain et al., 2014; Mather and Roberts, 1998).

This was followed by the work of Carrel and his co–worker who succeeded in growing adult and embryonic tissues of warm-blooded mammalian cells in vitro. Carrel and his co–worker in 1923, has become one of the important items of cell culture laboratories enabling the subculture of cells (Swain et al., 2014; Mather and Roberts, 1998).

The developments that occurred in between 1940 and 1950 made cell culture available so wide and accepted as a tool for scientists. Notable among these are use of antibiotics to avoid contamination problems in cell culture and development of chemically defined culture media by Eagle in 1955 (Swain et al., 2014; Mather and Roberts, 1998). These advancements in technology of cell culture provided more scope to researchers to use cell, tissue and organ culture in their research as a result of which cell culture is routinely carried out in many laboratories throughout the world.

2.4.2. Establishing of Cell Culture System.

2.4.2.1. Isolating Cells

The suspension cells are isolated from the blood system. For example, white blood cell lymphocytes are suspended in plasma (Bhatia et al., 2019; Glade and Hirschhorn, 1970). Peripheral blood mononuclear cells (PBMCS), which include lymphocytes (T cells, B cells, and NK cell), monocytes, and dendritic cells. The most common isolation method for these cells involves using a density gradient medium (e.g. Ficoll) and centrifugation.

The main advantage of this method for lymphocyte is the removal of most granulocytes from the sample. Also, removing these cells by density gradient centrifugation decreases the time for acquisition of the samples since these comprise over 60% of the leukocytes in peripheral blood. Other advantage for this method is the removal of non-viable cells from the sample (Dagur and McCoy, 2015).

In this procedure, after collect peripheral blood into a tube containing an anticoagulant, the whole blood is diluted first with phosphate buffered saline (PBS) and then layered over the density gradient medium carefully. During centrifugation, the cells with higher densities (granulocytes and erythrocytes) sediment through the density gradient medium. The PBMCs settle at the interface between the density gradient medium and the plasma, from which they can be carefully collected by using a sterile Pasteur pipet (Dagur and McCoy, 2015) (**Figure 2.4**).

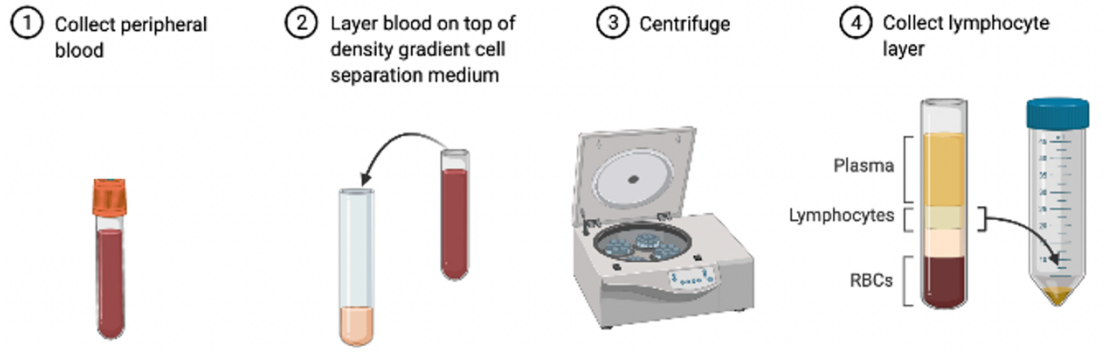


Figure 2.4. How to isolate peripheral blood mononuclear cells (PBMCS) from whole blood by density gradient centrifugation (Source: modified from bionere.com)

Animal cell culture involves isolation of cells from a tissue before establishing a culture in a suitable artificial environment. Initial isolation of the cells from the tissues can be achieved by disaggregation using enzymatic or mechanical methods. However, the source of the isolated cells is sometimes an in vivo environment, but usually the cells are also derived from an existing cell line or cell strain (Bhatia et al., 2019).

As mentioned above, the process begins with primary culture of cells to achieve confluence or formation of monolayer in a culture flask that supplemented with proper nutrients and growth factors. After achieving confluence, cells are sub-cultured or passaged routinely from primary to secondary and secondary to tertiary till establishment of a continuous cell line (Swain et al., 2014).

The primary cell and organ cultures have an advantage in that they are recently removed from the in vivo situation and might therefore be expected to more closely resemble the function of that cell or tissue in vivo. The disadvantage is that these cultures are reacting to a constantly changing environments over the first day or weeks in vitro including the damage sustained during the removal of cells from the animal and tissue and partial recovery from this damage, the change in environment from the animal to the in vitro culture, and the changing composition of the culture as some cells in the mixed cultures die and others proliferate and/or differentiate (Mather and Roberts, 1998).

To obtain primary cultures, explant and tissue dissociation (mechanical and enzymatic) methods are employed (Bhatia et al., 2019; Swain et al., 2014).

2.4.2.2. Explant Method

This technique is mainly used for disaggregation of small quantities of tissue (Bhatia et al., 2019). This is one of the simplest procedures, which involve finely chopping (explants) the tissue into fragments of no larger than 1–2 mm^3 (Bhatia et al., 2019; Dils, 1984). The tissue fragments (explants) are placed to a glass or treated culture flask. Next step is the addition of a suitable medium and followed by incubation for 3–5 days. Old medium is replaced by fresh medium unless desired growth. Once optimum growth is achieved the explants are separated and transferred to new culture vessels which contain fresh medium (Bhatia et al., 2019; Dils, 1984). One of the principal advantages of this method is that some aspects of the tissue's architecture can be preserved within the explant and the biochemical functions and hormonal responses of the tissue as closely as possible to those in vivo (Dils, 1984).

Explanting is done usually with microsurgical instruments such as eye-surgeon’s spring-loaded scissors and watch-maker’s forceps under a dissecting microscope in a sterile cabinet (Dils, 1984). The tissue to be explanted should be placed rapidly in liquid medium and depending on the size, fragility of the tissue some ingenuity may be required to hold the tissue while it is being explanted, but it is important to keep the tissue and explants moist with culture medium (Dils, 1984).

2.4.2.3. Enzymatic Dissociation

It is the most common method for cell isolation. In this method, suspension of cells is obtained by chopping the tissue or organs into small fragments followed by treatment with proteolytic enzymes such as trypsin, collagenase, dispase, protease,

pronase E, elastase, and others. These enzymes are commercially available in the market to detach the cultured cells, cell dissociation and cell component or membrane–associated protein isolation, but not suitable for each tissue type (Bhatia et al., 2019; Swain et al., 2014). These enzymes allow the hydrolysis of fibrous connective tissue and the extracellular matrix (Bhatia et al., 2019). This method offers high recovery of cells without affecting the viability of cells.

2.4.1.4. Mechanical Disaggregation

This method is necessary to disaggregate soft tissues such as brain, spleen, and soft tumors (Bhatia et al., 2019; Swain et al., 2014). By sieving, syringing and pipetting, you can obtain individual or small clumps of cells. This procedure is inexpensive, rapid and simple; however, this procedure involves the risk of cell damage, thus mechanical disaggregation is only used when the viability of the cells in the final yield is not very important (Bhatia et al., 2019; Swain et al., 2014).

2.5. Condition and Environment Required for Cell Culture

In mammalian, cell culture requires an optimal environment for growth. These environmental conditions are divided into physicochemical requirements and nutritional requirements. Nutritional requirements include a substrate or medium that provides support and important nutrients such as amino acids, carbohydrates, vitamins, minerals, growth factors, hormones and gases like oxygen and carbon dioxide, and factors that control physical and chemical factors such as pH, osmotic pressure and temperature (Bhatia et al., 2019). However, there is many challenges like genetic

complexity which make it so difficult to determine the optimum nutrient requirements of cells cultured under in vitro conditions, but it can be cultured successfully.

Contamination by microorganisms cause a major problem in tissue culture such as bacteria, mycoplasma, yeast, and fungal spores that may be introduced via the operator, the atmosphere, work surfaces, solutions, and many other sources. Aseptic technique aims to exclude contamination. Below some of environment conditions that required for get optimized cultures will be discussed.

2.5.1. Laminar Flow Cabinet or Tissue Culture Hood

The hood is an enclosed bench designed carefully to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials. The air is drawn through a high efficiency particle (HEPA) filter and blown in a very smooth, laminar flow towards the user. Due to the direction of air flow, the sample is protected from the user but the user is not protected from the sample.

Biological safety cabinet create a unidirectional laminar flow across the surface of work, but the laminar flow cabinet is not biological safety cabinet. Laminar flow cabinets are configured to protect the work on the work surface, but do not protect the operator as the airflow pushes aerosols or particulates from the work surface toward the operator. Incoming air is captured at the opening and passed through a HEPA filter. The air flows down to the work surface in a laminar flow pattern, where it splits and re-enters the plenum. 70% of the air is recirculated and 30% is passed through another HEPA filter to be exhausted to the room (Anonymous, n.d[5]).

The hood is equipped with a short-wave UV light that can be turned on for ~ 30 minutes to sterilize the surfaces of the hood after swabbing with disinfectant such as 70% alcohol (Freshney, 2010). The idea from using 70% alcohol instead 100 % or other concentration. First it is the most commonly disinfectant used in pharmaceutical industries. The 70 % alcohol solution kills microorganisms by dissolving plasma membrane of the cell wall completely and water is also required to denature the proteins of the cell membrane so, it acts as catalyst for the reaction. In addition to the contact time of alcohol with organism, extra water content slows evaporation, therefore increasing surface contact time and enhancing effectiveness. Should be aware that only exposed surfaces will be accessible to the UV light and do not put your hands or face near the hood when the UV light is on because this wave light can cause skin and eye damage.

2.5.2. Culture Flasks, Dishes, and Multi-Well Plates

Tissue culture flasks are available with vented caps that have filters incorporated with them for gas exchange without having to loosen the cap itself (Mather and Roberts, 1998). In 1920s, Alexis Carrel developed the first glass flasks. In the 1940s, Harry Earle developed the more traditional straight neck rectangular or hexagonal glass T-flasks. These days, many of plastic flasks with different size and designs are available depends on range with the growing area, the used. cell culture techniques (Anonymous, n.d[6]). The more common sizes are listed below (**Table 2.3**).

Cell culture dishes are the vessels of choice for cloning or other manipulations such as scraping that require direct access to the cell monolayer and offer the best economy, but they must be used with incubators that control CO_2 and humidity. There

are many kinds of dishes comes in different diameters but the most manufacturers offer dishes in four diameters including 35 mm, 60 mm, 100 mm, and 150 mm (**Table 2.4**). Cell culture dishes are available with either specially treated surfaces for growing anchorage-dependent cells, or untreated (native) surfaces for growing suspension cultures where attachment is not required (Anonymous, n.d[6]).

The multi-well plate is a flat plate that looks like a tray with multiple wells that are used as small test tubes. The main advantage of multi-well plates is the possibility to grow many cultures under identical conditions in the same culture plate. It is also beneficial for multiple reactions carried in a single plate. In addition to that, these plates offer significant savings in space, media, and reagents and more convenient to handle especially if the pipettors, plate washers, readers, and other equipment for processing these plates are used (Anonymous, n.d[6]). The wells are available in different shapes: F-Bottom: flat bottom, C-Bottom: bottom with minimal rounded edges, V-Bottom: V-shaped bottom, and U-Bottom: U-shaped bottom (Anonymous, n.d[6]).

The U-Bottom plates where is no edges, they are well suited or stirring and washing of samples. This bottom shape is used for agglutination and other assays requiring these tasks. The F-bottom is designed for precise optical measurements and is well suited for colorimetric determinations as well as microscopy applications. V-shaped bottom plates are especially suited for precipitation assays and storage. The C-bottom combines the advantages of U-bottom and F-bottom. Because of the curved edges, better sample mixing is obtained. The flat bottom in the middle area of the wells at the same time allows the realization of precise optical measurements. It is especially

suited for immunological assays, as sample stirring has a major impact on precise assay results as well as optical measurement to determine those results (Anonymous, n.d[7]). The more common sizes are listed below (**Table 2.5**). In our laboratory, the most common plate use is 96-well plates (U-shaped bottom).

2.5.3. Media

Media has an important role to play as cultured cells need either a completely natural or artificial medium supplemented with some natural products for growth. Selecting the appropriate media for a particular cell type is because their characteristics and compositions is varied. The source of culture medium has a major effect on the health and viability of cultured cells it must be determined the basal nutrient medium that fits the best to the cell type depending upon the objective of culture including growth, survival, differentiation, production of desired proteins.

For example, RPMI, also known as RPMI 1640 or Roswell Park Memorial Institute Medium. It was first described by Moore in 1967 and it was successfully cultured and expanded human lymphoid cells. RPMI Medium is unique from other media because it contains the reducing agent glutathione and high concentrations of vitamins. contains biotin, vitamin B_{12}, and PABA, which are not found in Eagle's Minimal Essential Medium or Dulbecco's Modified Eagle Medium. It also contains 19 essential and non-essential amino acids including glycine, 11 vitamins, 6 salts, and sodium bicarbonate. It is designed for an environment containing 5% CO2. RPMI is suitable for cells in suspension or monolayer and is widely used to grow a variety of mammalian cell types. These include primary human leukocytes, bone marrow cells,

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
T-25	25	5 to 10	2.5×10^6
T-75	75	15 to 25	7.5×10^6
T-150	150	30 to 50	15.5×10^6
T-175	175	35 to 60	17.5×10^6
T-225	225	45 to 75	22.5×10^6

Table 2.3. The more common sizes of tissue culture flasks. *Cell line dependent. Based upon a density of 1×10^5 cells/cm^2.

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
35 mm	8	1 to 2	0.8×10^6
60 mm	21	4 to 5	2.1×10^6
100 mm	55	10 to 12	5.5×10^6
150 mm	148	28 to 32	14.8×10^6

Table 2.4. The more common sizes of tissue culture dishes. *Cell line dependent. Based upon a density of 1×10^5 cells/cm^2.

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
96-well	0.32	0.32×10^5	0.32×10^5
48-well	1.00	0.3 to 0.6	0.8×10^5
24-well	1.88	0.5 to 1.2	1.9×10^5
12-well	3.83	1.0 to 2.4	3.8×10^5
6-well	9.40	2.0 to 3.0	9.5×10^5

Table 2.5. The more common sizes of tissue culture multi-well plates. *Cell line dependent. Based upon a density of 1×10^5 cells/cm^2.

hybridomas, and common cell lines. RPMI typically requires supplementation with serum protein such as fetal bovine serum (FBS) (Arora, 2013). **See serum section.**

Most of the life sciences companies are providing complete and ready to use, fully supplemented conditioned medium and other culture media needs to add supplements like proteins and antibodies. The media can be also prepared in lab. As an example, to make RPMI 1640. To prepare this kind of media, add distilled water in clean glass beaker, then add the media powder to the water and stir gently till completely dissolved. After that, add sodium bicarbonate followed by adjust pH to 0.2-0.3 units below the required pH by using HCl or NaOH. Lastly, filter for sterility into sterile bottles.

Another example of media, Ham's F-12 Nutrient Mixture (F-12), which was designed for serum-free single-cell plating of Chinese Hamster Ovary (CHO) cells. It has been used for serum-free growth of CHO cultures as well as serum-supplemented growth of other mammalian cells including chondrocytes and rat prostate epithelial cells. The serum albumin and fetuin in this media are replaced by two compounds with important composition like linoleic acid and putrescine that enables colony formation by a single CHO cell under protein-free conditions (Tatsuma and Asayama, 2017). Compared to other basal media, F-12 contains a variety of other components including zinc, hypoxanthine, and thymidine. F-12 comes with no proteins or growth factors. Therefore, it requires supplementation including 10% Fetal Bovine Serum (FBS). F-12 uses a sodium bicarbonate buffer system so, it requires a 5-10% CO2 environment to maintain physiological pH at the time of preparation (Anonymous, n.d[8]).

As mentioned previously, one of the major advantages of cell culture is the capability to manipulate the physicochemical such as temperature, pH, osmotic pressure, O_2, and CO_2 tension. Also, the physiological environment including hormone and nutrient concentrations in which the cells proliferate. Some specific parts will be discussed below:

2.5.4. Serum

Serum is a mixture of albumins, growth factors that promote cell proliferation, and adhesion factors, that promote cell attachment and antitrypsin activity. Serum is also a source of minerals, lipids, and hormones, which may be bound to protein (Freshney, 2010). The sera used most in tissue culture are bovine calf, fetal bovine, adult horse, and human serum. Fetal bovine (FBS) serum and newborn calf serum (NBCS) are the most widely used for more demanding cell lines and for cloning (Freshney, 2010). The role of serum in cell culture is very complex due to the presence of both growth factors and inhibitors in it. The use of serum in tissue culture applications has several limitations (Mather and Roberts, 1998; Arora, 2013) (**Table 2.6**). In our laboratory, FB Essence has been used, it is a nutritionally rich, alternative to Fetal Bovine Serum (FBS), and effective of all cell types including both suspension and adherent cell types. FB Essence contains FBS, Bovine Calf Serum, Equine Serum, and a proprietary blend of supplements including vitamins, minerals, and growth factors.

Advantages of serum in media	Disadvantages of serum in media
Serum contains many growth factors and hormones which stimulates cell growth and functions.	Lack of uniformity in the composition of serum
Acts as a spreading factor	May contain some of the growth inhibiting factors
Helps in the attachment of cells	Testing needs to be done to maintain the quality of each batch before using
Functions as a binding protein	Presence of serum in media may interfere with the purification and isolation of cell culture products
Acts as a buffering agent which helps in maintaining the pH of the culture media	Increase the risk of contamination
Minimizes mechanical damages or damages caused by viscosity	

Table 2.6. shows the advantages and disadvantages of using serum in the media.

2.5.5. pH

Most normal mammalian cell lines grow well at pH 7.4, However, a slight variation in pH level is essential depending on the type and origin of cells (Nema and khare, 2012; Swain et al., 2014). However, some transformed cell lines have been shown to grow better at slightly more acidic environments (pH 7.0 - 7.4), and some normal fibroblast cell lines prefer slightly more basic environments (pH 7.4 - 7.7) (Nema and khare, 2012). It is important to measure the exact pH of the culture media to overcome any problem. The most culture media are buffered by using bicarbonate and the pH level of the medium checked by a pH indicator like phenol red. Therefore, the media may be changed / replenished. During cell culture, if the color of media turns yellow (acid) or purple (alkali). Also, it is recommended that not to use media with phenol red for studies using serum–free media formulations, as it is reported to interfere with the sodium potassium homeostasis (Swain et al., 2014).

2.5.6. Carbon dioxide

The pH of the medium is dependent on the delicate balance of dissolved carbon dioxide (CO_2) and bicarbonate (HCO_3). Therefore, changes in the atmospheric CO_2 can alter the pH of the medium that's why should consider measure it. Most researchers usually use 5% - 7% CO_2 in air and 4% - 10% CO_2 is common for most cell culture experiments. However, each medium has a recommended CO_2 tension and bicarbonate concentration to achieve the correct pH and osmolality (Nema and khare, 2012).

2.5.7. Temperature

Like pH, optimized temperature is required for proper growth of cells. The majority human and mammalian cell lines are maintained at 36˚C to 37˚C for optimal growth while in Avian, cell lines need 38.5˚C for maximum growth. Cell lines derived from cold-blooded animals (e.g., amphibians, cold-water fish) bear an extensive temperature vary between 15˚C and 26˚C (Nema and khare, 2012). At higher incubation temperature (>37^0 C), cultured cells will lose their viability and the enzymes of the cell denature or become inactive whereas at low temperature the CO_2 concentration increases and decreasing pH level), thereby influencing growth of cells (Freshney, 2010).

2.6. Adherent vs. Non-adherent Cells

In mammalian, most cells grow attached to some structure like connective tissue, basement membrane, or mineral matrix such as bone. In blood, lymph, and other fluids, cells are the only ones that normally grow "in suspension". However, many of these, such as lymphocytes have the ability to move into solid tissue when it is required. Many cells that grow in vivo as attached cells cannot grow in vitro as a single cell suspension and if they are placed in culture in such a way that they cannot attach to a surface or another cell, they will die. Cells from tumors and those transformed in vitro frequently lose this need for attachment. Lymphocytes grow readily in suspension and many cell types can survive and even function better when grown in conditions in which they do not attach and spread on a cell surface. In cluster where the cells are attached to each other are not growing as a single cell suspension, but do have a more rounded shape than they would if they were attached to surface. That’s why must be

take care about the differention between the effects of changes in culture surface and the effects of changes in cell shape because it may result from altering the surface on which they are grown (Mather and Roberts, 1998).

2.6.1. Anchorage-dependent or Adherent Cells

Adherent cells are those cells which require attachment for growth and are also called anchorage-dependent cells. In other words, these cells are capable of attaching on the surface of the culture vessel. These types of cells are often derived from the tissues of organs, for example from the kidney, where the cells are immobile and embedded in connective tissue.

2.6.2. Anchorage-independent or Suspension Cells

Suspension cells do not require attachment or any support for their growth and are also called anchorage-independent cells. All suspension cells are isolated from the blood system, for example white blood cell lymphocytes, and are suspended in plasma.

2.6.3. Different of Passaging Between Adherent Cells and Suspension Cells

The initial step in subculturing of monolayers adherent cells is to remove cells from the interface of the vessel by trypsinization or mechanical means. The final dispersion is then subdivided and transferred to fresh cultures. The growth of the secondary cultures is periodically monitored and further sub-cultured to produce tertiary cultures, etc. (Mather and Roberts, 1998; Harris et al., 2012).

Subculturing suspension cells is less complicated somewhat than passaging adherent cells because the cells are already suspended in growth medium so, there is no need to treat them enzymatically to detach them from the surface of the culture vessel. The replacement of growth medium is not achived in suspension cultures; instead, the cells are maintained by feeding them every 2 to 3 days until they reach their confluence. This can be done by directly diluting the cells in the culture flask and continue expanding them or by withdrawing a portion of the cells from the culture flask and diluting the remaining cells down to a seeding density appropriate for the cell line.

2.7. Possible Problems of Cell Cultures

One of the most complex challenges in tissue culture is protection of aseptic condition. There are possible problems that can cause contamination, which involves in malfunction in the sterilization procedures used for glassware, pipettes, particulates cross contamination of air inside the room, weakly maintained incubation, and inappropriate handling. The possibilities for unexpected infection with viruses or microorganisms or even cross-contamination with other cell types. Cell culture contaminants can be divided into two main categories: chemical contaminants such as impurities in media, sera, and water, endotoxins, plasticizers, and detergents. Biological contaminants are other category such as bacteria, molds, yeasts, viruses, mycoplasma, as well as cross contamination by other cell lines. Another difficulty with some cultured cells is their tendency to change their morphology, functions, or the range of genes they express.

The other possible problem is that the cells in suspension may attach to one another and can form clumps. the presence of free DNA and cell debris in the culture medium is the most common cause of cell clumping. There are some causes of DNA release into culture media including over digestion, which can happen to an excessive treatment with proteolytic enzymes like trypsin that used for cell detachment. Environmental stress like repeated freeze or thaw cycles also can cause cells to clump, Disaggregation of tissue can lead to some cell rupture. Overgrowth, when cells reach confluency, there is excessive buildup of free DNA from cell lysis and cell debris. Depending on the underlying cause of clumping, there is some solutions for cell clumping problems. DNase I, which is maximally active in the presence of bivalent ions (e.g., Mg^{2+} and Ca^{2+}), this can be used during tissue disaggregation in cell culturing to prevent clumping in a variety of cell types.

2.8. References

Abbondanzo SL, Allred DC, Lampkin S, et al. (1991). Enhancement of immunoreactivity among lymphoid malignant neoplasms in paraffin-embedded tissues by refixation in zinc sulfate formalin. Arch Path Lab Med,115:31–33.

Aziz S, Mehta, et al. (2016). Technical aspects of toxicological immunohistochemistry: system specified biomarkers. Springer.

Anonymous. (n.d)[1]. Immunohistochemistry (IHC) principle. Guide https://www.bosterbio.com/protocol-and-troubleshooting/immunohistochemistry-ihc-principle

Anonymous. (n.d)[2]. Overview of immunohistochemistry (IHC). https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-immunohistochemistry.html.

Anonymous. (n.d.)[3]. IHC/ICC sample fixation (Formalin vs. alcohol). Novus Biologicals. Retrieved November 29, 2021, from https://www.novusbio.com/sample-fixation-for-ihc-icc.

Anonymous. (2017)[4]. Immunocytochemistry vs. immunohistochemistry. Study.com. https://study.com/academy/lesson/immunocytochemistry-vs-immunohistochemistry.htm

Anonymous. (n.d)[5]. Vertical laminar flow hoods vs. biological safety cabinets. https://www.airscience.com/briefs/briefnum/9/vertical-laminar-vs-bsc.

Anonymous. (n.d.)[6]. ATCC animal cell culture guide. https://www.atcc.org/resources/culture-guides/animal-cell-culture-

guide?matchtype=&network=g&device=c&adposition=&keyword=&gclid=Cj0KCQiAk4aOBhCTARIsAFWFP9Hy0tvuDlJnbLAMbWa76r60fWgvbuGgJkLUga12yw1xGIVNK1ajFZkaAlXpEALw_wcB.

Anonymous. (n.d)[7]. Difference between: 96-well plate bottom shapes. https://www.wellplate.com/96-well-plate-bottom-shapes/.

Anonymous. (n.d)[8]. Ham's F-12 nutrient mix, powder. https://www.thermofisher.com/order/catalog/product/21700075?ef_id=Cj0KCQiAoNWOBhCwARIsAAiHnEhe5r0oJHQ3poP_Hn1y9dKI27rOY9dufMGQUpJSBf4zJPITMuXc1fAaAnqjEALw_wcB:G:s&s_kwcid=AL!3652!3!305473461783!!!g!!&cid=bid_clb_cce_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!305473461783!!!g!!&gclid=Cj0KCQiAoNWOBhCwARIsAAiHnEhe5r0oJHQ3poP_Hn1y9dKI27rOY9dufMGQUpJSBf4zJPITMuXc1fAaAnqjEALw_wcB.

Arora M. (2013). Cell culture media: A review. Mater Meth doi: dx.doi.org /10.13070 /mm.en.3.175.

Bhatia S. (2019). Introduction to pharmaceutical biotechnology. IOP Publishing.

Beesley. JE. (2001). Immunocytochemistry and in situ hybridization in the biomedical sciences. Birkhäuser. 1:P XI, 267. https://doi.org/10.1007/978-1-4612-0139-7.

Boenisch T. (1999). Diluent buffer ions and ph: Their influence on the performance of monoclonal antibodies in immunohistochemistry. Applied Immunohistochem Molecul Morphol. 7(4). P 300.

Burry RW. (2011). Controls for immunocytochemistry. J Histochem Cytochem, 59(1), 6–12.

Conrad B. (2020). https://www.enzolifesciences.com/science-center/technotes/2020/september/ihc-counterstains-for-enzyme-chromogen-and-fluorescent-immunostaining/.

Dils RR. (1984). Explants and disaggregated tissue preparations as model systems in nutritional research: advantages and pitfalls. Proc Nutr Soc, 43(2):133-40.

Dagur PK, McCoy JP. (2015). Collection, storage, and preparation of human blood cells. Curr Protoc Cytom, 1;73:5.1.1-5.1.16.

Elias JM. (2003). Immunohistopathology: a practical approach to diagnosis. Chicago: American Society for Clinical Pathology Press.

Elias JM. (2003). Immunohistochemical methods. In: Immunohistopathology. A Practical Approach to Diagnosis, ed. Elias JM, 2nd ed., P 1–110. American Society for Clinical Pathology Press, Chicago, IL.

Freshney. (2010). Culture of animal cells: a manual of basic technique and specialized applications (6th ed.). Wiley-Blackwell.

Hayat MA. (2002). Fixation and embedding. In: Microscopy, immunohistochemistry, and antigen retrieval methods for light and electron microscopy, ed. Hayat MA, pp. 71–93. Kluwer Academic, New York, NY, 2002.

Harris AR, Peter L, Bellis J, et al. (2012). Characterizing the mechanics of cultured cell monolayers. Proc Natl Acad Sci USA, 109(41):16449-54.

Hausen P, Dreyer C. (1982). Urea reactivates antigens in paraffin sections for immunofluorescent staining. Stain Technology, 57:321–324.

Igor B. Buchwalow., Werner Böcker. (2010). Immunohistochemistry: Basics and Methods. Springer-Verlag Berlin Heidelberg. 1: X, 153.

Jamur MC, Oliver C. (2010). Permeabilization of cell membranes. Methods Mol Biol., 588:63-6.

Kitamoto T, Ogomori K, Tateshi J, Prusiner SB. (1987). Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloidosis. Lab Invest, 57; 230–236.

Linthicum FH, Tian Q, Milicic M. (1995). Constituents of the endolymphatic tubules as demonstrated by three-dimensional morphometry. Acta Laryngolaryngologica. 115, 246–250.

Magaki S, Hojat SA, Wei B, et al. (2019). An Introduction to the Performance of Immunohistochemistry. Methods Molecul Biol. 1897: 289-298.

Mather, Jennie P, Penelope E. (1998). Roberts. Introduction to Cell and Tissue Culture: Theory and Technique / Jennie P. Mather and Penelope E. Roberts. New York: Plenum Press, 1998. Print.

McNicol AM, Richmond JA. (1998). Optimizing immunohistochemistry: antigen retrieval and signal amplification. Histopathol,32(2):97-103.

Nema, Khare, S. (2012). An animal cell culture: Advance technology for modern research. Advances in Bioscience and Biotechnology, *3*(3):219–226.

Polak JM, Van Noorden S. (2003). Introduction to Immunocytochemistry, 3rd ed. Bios Scientific Publishers Ltd, Oxford, UK,

Puchtler H, Meloan SN. (1985). On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. Histochemistry, 82:201–204.

Ramos-Vara JA. (2017). Principles and Methods of Immunohistochemistry. Methods Molcul Biol,1641:115-128.

Ramos-Vara JA, Beissenherz ME. (2000). Optimization of immunohistochemical methods using two different antigen retrieval methods on formalin-fixed, paraffin-embedded tissues: experience with 63 markers. J Vet Diag Invest, 12:307–311.

Ramos-Vara JA. (2005). Technical aspects of immunohistochemistry. Vet Pathol, 42(4):405-26.

Rodig SJ. (2019). Counterstaining, Mounting, and Photographing Stained Cells. Cold Spring Harb Protoc, 1;2019(4).

Straus W. (1979). Peroxidase procedures: technical problems encountered during their application. J Histochem Cytochem, 27:1349–1351.

Titus JA, Haugland R, Sharrow SO, et al. (1982). Texas Red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. J Immunol Methods, 50(2):193-204.

Yong WH, Dry SM, Shabihkhani M. (2014). A practical approach to clinical and research biobanking. Methods Molcul Biol, 1180: 137–162.

Yao T, Asayama Y. (2017). Animal-cell culture media: History, characteristics, and current issues. Reprod Med Biol, 16(2):99-117.

CHAPTER 3

EXPRESSION OF LUTEINIZING HORMONE RECEPTOR (LHR) IN CANINE LYMPHOMA IS NOT AFFECTED BY BODY WEIGHT, SEX, IMMUNOPHENOTYPE, OR TUMOR STAGE

Zwida KH, Vedus AV, Kutzler AM

Clinical Theriogenology

Volume 13, Issue 1

March, 2021

3.1. Abstract

The purpose of this investigation was to determine if luteinizing hormone receptor (LHR) expression in canine lymphoma was affected by body weight, sex, immunophenotype (B-cell, T-cell) or tumor stage. Formalin-fixed, paraffin-embedded lymphoma tissue samples from spayed and neutered dogs representing multiple breeds and mixed breeds (n=40) were subjected to routine immunohistochemical techniques using a polyclonal LHR antibody. The percentage of cells positive for LHR and the staining intensity (scored 0-3) were determined at 400X magnification. Data were expressed as mean ± standard deviation and significance was defined as $p<0.05$. Differences between sex and tumor phenotype were compared using a Welch two-sample t-test and differences in body weight and tumor stage were compared using a simple linear regression. All tumor samples contained cells positive for LHR but the percentage of cells expressing LHR and the LHR immunostaining intensity varied between individual. There were no significant differences in the percentage of LHR positive cells or staining intensity in canine lymphoma when compared to sex and immunophenotype. In addition, there was no significant correlation between the percentage of LHR positive cells or staining intensity in canine lymphoma when compared with body weight or tumor stage. These results indicate that the increased risk of lymphoma in spayed and neutered dogs is not related to body weight, sex, immunophenotype or tumor stage, but more likely related to increased LHR activation following gonadectomy.

3.2. Introduction

Canine lymphoma is the most common hematopoietic neoplasm in the dog, accounting for up to 24% of all canine cancers (MacEwen et al., 1987; Vail et al., 2001). Lymphoma can be derived from either B-cell or T-cell lymphocytes. Immunophenotyping is possible because B-cell lymphomas typically express cell surface proteins CD79a, CD20, CD21, and PAX5, whereas T-cell lymphomas typically express the unique cell surface protein CD3 (Caniatti et al., 1996; Fulkerson, 2016). Symptoms and treatment options vary depending on lymphoma predominate cell type, i.e. B- or T-cells (Burke, 2016). Although canine lymphoma is typically treated by chemotherapy, B-cell lymphomas become resistant to chemotherapy after subsequent treatments, and T-cell lymphomas often do not respond well to chemotherapy from the onset of treatment (Marconato et al., 2013). Prognosis following diagnosis with canine lymphoma is also dependent on the immunophenotype of the lymphoma, with average survival times shorter with T-cell than B-cell lymphomas (183 days compared to 365 days, respectively) (Fan, 2018). In general, T-cell lymphomas also have shorter remission times than B-cell lymphomas (Greenlee, 1990; Teske et al. 1994; Valli et al. 2013).

The Basset Hound, Beagle, Boxer, Bulldog, Bull Mastiff, Cocker Spaniel, German Shepherd, Golden Retriever, Labrador Retriever, Rottweiler, Shih Tzu, St. Bernard, and any terrier breed are at the higher risk for developing lymphoma with lower relative risk including Dachshunds and Pomeranians (Fulkerson, 2016). In addition, breed also appears to influence the lymphoma immunophenotype. Boxers are more likely to develop T-cell lymphomas whereas Basset Hounds and Cocker Spaniels

are more likely to develop B-cell lymphomas (Dobson, 2013). Irrespective of breed, medium and large breed dogs are more likely to develop lymphoma compared to toy and small breed dogs (Valli et al., 2013). With respect to prognosis, smaller body weight dogs (<17 kg) are reported to have longer median survival times (Garrett et al., 2002; Valerius, 1997). There was no difference in median survival times (Deravi et al., 2017; MacEwen et al., 1987) when compared to dogs with larger body weights. However, body condition was not reported in these studies. It is important to mention that dogs with an underweight body condition are reported to have significantly shorter survival times than dogs in ideal or overweight body condition (Romano et al., 2016).

Several studies have shown differences in cancer survival by sex. In humans, men generally have shorter survival times than women for most cancers including lymphoma (Afshar et al., 2018; Cook et al., 2011; Radkiewicz et al., 2017). This is similar to what has been reported in dogs with advanced multicentric lymphoma, in that intact male dogs had significantly shorter remission and survival times than intact female dogs (Villamil et al., 2009).

Luteinizing hormone receptors (LHR) are expressed in canine lymphoma tissue and isolated canine lymphoma cells (Ettinger, 2019). However, the influence of body weight or sex on LHR expression was not evaluated. In addition, the influence of immunophenotype or tumor stage on LHR expression was not evaluated. Based upon what has been previously reported about canine lymphoma, it was hypothesized that larger body weight male dogs with advanced tumor stages and with T-cell lymphomas would have higher LHR expression in their lymphoma tissue than that of smaller body weight female dogs with earlier tumor stages and with B-cell lymphomas.

3.3. Materials and Methods

This study was conducted using archived tissue samples obtained from the Canine Comparative Oncology and Genomics Consortium (Frederick, MD, 2018). Samples were collected from forty purebred and mixed breed dogs (spayed females n=24; neutered males n=16) weighing 7-56 kg. Immunophenotyping was available on 26 of the 40 tumors. Tumors were staged using the World Health Organization system for tumor grading: stage I, single node or lymphoid tissue in single organ; stage II, regional involvement of multiple lymph nodes; stage III, generalized lymph node involvement; stage IV, stages I-III with the involvement of the liver and/or spleen; and stage V, stages I-IV with involvement of blood or bone marrow (Owen, 1980). In addition, samples from each primary tumor were formalin-fixed, paraffin embedded, and sectioned onto charged slides for LHR immunohistochemistry. All slides were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval (#S1700, Dako, Carpinteria, CA). Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide and nonspecific binding was blocked Protein Block Serum-Free (#X0909, Dako, Carpinteria, CA). Either rabbit polyclonal anti-human LHR antibody (#NLS1436, Novus Biologics, Centennial, CO) was applied at a 1:100 dilution or rabbit negative control (#NC495H, Biocare Medical, Pacheco, CA) was applied to slides. This antigen retrieval method and antibody concentration was previously found to be optimal in normal and neoplastic canine lymphatic tissue (Ettinger, 2019). Slides were reacted with One Step Horse Radish Peroxidase-Conjugated Polymer Anti-Rabbit IgG (#IH-8064-custom-OrSU, ImmunoBioScience, Mukilteo, WA) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories, Burlingame, CA). Slides were

counter-stained with hematoxylin, dehydrated, and mounted. The percentage of cells positive for LHR and the staining intensity (scored 0-3) was determined at 400X magnification by a single observer (AV) blinded to the identity of the samples.

Differences between sex and tumor phenotype were compared using a Welch two-sample t test in the free statistical package R (version 1.2.1355, Boston, MA). Differences in bodyweight and tumor stage were compared using a simple linear regression (Microsoft Excel, version 14.5.2, Redmond, WA). Data were expressed as mean ± standard deviation and significance was defined as $p < 0.05$.

3.4. Results

Although there were many purebred dogs included in the data set, mixed breed dogs were overrepresented (**Table 3.1**). Most breeds were only represented by one individual; with the exception of Labrador retrievers (n=3), German shorthaired pointers (n=2), poodles (n=2), miniature schnauzers (n=2), Boston terriers (n=2). Irrespective of breed, all lymphoma tissue samples contained cells positive for LHR (**Figure 3.1**), but the percentage of cellular expression and staining intensity varied between individuals. When the influence of body weight was examined, there was no correlation between the percentage of LHR positive cells (R^2=0.021) or staining intensity (R^2=0.077). There were also no significant differences in the percentage of LHR positive cells or staining intensity when compared by sex (**Figure 3.2**) or lymphoma phenotype (**Figure 3.3**). There was no correlation between the percentage of LHR positive cells (R^2= 0.0017) or staining intensity (R^2=0.063) when compared by tumor stage.

Breed	Sex	Weight (kg)	Tumor phenotype	Tumor stage	Percent positive	Staining intensity	h-score
Miniature Schnauzer	F	7	NA	3	10	2.5	25
Shih Tzu	F	7.2	NA	5	10.5	1	10.5
Boston Terrier	F	8	NA	2	10.5	3	31.5
Pembroke Welsh Corgi	F	9.5	NA	3	6.8	3	20.4
Miniature Schnauzer	F	11.4	NA	3	3	2.5	7.5
Shetland Sheepdog	F	12.6	B cell	3	23.5	3	70.5
Cocker Spaniel	F	14	T cell	3	10.3	3	30.7
Poodle	M	15.2	B cell	5	5.75	2.5	14.4
Boston Terrier	M	16	B cell	3	20	2	40
Mixed breed	F	17.3	NA	5	16.6	2	33.2
Vizsla	F	19	B cell	3	20.8	1.5	31.2
Mixed breed	F	19.7	NA	3	7.5	3	22.5
Labrador Retriever	F	21	T cell	5	7.5	2	15
Mixed breed	M	21.4	B cell	3	3	2	6
Poodle	F	22	B cell	4	3.6	2	7.2
Mixed breed	F	22.6	B cell	3	9	2.5	22.5
German Shorthair Pointer	F	24.5	NA	4	10.8	3	32.4
German Shorthair Pointer	F	26	NA	4	9.5	2	19
Mixed breed	M	26	B cell	4	21.8	2	43.6
Samoyed	F	26.7	NA	2	17.65	3	52.9
Boxer	M	28.6	T cell	3	15	2	30
Labrador Retriever	F	28.6	NA	2	19	2	38
Mixed breed	F	28.8	B cell	3	6.5	2	13
Dalmatian	M	29	B cell	3	9	2	18
Mixed breed	M	29.3	B cell	4	2	1.5	3
Mixed breed	M	29.5	B cell	4	10	1.5	15
Bernese Mountain Dog	M	30	T cell	3	4.3	1.5	6.4
Labrador Retriever	F	30	T cell	5	5.9	2	11.8
Mixed breed	M	31.6	B cell	4	13.4	2	26.8
Mixed breed	M	32	B cell	3	7.8	2	15.6
Basset Hound	F	34.6	NA	3	8.2	3	24.6
Australian Shepherd	M	35.2	T cell	5	10.3	2	20.6
Mixed Breed	M	35.2	B cell	3	10	1	10
Mixed breed	F	37.2	B cell	4	5	3	15
Black/Tan Coonhound	F	37.5	NA	3	3.6	2	7.2
Mixed breed	F	38	B cell	4	26.5	0.5	13.2
Irish Setter	M	46.5	B cell	4	7	3	21
Mixed breed	M	50	B cell	3	8	2	16
Bullmastiff	F	52	B cell	5	7.3	0.5	3.6
Saint Bernard	M	56	NA	5	6.3	2.5	15.6

Table 3.1. Gonadectomized dogs with lymphoma studied: body weight, sex, tumor phenotype, tumor stage on the percentage of LHR positive cells, staining intensity, and histology score (h-score). Female (F); Male (M); Not available (NA).

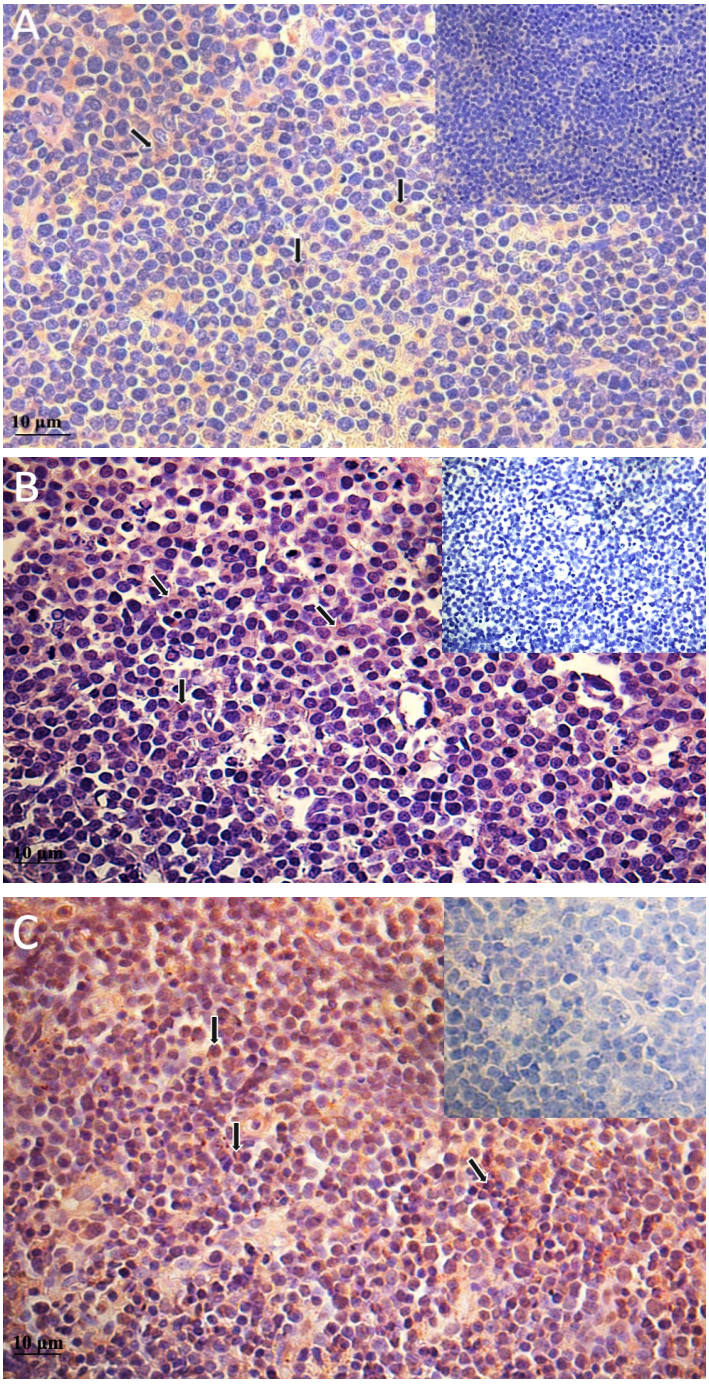


Figure 3.1. Representative images for luteinizing hormone receptor staining intensity in canine lymphoma. Arrows illustrate examples of cells stained positive. Negative control in upper right inset. **A**: staining intensity = 1; **B**: staining intensity = 2; **C**: staining intensity = 3. Bar = 10 µm.

3.5. Discussion

Several studies have shown breed predispositions for developing lymphoma (Dobson, 2013; Fulkerson, 2016; Valli et al., 2013). However, there were not enough dogs represented by any single breed in the current study to compare the expression of LHR, so the effect of body weight was examined. In agreement with previous research on LHR expression in the bladder (Reichler et al., 2007), body weight did not influence LHR expression in canine lymphoma. It is important to note that body condition score was not available in the current study, which could have been a confounding factor since underweight dogs with lymphoma are reported to have significantly shorter survival times (Romano et al., 2016).

Sex plays a role in remission and survival times following lymphoma diagnosis (Valli et al., 2013; Deravi et al., 2017; MacEwen et al., 1987; Keller et al., 1993). For this reason, we sought to determine if LHR expression in lymphoma differed by sex in dogs. Although there was no significant influence of sex on LHR expression, it is important to mention that the case material available for this study was unfortunately limited to only spayed and neutered dogs. Lymphoma is three to four times more common in spayed female and neutered male dogs compared to intact females and males (Torres de la Riva et al., 2013; Zink et al., 2014). In addition, lymphoma is rarely reported in intact females (Torres et al., 2013). The absence of gonadal hormones in the dogs sampled for the current study may have masked what effect sex has on LHR expression. Further research is needed to determine if LHR expression within lymphoma tissue is increased in gonadectomized compared to intact dogs.

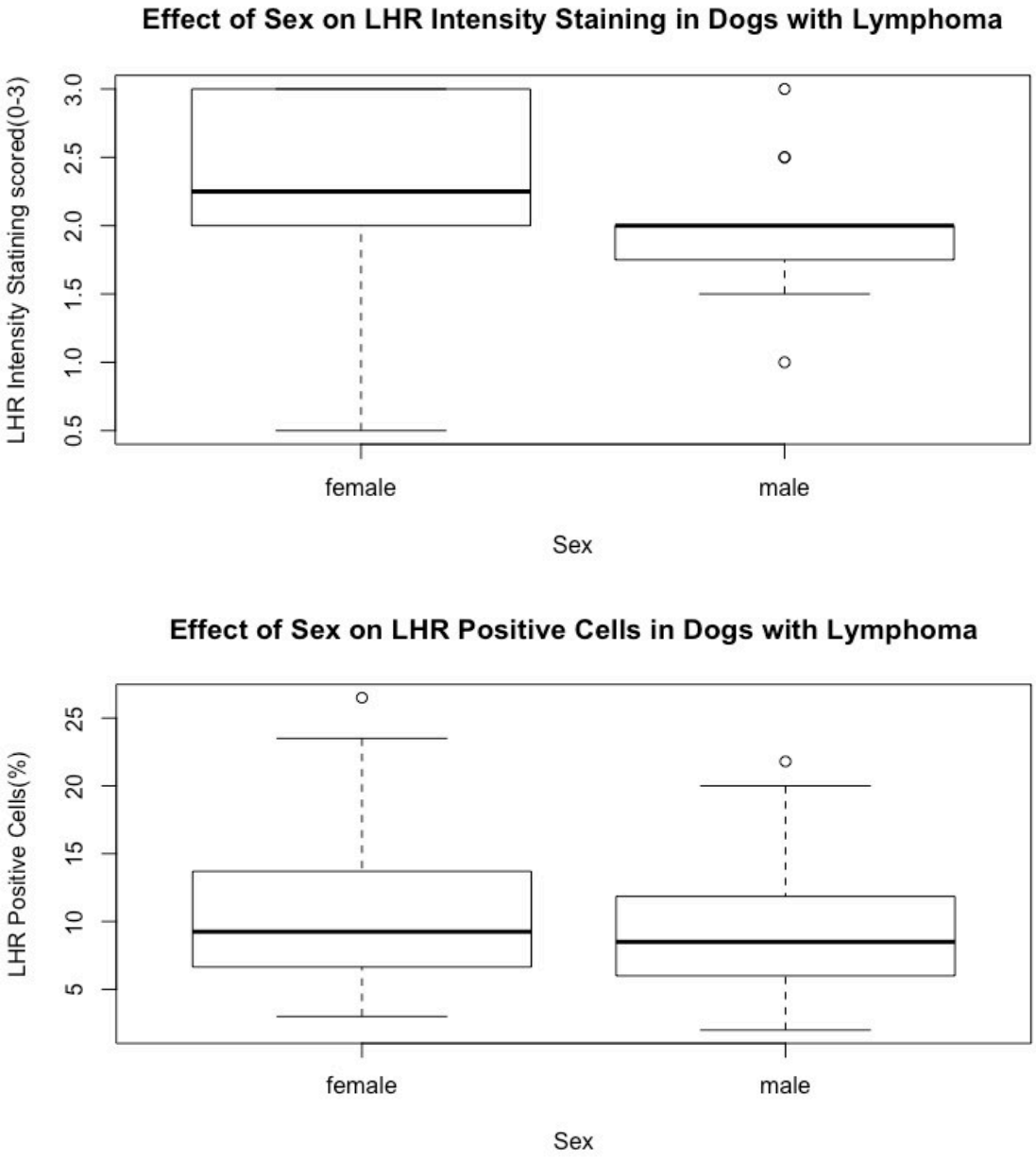


Figure 3.2. Effect of sex (neutered male vs. spayed female) on luteinizing hormone receptor (LHR) in canine lymphoma tissue. Staining intensity (top) and percentage of positive cells (bottom). There was no significant effect of sex on LHR expression ($p>0.05$) in the excised tumors.

Lymphoma phenotype often influences receptor expression. For example, most human B-cell lymphomas depend on the expression of a B-cell receptor for continued growth (Küppers, 2005). In dogs, the retinoid receptor is expressed higher in T-cell lymphomas compared to B-cell lymphomas (de Mello et al., 2014). Therefore, one of the objectives of the current study was to determine if LHR expression changed with lymphoma phenotype. Although more B-cell lymphomas were present in the data set (n=20) than T-cell lymphomas (n=6), there was no significant difference in the relative percentage of cells expressing LHR or in the relative intensity of LHR staining between phenotypes.

Receptor expression also can vary with advancing tumor stage. For example, estrogen receptor beta is expressed higher in human B-cell lymphoma with tumor stage of III or IV (Faknuam et al., 2017). Alternatively, LHR expression in human ovarian cancer is higher in stages I and II compared to stages III and IV (Cui et al., 1997). In the current study, LHR expression varied widely within tumor stage, which is similar to what has been reported with expression of steroid hormone receptors in canine and human mammary tumors (Millanta et al., 2005; Picon et al., 1996; Bardou et al.,2003). The lack of correlation between receptor expression and tumor stage could result from the non-static expression LH receptors in tumors, as has been reported with the lipoprotein receptors in canine lymphoma (Ceres et al., 2018).

Activation of LHR in luteal (Davis et al., 1996; Menon, 2014) and trophoblast cells (Reshef et al., 1990) as well as in canine T-cell lymphoma cells (Flint et al., 2019) results in dose-dependent cell proliferation. Because circulating LH concentrations are sustained at significantly higher concentration in spayed and neutered dogs (Beijerink

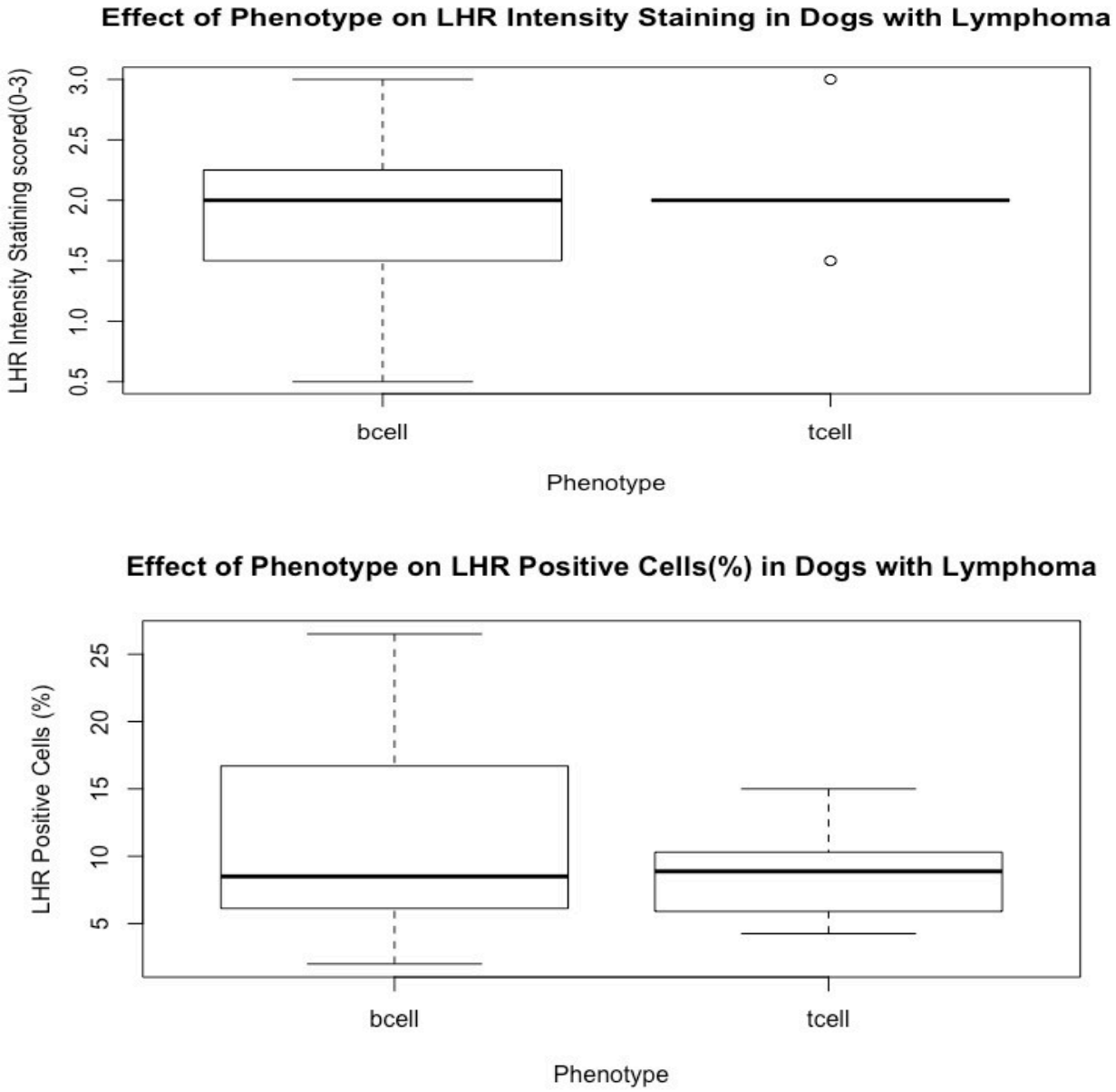


Figure 3.3. Effect of lymphoma phenotype (B-cell vs. T-cell) on luteinizing hormone receptor (LHR) staining intensity (top) and percentage of positive cells (bottom). There was no significant effect of phenotype on LHR expression ($p>0.05$).

et al., 2007), it is possible that LHR activation plays a role in the etiopathogenesis of canine lymphoma. Additional research is needed to determine if reducing LH concentrations in spayed and neutered dogs with a gonadotropin-releasing hormone agonist can prolong survival time in dogs with canine lymphoma

3.6. References

Afshar N, English DR, Thursfield V, et al. (2018). Differences in cancer survival by sex: a population-based study using cancer registry data. Cancer Causes Control, 29(11), 1059–1069.

Bardou VJ, Arpino G, Elledge RM, et al. (2003). Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. J Clin Oncol, 21(10), 1973–1979.

Beijerink NJ, Buijtels JJ, Okkens AC, et al. (2007). Basal and GnRH-induced secretion of FSH and LH in anestrous versus ovariectomized bitches. Theriogenology, 67(5), 1039–1045.

Burke A. (2016). Lymphoma in dogs: symptoms, diagnosis, and treatment. American Kennel Club. Accessed April 22, 2018. Available from: https://www.akc.org/expert-advice/health/common-conditions/lymphoma-in-dogs-symptoms-diagnosis-and-treatment/.

Caniatti M, Roccabianca P, Scanziani E, et al. (1996). Canine lymphoma: immunocytochemical analysis of fine-needle aspiration biopsy. Vet Path, 33(2), 204–212.

Ceres K, Fitzgerald H, Quiznon KS, et al. (2019). Immunohistochemical labeling of low-density lipoprotein receptor and scavenger receptor class B type 1 are increased in canine lymphoma. Front Vet Sci, 5, 340.

Cook MB, McGlynn KA, Devesa SS, et al. (2011). Sex disparities in cancer mortality and survival. Cancer Epidem Biomark Prev, 20(8), 1629–1637.

Cui J, Guo Y, You Z. (1997). LH-CG receptor protein expression in epithelial ovarian cancer. Zhonghua Fu Chan Ke Za Zhi, 32, 742-745.

Davis JS, May JV, Keel BA. (1996). Mechanisms of hormone and growth factor action in the bovine corpus luteum. Theriogenology, 45(7), 1351–1380.

Deravi N, Berke O, Woods JP, et al. (2017). Specific immunotypes of canine T cell lymphoma are associated with different outcomes. Vet Immunol Immunopathol, 191, 5–13.

de Mello Souza CH, Valli VE, Kitchell BE. (2014). Detection of retinoid receptors in non-neoplastic canine lymph nodes and in lymphoma. Can Vet J, 55(1), 1219–1224.

Dobson JM. (2013). Breed-predispositions to cancer in pedigree dogs. Internat Schol Res Notices, 2013, 941275. Doi.10.1155/2013/941275

Ettinger AM, Gust SK, Kutzler MA. (2019). Luteinizing hormone receptor expression by nonneoplastic and neoplastic canine lymphocytes. Am J Vet Res, 80(6), 572–577.

Faknuam S, Assanasen T, Ruangvejvorachai P, et al. (2018). Estrogen receptor beta expression and prognosis of diffuse large B cell lymphoma. Hematology, 23(4), 235–241.

Fan TM. (2018). Overview of canine lymphoma - circulatory system. Merck Veterinary Manual. Accessed April 21, 2018. Available from: https://www.merckvetmanual.com/circulatory-system/canine-

lymphoma/overview-of-canine-lymphoma.

Flint C, Gust S, Kutzler MA. (2019). Luteinizing hormone receptor mediated proliferation of isolated canine T lymphoma cells. Clin Theriogenology, 11: 477.

Garrett LD, Thamm DH, Chun R, et al. (2002). Evaluation of a 6-month chemotherapy protocol with no maintenance therapy for dogs with lymphoma. J Vet Intern Med, 16(6), 704–709.

Greenlee PG, Filippa DA, Quimby FW, et al. (1990). Lymphomas in dogs. A morphologic, immunologic, and clinical study. Cancer, 66(3), 480–490.

Keller ET, MacEwen EG, Rosenthal RC, et al. (1993). Evaluation of prognostic factors and sequential combination chemotherapy with doxorubicin for canine lymphoma. J Vet Intern Med, 7(5), 289–295.

Küppers R. (2005). Mechanisms of B-cell lymphoma pathogenesis. Nature reviews. Cancer, 5(4), 251–262.

Marconato L, Gelain ME, Comazzi S. (2013). The dog as a possible animal model for human non-Hodgkin lymphoma: a review. Hematol Oncol, 31(1), 1–9.

Millanta F, Calandrella M, Bari G, et al. (2005). Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. Res Vet Sci, 79(3), 225–232.

Menon KM, Menon B. (2014). Regulation of luteinizing hormone receptor expression by an RNA binding protein: role of ERK signaling. Indian J Med Res, 140(Suppl 1), S112–S119.

MacEwen EG, Hayes AA, Matus RE, et al. (1987). Evaluation of some prognostic factors for advanced multicentric lymphosarcoma in the dog: 147 cases (1978-1981). J Am Vet Med Assoc, 190(5), 564–568.

Owen LN. (1980). TNM classification of tumours in domestic animals. Geneva: World Health Organization. Document number: VPH/CMO/80.20. Available at: https://apps.who.int/iris/handle/10665/68618

Pichon MF, Broet P, Magdelenat H, et al. (1996). Prognostic value of steroid receptors after long-term follow-up of 2257 operable breast cancers. Brit J Cancer, 73(12), 1545–1551.

Romano FR, Heinze CR, Barber LG, et al. (2016). Association between Body Condition Score and Cancer Prognosis in Dogs with Lymphoma and Osteosarcoma. J Vet Intern Med, 30(4), 1179–1186.

Radkiewicz C, Johansson A, Dickman PW, et al. (2017). Sex differences in cancer risk and survival: A Swedish cohort study. Eur J Cancer (Oxford, England: 1990), 84, 130–140.

Reichler IM, Welle M, Sattler U, et al. (2007). Comparative quantitative assessment of GnRH- and LH-receptor mRNA expression in the urinary tract of sexually intact and spayed female dogs. Theriogenology, 67(6), 1134–1142.

Reshef E, Lei ZM, Rao CV, et al. (1990). The presence of gonadotropin receptors in nonpregnant human uterus, human placenta, fetal membranes, and decidua. J Clin Endocrinol Metabol, 70(2), 421–430. Doi.10.1210/jcem-70-2-421

Teske E. (1994). Canine malignant lymphoma: a review and comparison with human non-Hodgkin's lymphoma. Vet Q, 16(4), 209–219.

Torres de la Riva, G., Hart BL, Farver TB, et al. (2013). Neutering dogs: effects on joint disorders and cancers in golden retrievers. PloS One, 8(2), e55937. Doi.10.1371/journal.pone.0055937

Vail DM, MacEwen, EG, Young KM. (2001). Canine lymphoma and lymphoid leukemia. In: Withrow, S.J., MacEwen, E.G. (eds.), Small Animal Clinical Oncology, 3rd ed. WB Saunders Co, Philadelphia, PA 2001; 558-590.

Valli VE, Kass PH, San Myint M. (2013). Canine lymphomas. Vet Pathol, 2013; 50; 738-748.

Villamil JA, Henry CJ, Hahn AW, et al. (2009). Hormonal and sex impact on the epidemiology of canine lymphoma. J Cancer Epidemiol, 2009, 591753. Doi.10.1155/2009/591753

Valerius KD, Ogilvie GK, Mallinckrodt CH, et al. (1997). Doxorubicin alone or in combination with asparaginase, followed by cyclophosphamide, vincristine, and prednisone for treatment of multicentric lymphoma in dogs: 121 cases (1987-1995). J Am Vet Med Assoc, 210(4), 512–516.

Zink MC, Farhoody P, Elser SE, et al. (2014). Evaluation of the risk and age of onset of cancer and behavioral disorders in gonadectomized Vizslas. J Am Vet Med Assoc, 244(3), 309–319.

CHAPTER 4

LUTEINIZING HORMONE RECEPTOR EXPRESSION IN MASTOCYTOMA CELLS IS INCREASED IN SPAYED AND NEUTERED DOGS

Valerio Moccia, Khawla Zwida, Christiane Löhr, Michelle Kutzler

Journal of the American Animal Hospital Association

Accepted for publication in September 2021

Expected Publication in September 2022

4.1. Abstract

The expression of luteinizing hormone receptors (LHR) has been reported in canine lymphoma and hemangiosarcoma. We hypothesized that LHR would also be expressed in canine mastocytoma (MCT) and that more mastocytoma cells would express LHR in gonadectomized dogs compared to intact dogs. Eleven archived formalin-fixed paraffin-embedded cutaneous MCT tissue sections were processed using routine immunohistochemistry. For both KIT (a transmembrane tyrosine kinase receptor) and LHR, the percentage of positive cells for each staining pattern (I-III) was calculated. Comparisons were made between intact and gonadectomized dogs using a student's t test for the total percentage of positive cells expressing LHR and KIT. Comparisons were made using a one-way analysis of variance between the percentage of cells within each staining pattern for LHR and KIT in intact and gonadectomized dogs. All MCT expressed LHR. MCT from gonadectomized dogs had a significantly higher percentage of LHR-positive mastocytoma cells (84.2±8.7%) compared to MCT from intact dogs (64.3±4.2%). This is the first study to demonstrate the expression of LHR in canine MCT and to report that LHR expression is increased in mastocytoma cells from gonadectomized dogs when compared to LHR expression in intact dogs. Future studies are planned to evaluate the functionality of the LHR in canine mastocytoma cells.

4.2. Introduction

Mastocytomas (MCTs) are one of the most common neoplasms in dogs, accounting for 10–21% of all skin tumors (Villamil et al., 2011). The biologic behavior

of canine MCTs is extremely variable. Prognostic and therapeutic determinations of MCTs are based primarily on their histologic grade. Two grading systems (Patnaik and Kiupel) were developed for canine MCT (Patnaik et al., 1984; Kiupel et al., 2011). The cKit protooncogene encodes for the KIT protein (KIT, a transmembrane tyrosine kinase receptor) that normal mast cells and some mastocytoma cells express mainly on the cell membrane (Kiupel et al., 2004). However, in many mastocytoma cells, KIT accumulates in the cytoplasm, primarily adjacent to the nucleus (Kiupel et al., 2004). Immunoexpression of KIT has been evaluated in the context of MCT histologic grade, with well-differentiated tumors having weak KIT expression and poorly-differentiated tumors having high KIT expression (Reguera et al., 2000). In addition, increased cytoplasmic KIT immunoexpression is significantly associated with an increased rate of local MCT recurrence and decreased survival rate (Kiupelet et al., 2004). It is important to mention that mutations in KIT have been reported and these mutations may not be related to KIT immunoexpression. For example, juxtamembrane mutations in KIT have been reported in approximately 25-30% of canine MCTs, resulting in spontaneous phosphorylation and activation of KIT signaling which is associated with more aggressive biologic behavior (Ma et al., 1999). Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mastocytomas in dogs (Zemke et al., 2002).

A significantly increased risk for developing MCT in gonadectomized female dogs has been reported (White et al., 2011). Gonadectomy prevents negative feedback of gonadal hormones (e.g. estrogen, progesterone) to the anterior pituitary, resulting in sustained, supraphysiologic circulating concentrations of luteinizing hormone (LH), up

to twenty times the concentrations found in pre-ovulatory intact female dogs (Beijerink et al., 2007). In dogs, luteinizing hormone receptors (LHRs) are present in reproductive and non-reproductive normal tissues including the bladder and urethra (Ponglowhapan et al., 2007), skin (Welle et al., 2006), vascular smooth muscle cells (Welle et al., 2006), thyroid gland (Zwida and Kutzler, 2019), and adrenal cortex (Galac et al., 2010). In addition, LHR expression has been reported in several neoplastic canine tissues including lymphoma (Ettinger et al., 2019), hemangiosarcoma (Zwida and Kutzler, 2016), and adrenocortical tumor (Abdelbaset-Ismail et al., 2017). High LHR expression has been linked to increased metastatic potential of lung, endometrial, breast, and lymphoid cancers in humans (Abdelbaset-Ismail et al., 2017; Noci et al., 2008; Sanchez et al., 2016; Abdelbaset-Ismail et al., 2016).

The objective of this study was to determine if canine cutaneous mastocytoma (MCT) expresses LHR. We hypothesized that gonadectomized dogs would have a higher expression of LHR in MCT cells compared to LHR in cutaneous tissue in intact dogs.

4.3. Material and Methods

4.3.1. Samples

Archived formalin-fixed paraffin-embedded cutaneous MCT cases (n=11) submitted to a referral veterinary diagnostic laboratory were used for this study. Information regarding if (and when) the MCT recurred following surgical excision was provided from the referring veterinarians (**Table 4.1**). Canine MCT were graded by a board-certified pathologist using hematoxylin and eosin-stained sections. All cases

selected for inclusion in this study were completely excised as read out by a pathologist and were Patnaik grade 2 and Kiupel low grade to minimize variability associated with tumor grade. Sections (3 μm) were prepared from the same paraffin blocks for immunohistochemistry (IHC).

4.3.2. Immunohistochemistry

Briefly, sections were deparaffinized in xylene, rehydrated in graded ethanol series (100%, 75%, 50%), and subjected to heat-induced epitope retrieval in sodium citrate a for ten minutes. After inhibition of tissue-specific endogenous peroxidase activity with 3% hydrogen peroxide and blocking with goat serum, a rabbit anti-human LHR primary antibody was applied at a 1:50 dilution for 30 minutes at room temperature. For KIT IHC (immunohistochemistry), serial sections were subjected to heat-induced epitope retrieval in sodium citrate a for ten minutes and stained on the automatic stain using rabbit polyclonal anti-human CD117 at a 1:100 dilution. A universal negative rabbit antibody was applied to serial sections to serve as a negative control for each section.

Unbound primary antibody was removed by washing with a tris-buffer containing Tween. Slides were then incubated for seven minutes with horseradish peroxidase-conjugated polymer anti-rabbit IgG secondary antibody. Unbound secondary antibody was removed by washing with the same tris-buffer. Slides were then reacted with Nova Red Peroxidase substrate, counter-stained with hematoxylin, dehydrated in a graded ethanol series, and mounted with coverslips. Images were digitally captured using bright field microscopy at 400X magnification.

Breed	Age	Repro status	Recurrence?	When recurred?
Labrador Retriever	8	Female	No	
Crossbreed	10	Female	Yes	Less than 1 month
German Shorthair Pointer	9	Female	No	
Boxer	7	Male	No	
Australian Terrier	2	Male	No	
Labrador Retriever	3	Male	Yes	More than 6 months
Heeler	8	Spay	No	
Crossbreed	2	Spay	No	
Cocker Spaniel	6	Spay	Yes	2 to 5 months
Miniature Pinscher	11	Spay	No	
Lhasa Apso	11	Spay	No	

Table 4.1. Signalment of canine MCT cases, age at gonadectomy, recurrence rate, and when recurred.

4.3.3. Data collection

The percentage of positive LHR cells was determined from counting 200 cells within the tumor. Cells on the margins of the tissue sections were excluded to minimize variability in staining from tissue edge artifact.

4.3.4. Statistical analysis

For both KIT and LHR, the percentage of positive cells for each staining pattern (I-III) was calculated. Comparisons were made between intact and gonadectomized dogs using a Chi-squared test for MCT recurrence and Student's t test l for the total percentage of positive cells expressing LHR and KIT. In addition, comparisons were made using a one-way analysis of variance between the percentage of cells within each staining pattern (I-III) for LHR and KIT in intact and gonadectomized dogs. Significance was defined as $p<0.05$.

4.4. Results

4.4.1. Immunohistochemistry

All sections stained for LHR had immunolabeled cells. The keratinocytes in the epidermis and hair follicles adjacent to each MCT served as a positive control for LHR expression (**Figure 4.1**). There was no immunolabeling in the negative control tissues (e.g., collagen and eosinophils) treated with the LHR primary antibody as well as in the negative control serial sections (**Figure 4.1**). Three distinct expression patterns of LHR immunostaining were identified. The first staining pattern (LHR-type I; **Figure 4.2 A**) was characterized by intense staining confined to the cell membrane of the

mastocytoma cells. The second staining pattern (LHR-type II; **Figure 4.2 B**) was characterized by cytoplasmic granular staining, with low to medium staining intensity. The third staining pattern (LHR-type III; **Figure 4.2 C**) was characterized by a high staining intensity, with some cells having coarser, more closely grouped granules.

3.4.1. Group comparison

The MCT recurrence rate did not differ significantly between gonadectomized dogs (20%, 1/5) and intact dogs (33%, 2/6). All cutaneous MCT examined contained cells that expressed KIT and the percentage of KIT positive cells did not differ between gonadectomized and intact dogs (**Table 4.2**), which agrees with previous research (Reguera et al., 2000).

On the other hand, gonadectomized dogs had a significantly higher ($p<0.001$) percentage of LHR-positive cells (mean±SD: 84.2±8.7%) compared to intact dogs (mean±SD: 64.3±4.2%) (**Table 4.2**). In addition, the percentage of positive cells expressing the type II staining pattern for both KIT and LHR was significantly higher ($p<0.001$) than the other two staining patterns in both gonadectomized and intact dogs (**Figure 4.3**).

4.5 Discussion

Although LHR expression in canine skin has been previously reported (Welle et al., 2006), this is the first study to demonstrate the expression of LHR in canine cutaneous MCT and the first study to report that LHR expression is increased in mastocytoma cells in cutaneous MCT from gonadectomized dogs compared to intact

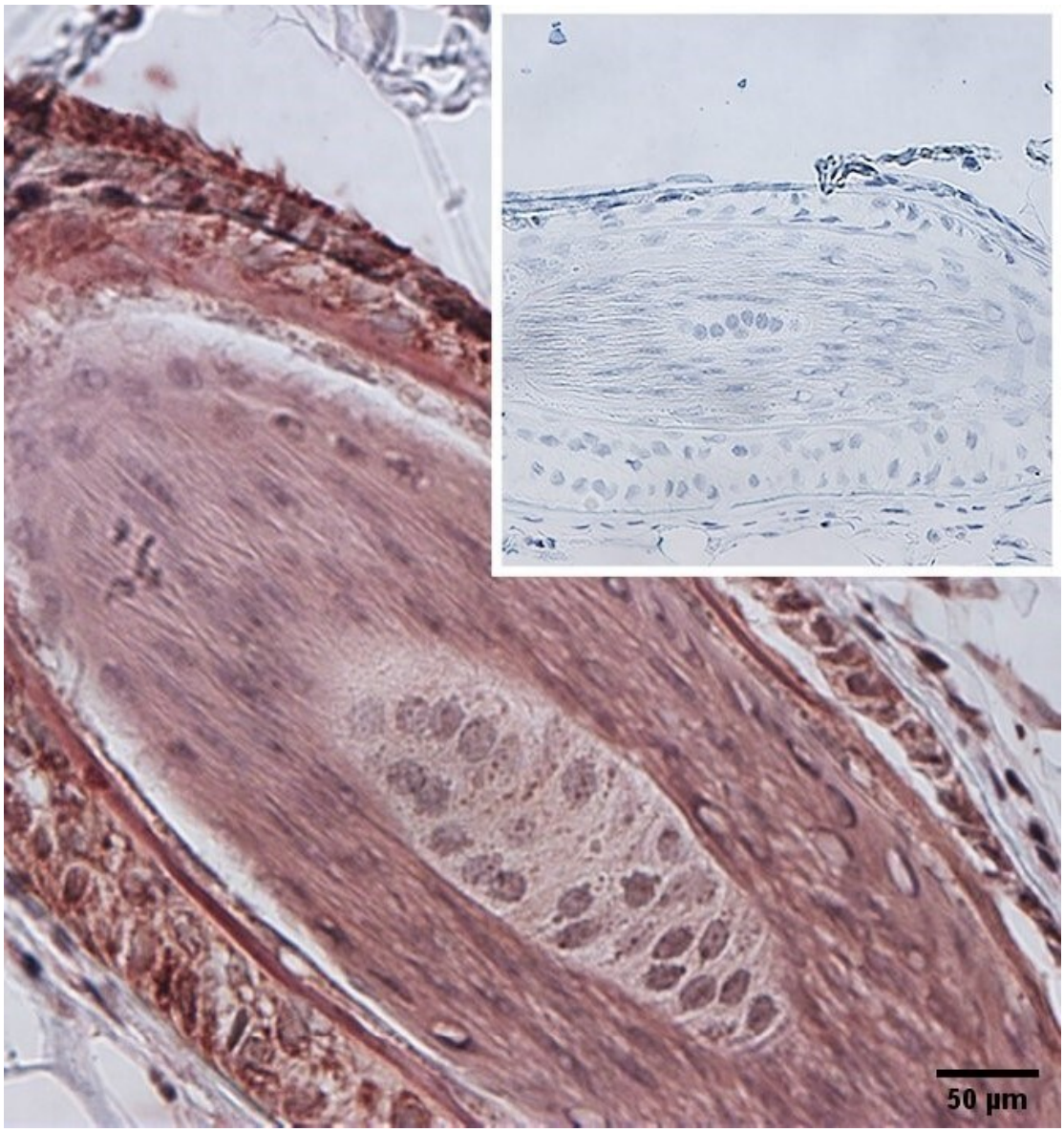


Figure 4.1. Skin; dog. Immunohistochemical localization of luteinizing hormone receptors in morphologically normal canine skin and hair follicle (positive control; scale bar = 50 µm). Inset: Negative control.

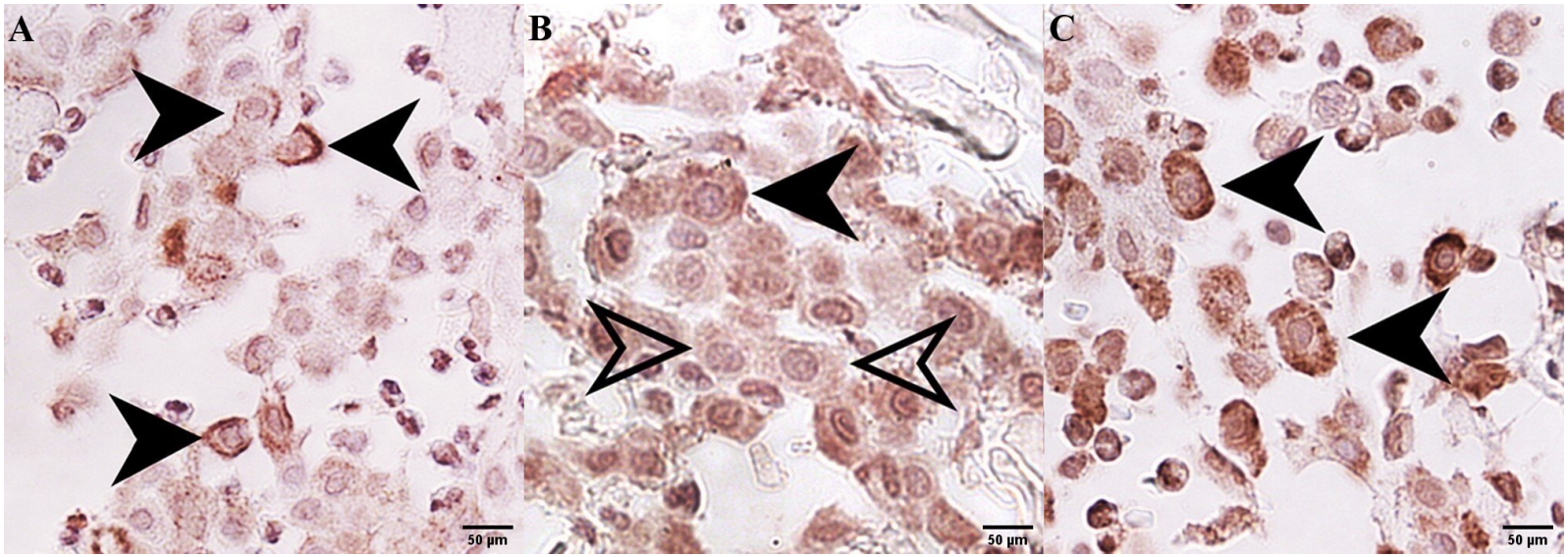


Figure 4.2. Cutaneous MCT, dog, immunolabeling for LHR. **A**: LHR- staining pattern I is characterized by membrane-associated staining (black arrowheads), with little cytoplasmic staining of mastocytoma cells. **B**: LHR- staining pattern II is characterized by low (open arrowheads) to medium (black arrowheads) cytoplasmic intensity staining with small granules in mastocytoma cells. **C**: LHR- staining pattern III is characterized by high staining intensity (black arrowheads). Some mastocytoma cells have staining of coalesced granules (scale bar = 50 µm).

	% Positive		% Pattern I		% Pattern II		% Pattern III	
	LHR†	**KIT‡**	**LHR**	**KIT**	**LHR**	**KIT**	**LHR**	**KIT**
Intact dogs (n=6)	64.3±4.2	95.8±2.7	1.9±1.4	13.3±15.8	49.2±8.4	81.3±15.4	13.2±9.7	1.7±1.9
Gonadectomized dogs (n=5)	84.2±8.7	95.7±2.0	1.9±0.8	17.6±11.2	66.6±15.3	77.3±8.9	15.7±12.1	1.2±0.6
	*p<0.001	p=0.487	p=0.255	p=0.305	p=0.424	p=0.324	p=0.452	p=0.308

Table 4.2. The percentage (mean ± SD) of cells expressing LHR and KIT from canine cutaneous mastocytomas (MCT).

†Luteinizing hormone receptor (LHR) cellular patterns of antigen distribution were categorized as follows: I- membranous; II- cytoplasmic (low to medium intensity); III- cytoplasmic (high intensity).

‡KIT cellular patterns of antigen distribution are different from those of LHR as previously described.

*There was a significantly higher percentage of cells from MCT in gonadectomized dogs positive for LHR compared to intact dogs.

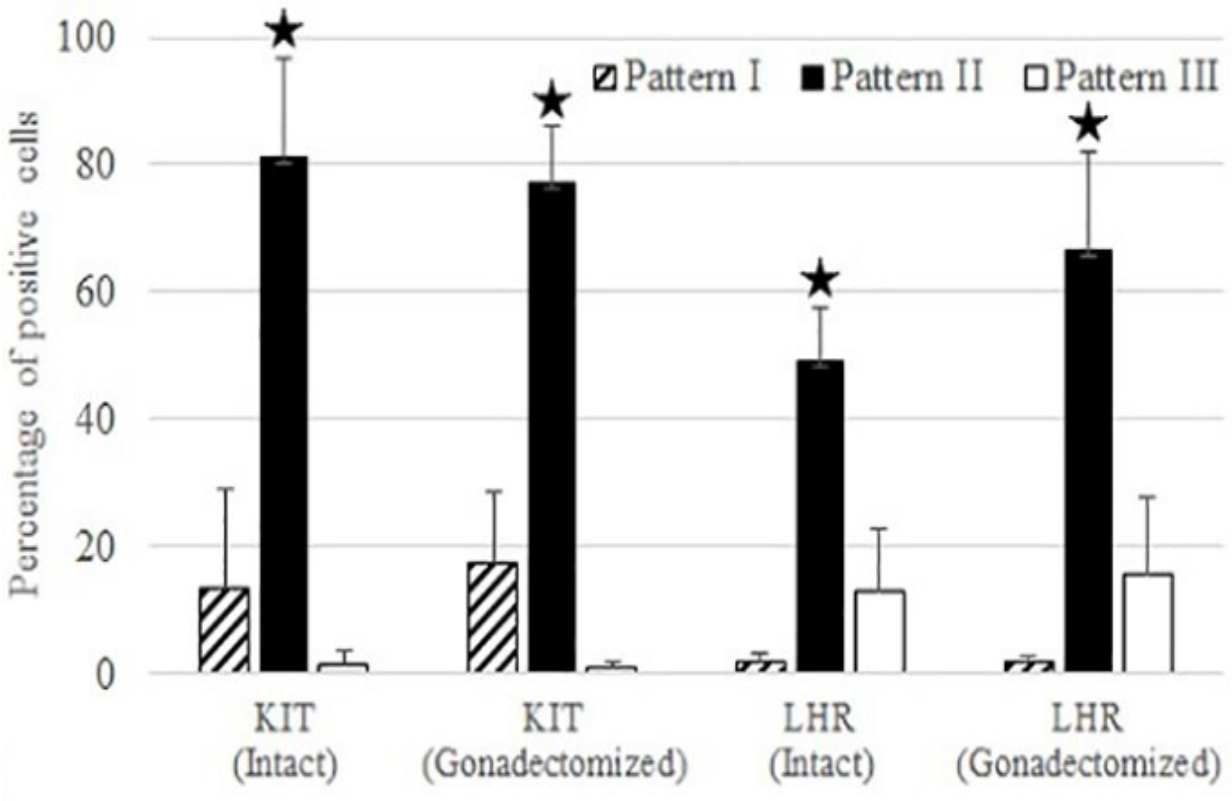


Figure 4.3. The percentage (mean ± SD) of cells positive for KIT and LHR in intact and gonadectomized dogs with cutaneous MCT varied by staining pattern. For both KIT and LHR, the percentage of cells expressing the type II staining pattern was significantly higher ($p<0.001$) than the other two staining patterns in both gonadectomized and intact dogs.

dogs. Previous research has demonstrated a significant increase in risk for development of MCT in gonadectomized female dogs (White et al., 2011), which may be due in part with an associated increase in LHR expression in gonadectomized dogs. The influence of sex was not examined in the current study, however, in both T-cell and B-cell canine lymphoma, there is no significant difference in the percentage of LHR positive cells or staining intensity when compared by sex (Zwida et al., 2021).

In canine splenic hemangiosarcoma, LHR stromal cells are immunolabeled in a cytoplasmic and granular pattern (Zwida and Kutzer, 2016). In adrenocortical adenomas and carcinomas, both zona glomerulosa and zona fasciculata LHR positive cells express a cytoplasmic granular pattern; immunolabeling varies from a homogeneous pattern characterized by the staining of the entire area, with only slight differences in staining intensity, to a more heterogeneous pattern, characterized by clusters of immunopositive areas close to areas with no staining. It is important to note that the heterogeneous pattern was observed mainly in adrenocortical carcinomas (Galac et al., 2010). In the current study, we found that in cutaneous MCT tissues, neoplastic cells were immunolabeled for LHR in three distinct cell-associated patterns, which are similar to the patterns described for KIT immunolabeling of mastocytoma cells in dogs, cats and horses (Reguera et al., 2000; Sabattini et al., 2013; Flores et al., 2017).

Both KIT and LHR are cell membrane receptors (KIT is a tyrosine kinase receptor; LHR is a G-protein coupled receptor (GPCR) and both display atypical cytoplasmic immunoexpression patterns. The cytoplasmic isoform of KIT may be activated by soluble stem cell factor or may harbor a constitutive activating mutation

leading to aberrant phosphorylation of KIT resulting in proliferation, migration, and inhibition of mast cell apoptosis (Reguera et al., 2000). Internalization of LHR from the cell membrane into the cytoplasm was initially thought to be from signal desensitization, an adaptation mechanism to prolonged receptor stimulation; However, several new theories regarding this function have recently emerged. Most notably, accumulating evidence indicates that internalization can induce prolonged receptor signaling on intracellular membranes, a required mechanism for at least some of the biological effects of GPCR hormones, including that of LH (Calebiro and Godbole, 2018). It is plausible that the membrane-associated pattern of LHR shows localization to the membrane-bound GPCR, and that the internal granular pattern shows GPCR internalization with continued signaling.

The majority of MCTs display KIT staining pattern II, with 43.9% more MCTs having staining pattern II comparing to staining pattern I (Kiupel et al., 2004). In the current study, we performed immunohistochemistry for KIT to identify if there was any overlap in the immunolocalization patterns with LHR. Although the expression patterns were similar, it is unknown if the two receptors are functioning synergistically in MCT development or maintenance. While our data suggest a difference in LHR expression in intact versus gonadectomized dogs, the overall impact on aberrant LHR signaling on the function of mastocytoma cells behavior remains unclear. Future studies aimed at understanding the impact of LHR signaling on mast cell biology are necessary to determine the role of LHR on MCT behavior. The aberrant expression of LHR in canine MCT is supported by previous findings on the aberrant expression of LHR in canine non-reproductive normal tissues (bladder, skin, adrenal gland, thyroid

gland, lymph node) (Ponglowhapan et al., 2007; Welle et al., 2006; Zwida and Kutzler, 2019; Galac et al., 2010; Ettinger et al., 2019) and neoplastic tissues (lymphoma, hemangiosarcoma, and adrenocortical tumors) (Galac et al., 2010; Ettinger et al., 2019; Zwida and Kutzler, 2019).

4.6. Conclusion

This research illustrates a relationship between canine gonadectomy and LHR expression in Patnaik Grade 2 and Kiupel low grade MCTs. Although the current study population was small, the percentage of mastocytoma cells expressing LHR was increased in MCTs from gonadectomized dogs as compared to MCTs from intact dogs. Although our data suggest that LHR expression is higher in MCTs from gonadectomized dogs, the relevance of LHR expression and its potential role in triggering cell proliferation, invasion, migration in MCTs remains unclear, especially since there was no difference in recurrence rate. Additional research on LHR expression in different histologic grades of MCTs and in a larger population of gonadectomized and intact dogs is necessary. To minimize possible variability associated with tumor grade, this study focused on LHR expression in Patnaik grade 2 and Kiupel low grade MCTs. Further research on LHR expression in different grades of MCT (especially in high grade and aggressive tumors) is also needed to further illustrate the interaction between LH and mastocytoma.

4.7. References

Abdelbaset-Ismail A, Borkowska S, Janowska-Wieczorek A, et al. (2016). Novel evidence that pituitary gonadotropins directly stimulate human leukemic cells-studies of myeloid cell lines and primary patient AML and CML cells. Oncotarget, 7(3), 3033–3046.

Abdelbaset-Ismail A, Pedziwiatr D, Schneider G, et al. (2017). Pituitary sex hormones enhance the pro-metastatic potential of human lung cancer cells by downregulating the intracellular expression of heme oxygenase-1. Int J Oncol, 50(1), 317–328.

Beijerink NJ, Buijtels JJ, Okkens AC, et al. (2007). Basal and GnRH-induced secretion of FSH and LH in anestrous versus ovariectomized bitches. Theriogenology, 67(5), 1039–1045.

Calebiro D, Godbole A. (2018). Internalization of G-protein-coupled receptors: Implication in receptor function, physiology and diseases. Best practice and research. Clin Endocrinol Metabol, 32(2), 83–91.

Ettinger AM, Gust SK, Kutzler MA. (2019). Luteinizing hormone receptor expression by nonneoplastic and neoplastic canine lymphocytes. Am J Vet Res, 80(6), 572–577.

Flores AR, Azinhaga A, Pais E, et al. (2017). Equine ocular mast cell tumor: histopathological and immunohistochemical description. J Equine Sci, 28(4), 149–152.

Galac S, Kars VJ, Klarenbeek S, et al. (2010). Expression of receptors for luteinizing hormone, gastric-inhibitory polypeptide, and vasopressin in normal adrenal glands

and cortisol-secreting adrenocortical tumors in dogs. Dom Anim Endocrinol, 39(1), 63–75.

Kiupel M, Webster JD, Bailey KL, et al. (2011). Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behavior. Vet Pathol, 48(1), 147–155.

Kiupel M, Webster J D, Kaneene JB, et al. (2004). The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. Vet Pathol, 41(4), 371–377.

Ma Y, Longley BJ, Wang X, et al. (1999). Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. J Invest Dermatol, 112(2), 165–170.

Noci I, Pillozzi S, Lastraioli E, et al. (2008). hLH/hCG-receptor expression correlates with in vitro invasiveness in human primary endometrial cancer. Gynecol Oncol, 111(3), 496–501.

Patnaik AK, Ehler WJ, MacEwen EG. (1984). Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. Vet Pathol, 21(5), 469–474.

Ponglowhapan S, Church D B, Scaramuzzi RJ, et al. (2007). Luteinizing hormone and follicle-stimulating hormone receptors and their transcribed genes (mRNA) are present in the lower urinary tract of intact male and female dogs. Theriogenology, 67(2), 353–366.

Reguera MJ, Rabanal RM, Puigdemont A, et al. (2000). Canine mast cell tumors express stem cell factor receptor. Am J Dermatopathol, 22(1), 49–54.

Sabattini S, Guadagni Frizzon M, Gentilini F, et al. (2013). Prognostic significance of Kit receptor tyrosine kinase dysregulations in feline cutaneous mast cell tumors. Vet Pathol, 50(5), 797–805.

Sanchez AM, Flamini MI, Russo E, et al. (2016). LH and FSH promote migration and invasion properties of a breast cancer cell line through regulatory actions on the actin cytoskeleton. Molecul Cell Endocrinol, 437, 22–34.

Villamil JA, Henry CJ, Bryan JN, et al. (2011). Identification of the most common cutaneous neoplasms in dogs and evaluation of breed and age distributions for selected neoplasms. J Am Vet Med Assoc, 239(7), 960–965.

Welle MM, Reichler I M, Barth A, et al. (2006). Immunohistochemical localization and quantitative assessment of GnRH-, FSH-, and LH-receptor mRNA Expression in canine skin: a powerful tool to study the pathogenesis of side effects after spaying. Histchem Cell Biol, 126(5), 527–535.

White CR, Hohenhaus, AE, Kelsey J, et al. (2011). Cutaneous MCTs: associations with spay/neuter status, breed, body size, and phylogenetic cluster. J Am Anim Hosp Assoc, 47(3), 210–216.

Zemke D, Yamini B, Yuzbasiyan-Gurkan V. (2002). Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. Vet Pathol, 39(5), 529–535.

Zwida K, Kutzler M. (2016). Canine hemangiosarcoma expresses luteinizing hormone (LH) receptors. J Anim Sci, 94(Suppl 5):205-206.

Zwida K, Kutzler M. (2019). Luteinizing hormone receptor is immunoexpressed within the canine thyroid. Clin Theriogenology, 11(1):23-29.

Zwida KH, Vedus A, Kutzler M. (2021). Expression of luteinizing hormone receptor (LHR) in canine lymphoma is not affected by body weight, sex, lymphoma phenotype, and lymphoma stage. Clin Theriogenology, 13(1).

CHAPTER 5

LUTEINIZING HORMONE RECEPTOR ACTIVATION IN CANINE HEMANGIOSARCOMA CELLS WILL INDUCE CELL PROLIFERATION IN VITRO

Khawla Zwida, Michelle Kutzler

American Journal of Veterinary Research

In preparation for submission in April 2022

5.1. Abstract

Hemangiosarcoma is a rapidly growing, highly invasive cancer arising from the lining of blood vessels of any tissue. More than half of all canine hemangiosarcoma primary tumors arise within the spleen. German shepherds, Golden Retrievers, and Labrador Retrievers are overrepresented compared to other breeds, making up 34.33% of all purebred dogs with splenic hemangiosarcoma. In addition to breed, gonad removal (spaying/neutering) also increases the likelihood for developing hemangiosarcoma. Spayed female dogs are reported to have two to ten times the risk for developing splenic hemangiosarcoma compared to intact female dogs. Following gonad removal, luteinizing hormone (LH) concentrations are significantly and persistently elevated. The effects of elevated LH concentrations can be observed in the bladder (urinary incontinence), thyroid (hypothyroidism), skin (alopecia X, puppy coat syndrome), and other tissues that have LH receptors (LHR). LH is a potent mitogen and our laboratory has previously shown that LH activation induces cell proliferation in canine T-cell lymphoma cells in vitro. Our laboratory has also demonstrated that canine splenic hemangiosarcoma tissues express LHR. However, it is not yet known what function (if any) activation of these receptors would have. The results of this research will allow for a better understanding of the relationship between gonad removal and the development of hemangiosarcoma in dogs. Additionally, if activation of LHR is shown to induce splenic hemangiosarcoma cells to proliferate, then reducing LH concentrations in spayed or neutered dogs may reduce the risk for development of hemangiosarcoma in breeds with a higher reported incidence. We hypothesized that the isolated canine splenic hemangiosarcoma cells will express LHR and the activation of

LHR will result in an increase in splenic hemangiosarcoma cells in vitro. To test these hypotheses, immunocytochemistry was used to determine percentage of cells expressing LHR from four commercially available canine splenic hemangiosarcoma cell lines. Canine hemangiosarcoma cell lines were cultured with increasing concentrations of human chorionic gonadotropin (hCG; an LH receptor agonist) or recombinant canine LH (cLH). LH receptor expression in isolated canine hemangiosarcoma was detected and varies between cell lines. The percentage of cells positive for LHR was 6.9± 2.5%, 8.5 ± 1.0%, 11.8±3.1%, and 17.2± 4.5% in DAL-4, DHSA, GRACE-HSA, and EFS, respectively. There also a significant effect of hCG and cLH exposure time on cell count and the interaction between hCG and cLH concentration X exposure time on cell count in all cell lines. Research into the effect of downregulation of LH receptor activation in dogs with hemangiosarcoma is needed.

5.2. Introduction

Hemangiosarcoma is a rapidly growing, highly invasive cancer arising from the lining of blood vessels of any tissue (Shiu et al., 2011). More than half of all canine hemangiosarcoma primary tumors arise within the spleen (Clifford et al., 2000; Brown et al., 1985; Hammer et al., 1991). Canine hemangiosarcoma occurs in 0.3 to 2% of all dogs (Brown et al., 1985; Ng and Mills, 1985). Affected dogs are typically older than 5 years but the disease can occur at younger ages (Brown et al., 1985; Ng and Mills, 1985). German shepherds, Golden Retrievers, and Labrador Retrievers are overrepresented compared to other breeds, making up 34.33% of all purebred dogs with splenic hemangiosarcoma (Brown et al., 1985; Hammer et al., 1991; Ng and

Mills, 1985). According to the American Kennel Club, these are the three most popular breeds of dogs in the United States (American Kennel Club, 2020).

In addition to age and breed, gonad removal also increases the likelihood for developing splenic hemangiosarcoma. In comparison to intact female dogs, spayed female dogs have two times the risk (Prymak and Mckee, 1988), four times the risk (Torres de la Riva et al., 2013), or up to ten times the risk (Zink et al., 2014) for developing splenic hemangiosarcoma. Because of its aggressive biological behavior, canine hemangiosarcoma is associated with a poor prognosis (Shiu et al., 2011). Despite surgical removal of the primary tumor, median survival times in dogs with splenic hemangiosarcoma is two months or less (Brown et al., 1985; Hammer et al., 1991). Adding chemotherapy with vincristine, doxorubicin, epirubicin, cyclophosphamide, anthracycline or metronomic-based protocols may prolong survival medians to 6-12 months (Hammer et al., 1991; Kim et al., 2007; Treggiari et al.,2020).

Luteinizing hormone (LH) is secreted by the anterior pituitary gland in brief low-amplitude pulses in sexually intact female and male dogs to stimulate synthesis of estrogen and testosterone, respectively (Concannon, 2011). These gonadal steroid hormones then negatively feedback to decrease the secretion of LH. However, following removal of the gonads with spaying or neutering, there is a loss of negative feedback and LH is continuously secreted at concentrations up to 20 times higher than what is observed at peak concentrations in estrous female dogs prior to ovulation (Beijerink et al., 2007).

The effect of persistent and supraphysiologic LH concentrations on gonadectomized dogs had not been seriously considered until LH receptors (LHR) were found in the bladder (Welle et al., 2006), skin (Ponglowhapan et al., 2007; Ponglowhapan et al., 2012), adrenal gland (Glac et al., 2010), thyroid gland (Zwida and Kutzler, 2019), lymph node (Ettinger et al., 2019), and musculoskeletal tissues (Kiefel and Kutzler, 2020). In addition, there is growing evidence that extragonadal LHR are functional when stimulated in vivo or in vitro with human chorionic gonadotropin (hCG) and/or recombinant canine LH (cLH). In incontinent spayed female dogs, LHR activation stimulates the release of nitric oxide within the bladder smooth muscle cells, resulting in urethral sphincter mechanism incompetence (Reichler et al., 2008). In the canine skin, LHR activation inhibits shedding by interfering with the hair growth cycle, specifically by significantly altering the anagen to telogen ratio within the first year following gonad removal (Reichler, 2010). In isolated canine T-lymphoma cells, LHR activation results in dose-dependent cell proliferation and increased expression of the LHR (Flint et al., 2019; Li and Kutzler, 2020).

In a previous study, we investigated the expression of LHR in formalin-fixed paraffin embedded splenic hemangiosarcoma tissues. Using routine immunohistochemistry, 62.5% of cases of canine splenic hemangiosarcoma were positive for LHR expression (Zwida et al., 2018). LHR cellular localization in splenic hemangiosarcoma was cytoplasmic and granular. However, the effect of LHR activation in canine splenic hemangiosarcoma is not known. The objective of the current study was to examine LHR expression and response to LHR activation in isolated canine splenic hemangiosarcoma cell lines. It was hypothesized that the

isolated canine splenic hemangiosarcoma cells will express LHR and the activation of LHR will result in cell proliferation in vitro.

5.3 Materials and Methods

5.3.1. Source of Animal Tissues

Immortalized cell lines isolated from four dogs with a primary splenic hemangiosarcoma (HSA) were purchased from Kerafast, Inc. (Boston, MA) for used in this research (**Table 4.1**).

5.3.2. Immunocytochemical Assay

Splenic hemangiosarcoma (HSA) cells (500,000/dish) were plated on sterile 22 mm^2 coverslips in 35 mm diameter dishes. Cells were cultured at 37°C in a humidified 5% CO_2 incubator with media (Canine Endothelial Cell Growth Medium, #Cn211-500, Cell Application, Inc., San Diego, CA) containing 10% FB essence (#10803-034, Avantor Seradigm, Visalia, CA), 10,000 µg/mL penicillin, and 10,000 µg/mL streptomycin. Media was replaced every 1-2 days until reaching 70-80% confluence.

The HSA cells then fixed onto the coverslips following removal of the culture media by washing with phosphate buffered saline (PBS) and fixing in 70% ethanol with 50 mM glycine (pH 2.0) for 30 minutes at -20°C. The coverslips washed again with PBS and stored at 4°C in PBS with 1% sodium azide until staining. Either rabbit polyclonal anti-human LHR antibody (#NLS1436, Novus Biologics, Centennial, CO) was applied at a 1:200 dilution or negative control (#NC495H, Biocare Medical, Pacheco, CA) was applied to coverslips for incubation overnight at 4°C. Coverslips

were then washed in PBS and a secondary antibody (FITC conjugated horse anti-rabbit IgG (Life Technologies, Eugene, OR) applied at a 1:200 dilution in a dark room for a 2-hour incubation at 20°C. Coverslips were then washed in PBS and then inverted and mounted onto slides with mounting medium containing DAPI (#H-1500, Vectashield® HardSet™ Antifade Mounting Medium with DAPI, Burlingame, CA). Three randomly selected fields from each cell line were captured using fluorescence microscopy (DMI6000B, fully motorized, Leica Microsystems, Germany) at 400X magnification. This experiment performed in triplicate for each cell line.

5.3.3. MTT Assay

Standard curves were established for each cell line by determining the absorbance of known cell numbers as described in the instructions of the MTT assay (#10009365, Cayman Chemical, Ann Arbor, MI). Briefly, after passaging the cells, the cell concentrations were determined by hemocytometry. The DAL-4 cells were diluted with protein-free media (Canine Endothelial Cell Growth Medium, # Cn211-500, Cell Application, Inc., San Diego, CA) to final concentration of 75,000, 150,000, 310,000, 620,000, 1,250,000, and 2,500,000 cells/mL. The EFS and DHSA cells were diluted with the same protein-free media to final concentration of 93,750, 187,500, 375,000, 750,000, and 1,500,000 cells/mL. The GRACE-HSA cells were diluted with protein-free media to final concentration of 8,000, 40,000, 200,000, 1,000,000, and 5,000,000 cells/mL. One hundred microliters of each cell concertation for each cell line were added to four wells in a 96-well plate (CLS3841, Sigma Aldrich). Four wells containing only media (no cells) served as a control. Then 10 µL MTT reagent ((3-) 4,5-

Cell line	Breed	Age	Sex and Status
DAL-4	Dalmatian	7 years	Intact male
EFS	Golden Retriever	9 years	Spayed female
DHSA-1426	Golden Retriever	10 years	Neutered male
GRACE-HSA	Portuguese Water Dog	8 years	Intact female

Table 5.1. Commercially available immortalized cell lines isolated from four different dogs with a primary splenic hemangiosarcoma that used in the proposed research.

dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; Cell growth determination kit, MTT based, Sigma) was added to each well. The 96-well plate was incubated at 37°C in 5% CO_2 for 3.5 hours. After incubation, 100 µL of detergent reagent from the MTT kit was added to all wells (including controls). The plate was then swirled gently for 60 seconds and returned to the incubator for 24-hours at which point it was read on plate reader (SpectraMax 190, Molecular Devices, LLC) at 570 nm.

5.3.4. Response LH Receptor Stimulation

After passaging cells, the cell concentrations were determined by hemocytometry for each cell line. The cells were diluted in serum containing culture media to the final concentration of 2 million cells/mL. Serial dilutions were made to get 100,000 cells per well added to 96-well plates in triplicate. The plate suspended in 100 µl of media without hCG or cLH (control) or with increasing concentrations of hCG (0-10,000 IU/mL) or cLH (0.5-7,500 ng/mL) and incubated at 37°C in 5 % CO_2 for 24, 48, 72, and 96 hours. Wells containing only media (no cells) served as a blank. The MTT reagent was added to each well and incubated at 37°C in 5 % CO_2 for 3.5 hours. The detergent reagent (100 µL) was aliquoted into all wells, including controls. The plate was then swirled gently for 60 seconds and returned to the incubator for 24-hours. It was then read on plate reader (SpectraMax 190, Molecular Devices, LLC) at 570 nm. This experiment was performed in triplicate.

5.3.4. Data Analysis

For immunocytochemistry, the mean±SD percentage of LHR positive cells for each cell line was compared by one-way analysis of variance (ANOVA; GraphPad Prism 8.4.3).

The best-fit line equation for the standard curves was calculated (Microsoft Excel®; Redmond, WA). Briefly, the absorbance of wells containing only media (no cells; blank) was subtracted from the average absorbance at each concentration for each cell line. The data were graphed and an equation for the best fit line for each cell line was determined. The correlation coefficient ($R^{2)}$ for each best fit line was greater than 0.97. The equation determined for best fit line was used to calculate the cell number from each absorbance following hCG or cLH incubation (**Table 5.2**).

Data from the LHR activation assay were managed in a similar way. Briefly, the absorbance of each triplicate was averaged. Then, the absorbance of the average of the wells containing only media (no cells, blank) was subtracted from the average absorbance at each hCG or cLH concentration for each incubation time. This correction was performed due to imperfections in the 96-well plate. This was repeated for each cell line. To determine the number of cells, the corrected absorbance was substituted for "x" in the best fit line equation for each cell lines (**Table 4.2**). The percentage relative to the control (0 IU/mL or 0 ng/mL for hCG or cLH, respectively) was calculated by dividing the average number of cells at each concentration of hCG or cLH by the average concentration of control (0 IU/mL or 0 ng/mL) and then multiplied by 100. The results were reported as mean±SD percentage of the control.

Two-way ANOVAs followed by Dunnett's post-hoc tests were used to compare the effect of hCG or cLH concentration, exposure time, and the interaction concentration X time. Because LHR expression differed between cell lines, a one-way ANOVA was also performed to examine the variation in cell number between cell lines at the different hCG or cLH concentrations and exposure times. Significance was defined as $p<0.05$.

5.4. Results

For isolated canine hemangiosarcoma cells, EFS cells expressed more LH receptors compared DHSA and DAL-4 cells ($p=0.0142$; **Figures 5.1 and 5.2**). The percentage of cells positive for LHR was 6.9± 2.5%, 8.5 ± 1.0%, 11.8±3.1%, and 17.2± 4.5% in DAL-4, DHSA, GRACE-HSA, and EFS, respectively (**Figure 5.3**).

There was significant effect of hCG concentration, exposure time, and the interaction between hCG concentration X exposure time on cell count in all cell lines ($p<0.005$; **Table 5.3; Figures 5.4 through 5.7**). There was significant effect of cLH concentration, exposure time, and the interaction between cLH concentration X exposure time on cell count for DAL-4, EFS, and Grace-HSA ($p<0.005$; **Table 5.3; Figures 5.8 through 5.10**). For DHSA, there was significant effect of hCG concentration on cell count ($p< 0.005$), but not of cLH concentration on cell count ($p=0.2204$; **Figure 5.11**).

When comparing between different canine splenic hemangiosarcoma cell lines, there were significantly different cell counts in response to hCG ($p=0.00320$ and $p=0.0157$) and cLH ($p=0.0109$ and $p=0.0035$) concentrations at 24- and 72-hours,

Cell line	Best fit line equation
DAL-4	y=132456x-17004
EFS	y=57081x-112787
DHSA	y=51902x-3684.8
GRACE-HSA	y=1E+6x-17863

Table 5.2. Best fit line equation used to calculate the cell number from each absorbance following hCG or cLH incubation (y=absorbance at 570 nm, x=cell number).

hCG	Concentration	Time	Concentration X time
DAL-4	0.0039	<0.0001	<0.0001
DHSA	<0.0001	<0.0001	<0.0001
EFS	<0.0001	0.0011	<0.0001
GRACE-HSA	<0.0001	<0.0001	<0.0001
cLH	Concentration	Time	Concentration X time
DAL-4	0.0005	0.0033	<0.0001
DHSA	0.2204	<0.0001	<0.0001
EFS	<0.0001	<0.0001	<0.0001
GRACE-HSA	<0.0001	<0.0001	<0.0001

Table 5.3. Results from two-way analysis variance comparing human chorionic gonadotropin (hCG) or canine luteinizing hormone (cLH) concentration, exposure time, and the interaction between hCG or cLH concentration X exposure time on cell count of canine splenic hemangiosarcoma cell lines (DAL-4, EFS, DHSA, and GRACE-HSA).

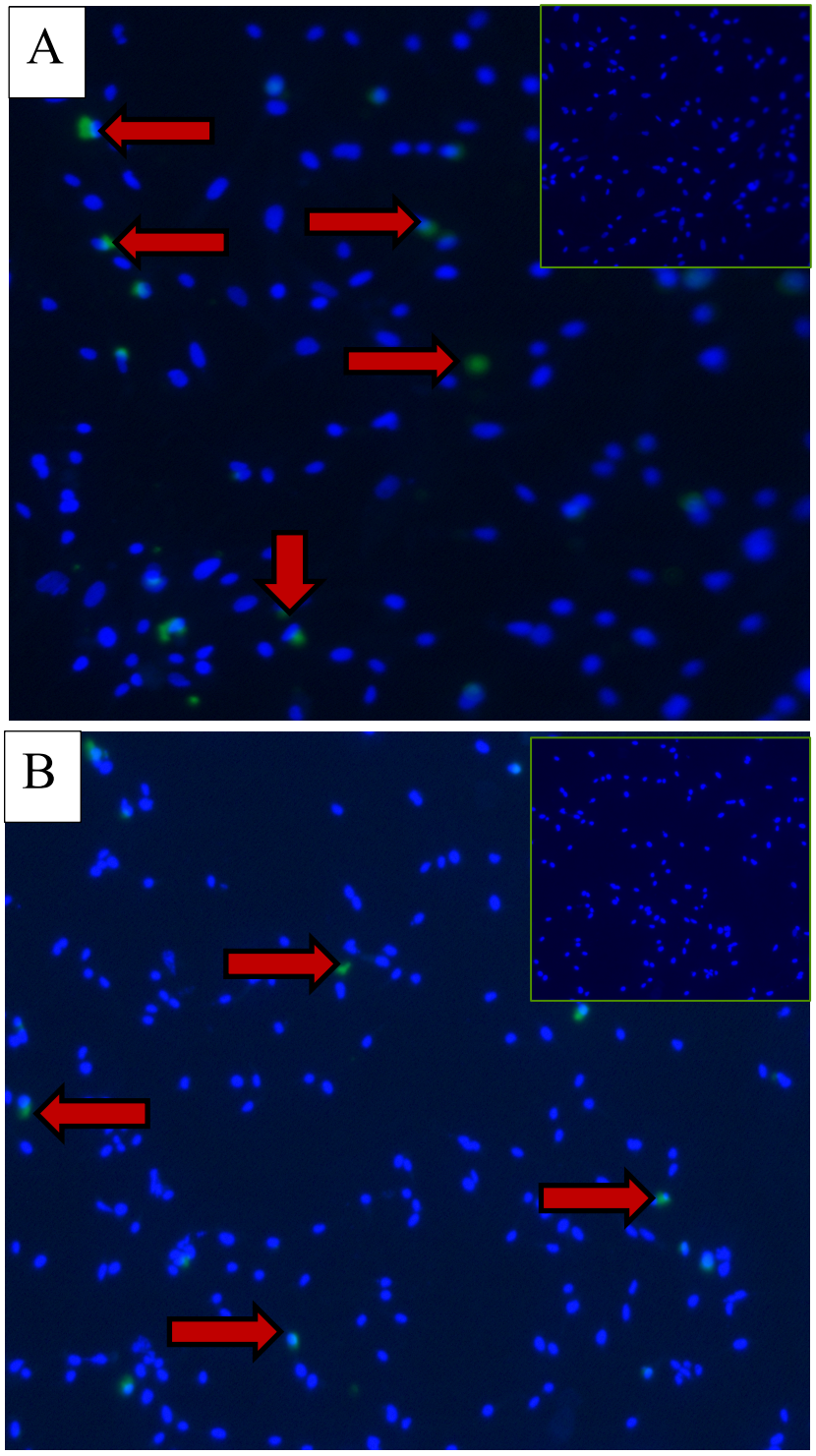


Figure 5.1. Immunocytochemical localization showing the expression of LH receptors in isolated hemangiosarcoma cells **A**: EFS cell line, **B**: GRACE-HSA cell line (inset: negative control).

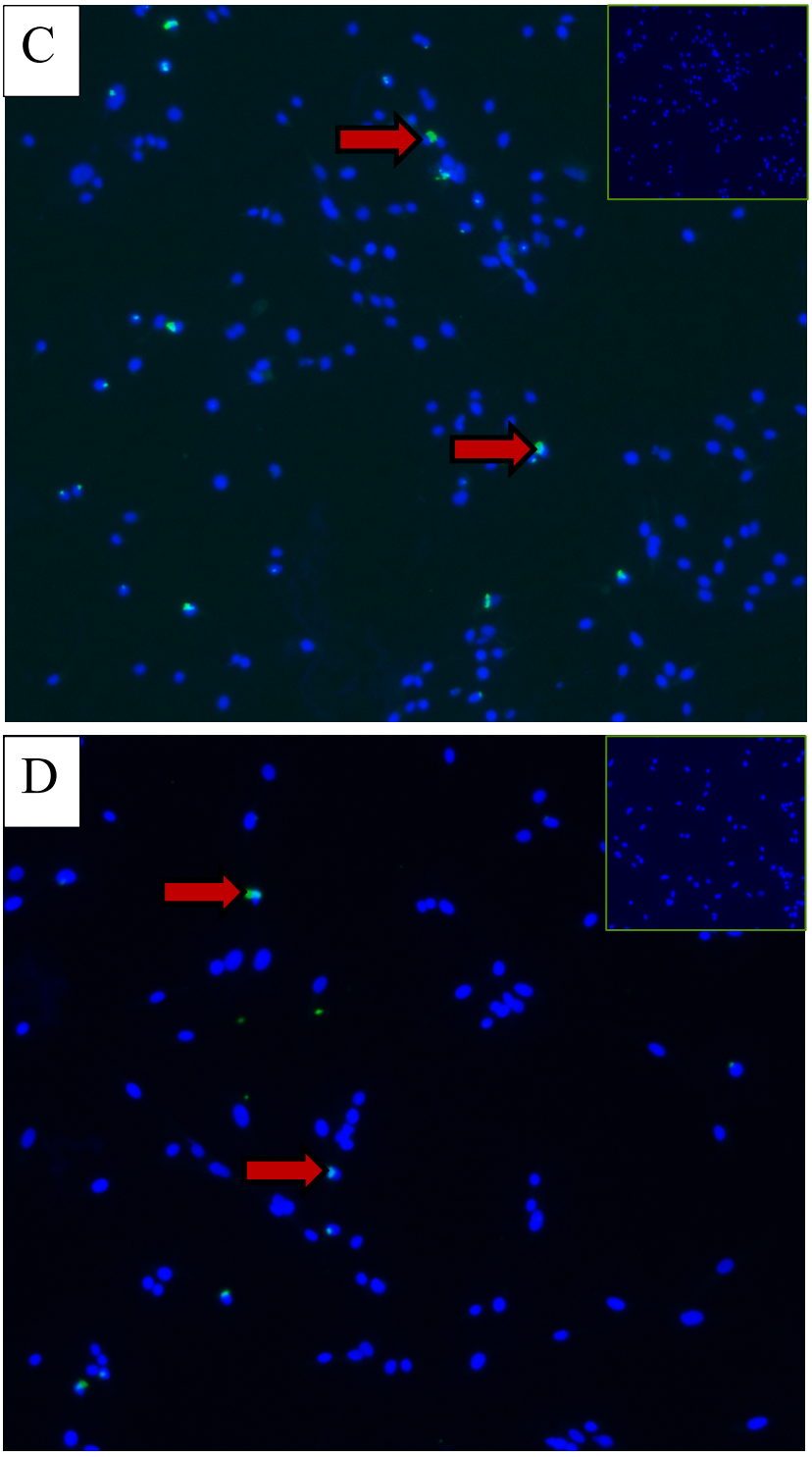


Figure 5.2. Immunocytochemical localization showing the expression of LH receptors in isolated hemangiosarcoma cells **C**: DHSA cell line, **D**: DAL-4 cell line (inset: negative control).

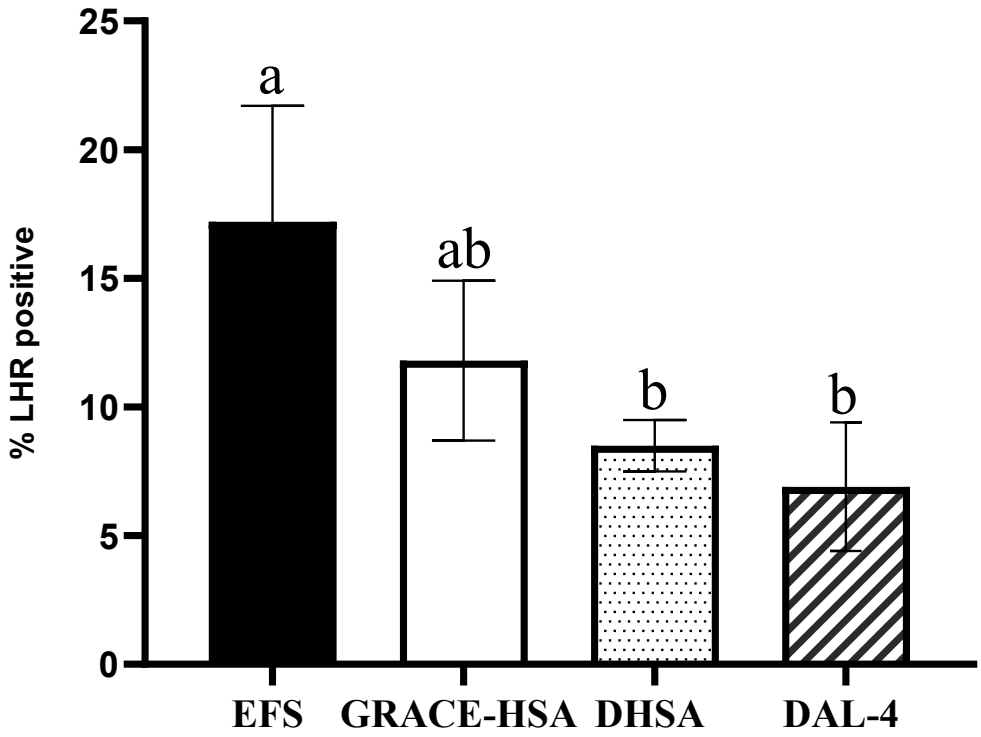


Figure 5.3. Percentage of luteinizing hormone receptors (LHR) in four canine splenic hemangiosarcoma cell lines as determined by immunocytochemistry. Cell lines characterized by different letters (a,b) are significantly different ($p<0.05$).

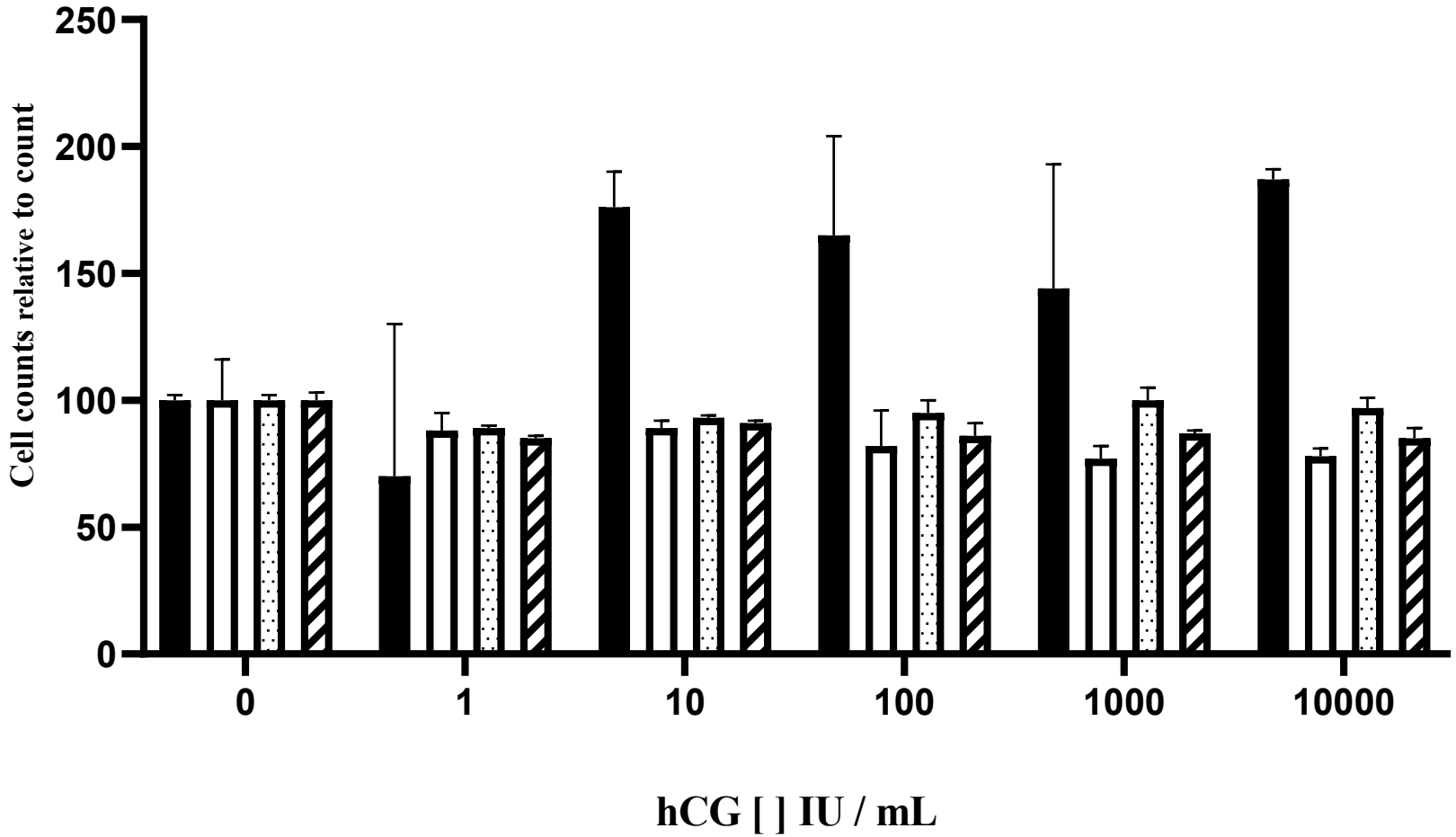


Figure 5.4. Significant effect of hCG concentration, exposure time, and interaction between hCG concentration X exposure time on cell count for DAL-4 ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

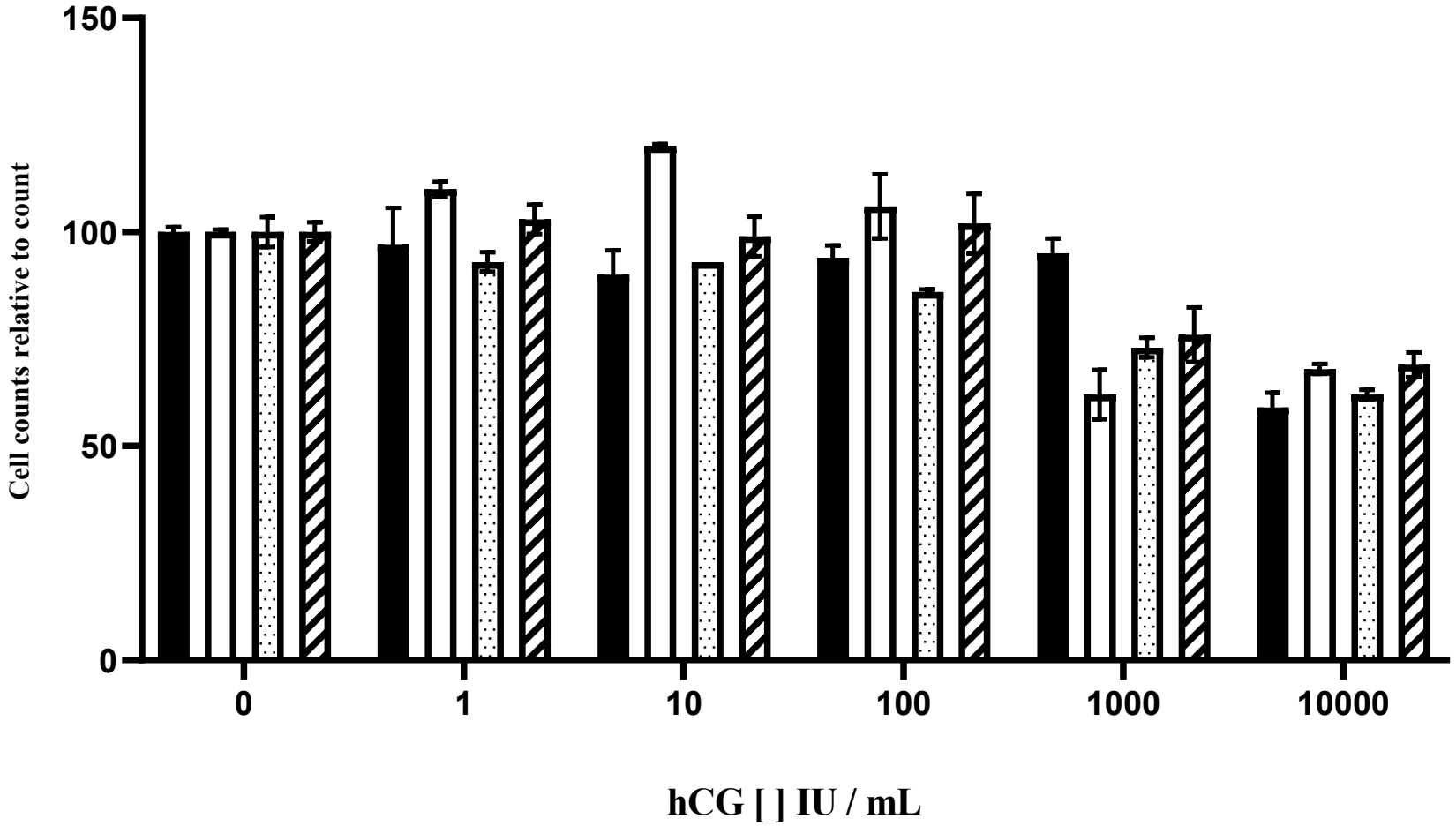


Figure 5.5. Significant effect of hCG concentration, exposure time, and the interaction between hCG concentration X exposure time on cell count for EFS ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

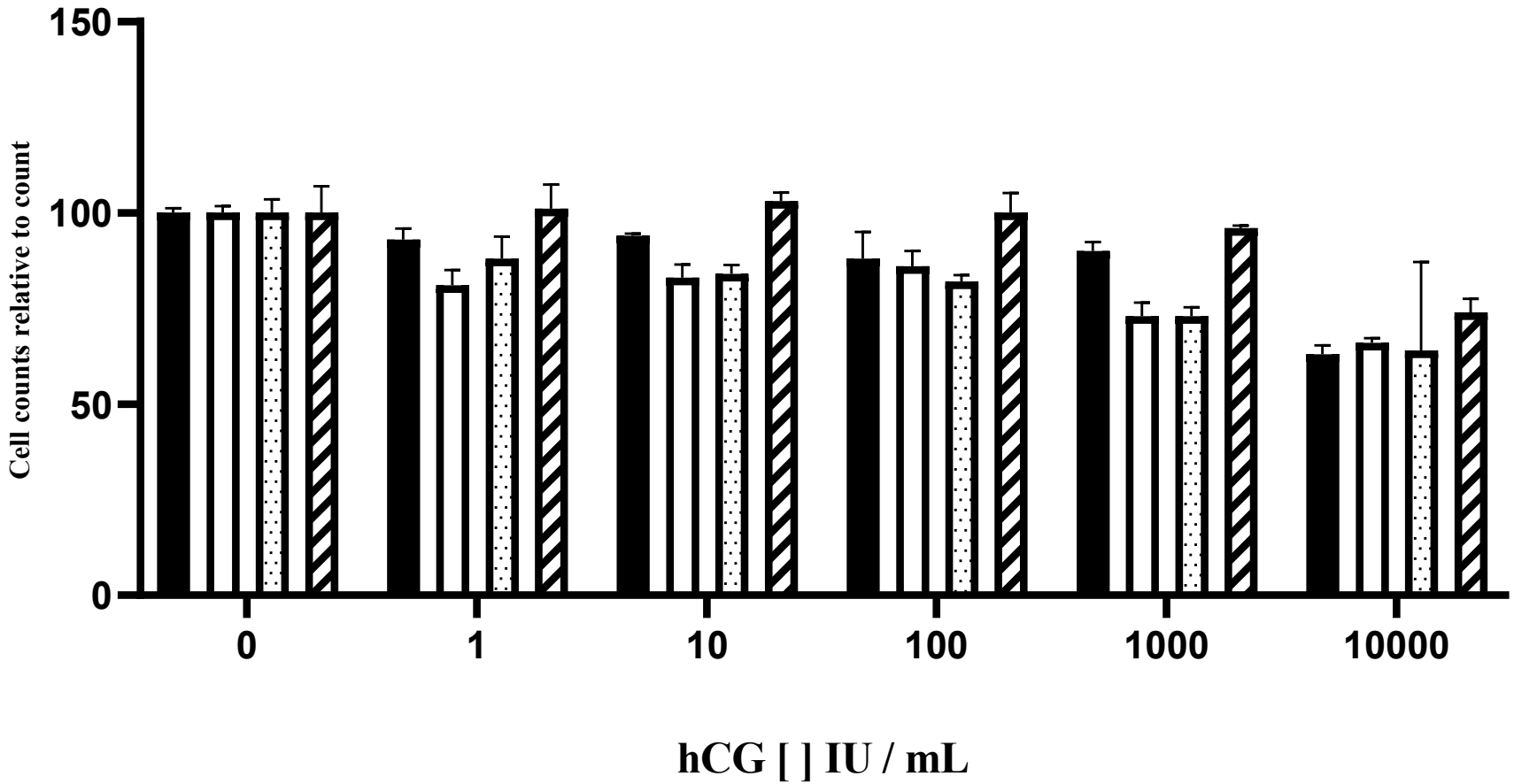


Figure 5.6. Significant effect of hCG concentration, exposure time, and the interaction between hCG concentration X exposure time on cell count for DHSA ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

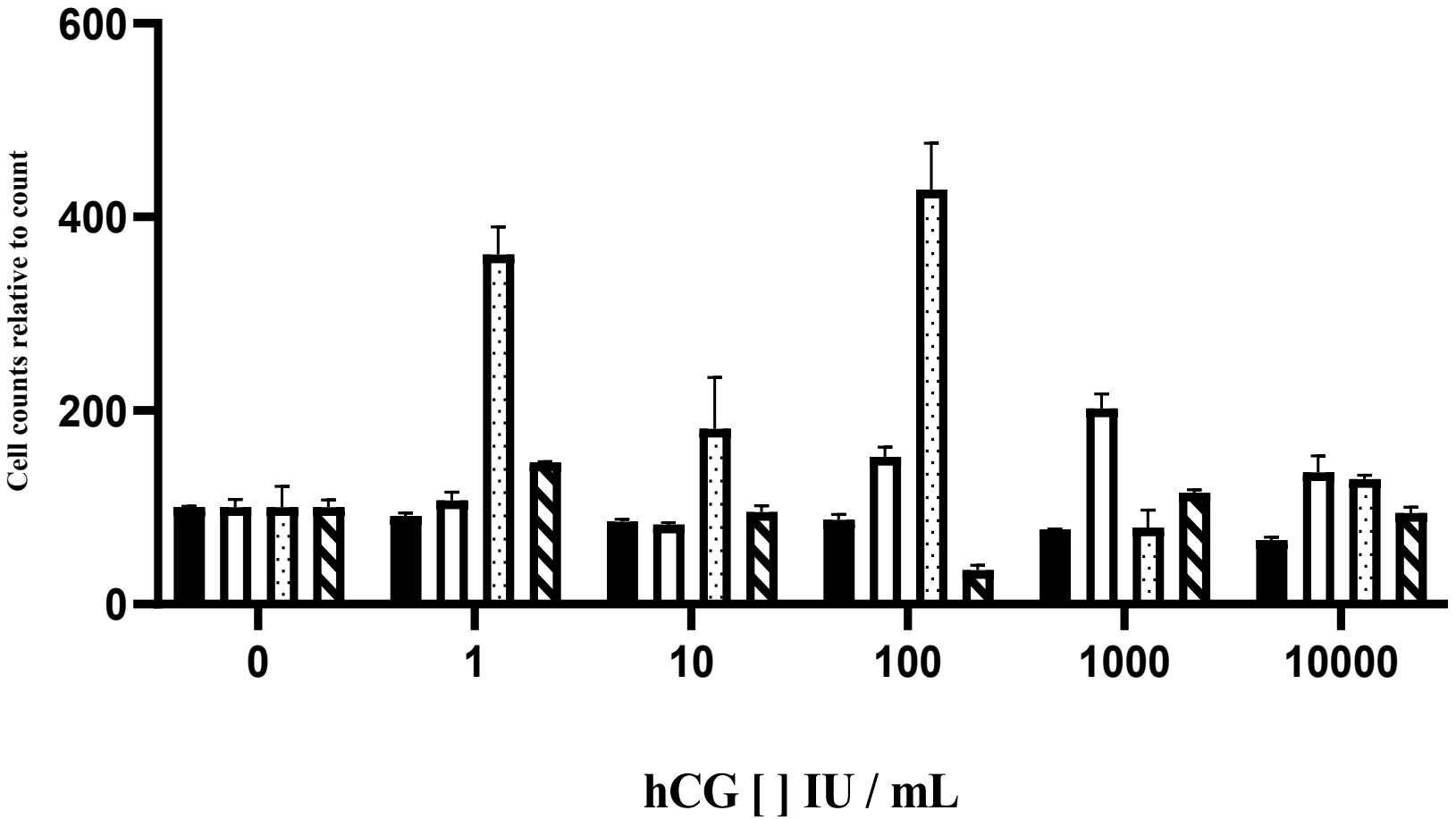


Figure 5.7. Significant effect of hCG concentration, exposure time, and the interaction between hCG concentration X exposure time on cell count for GRACE-HSA ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

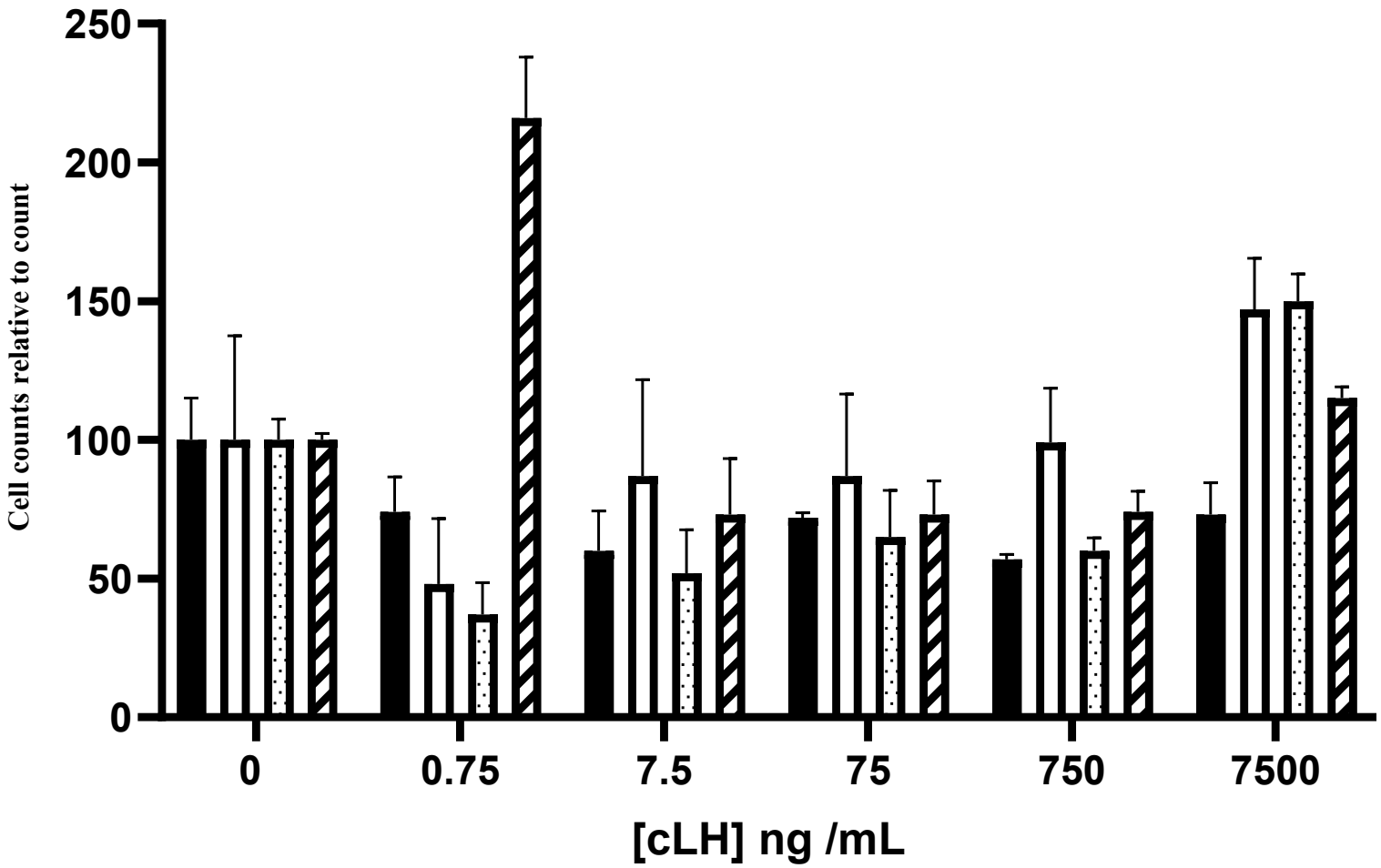


Figure 5.8. Significant effect of cLH concentration, exposure time, and the interaction between cLH concentration X exposure time on cell count for DAL-4 ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

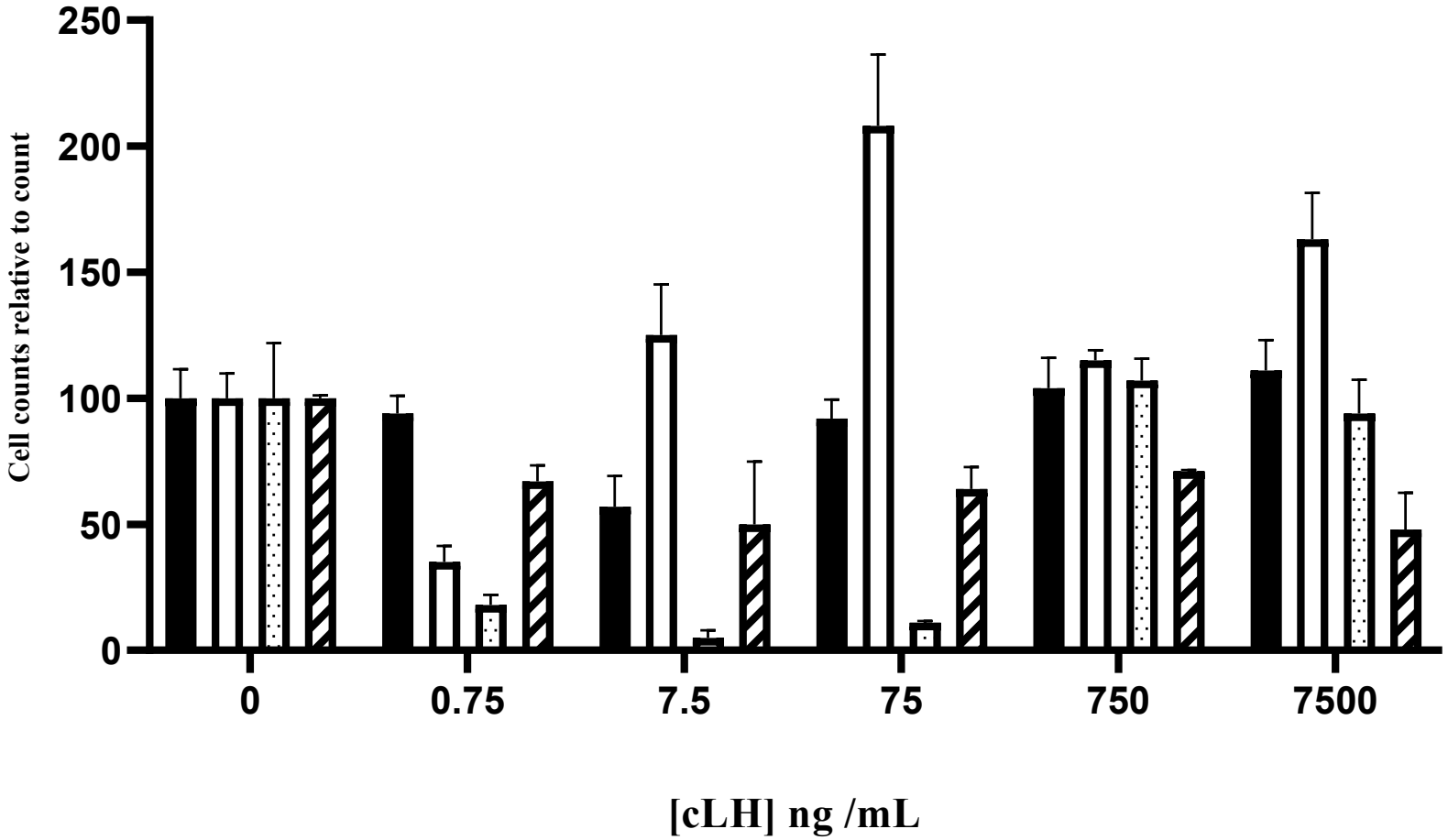


Figure 5.9. Significant effect of cLH concentration, exposure time, and the interaction between cLH concentration X exposure time on cell count for EFS ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

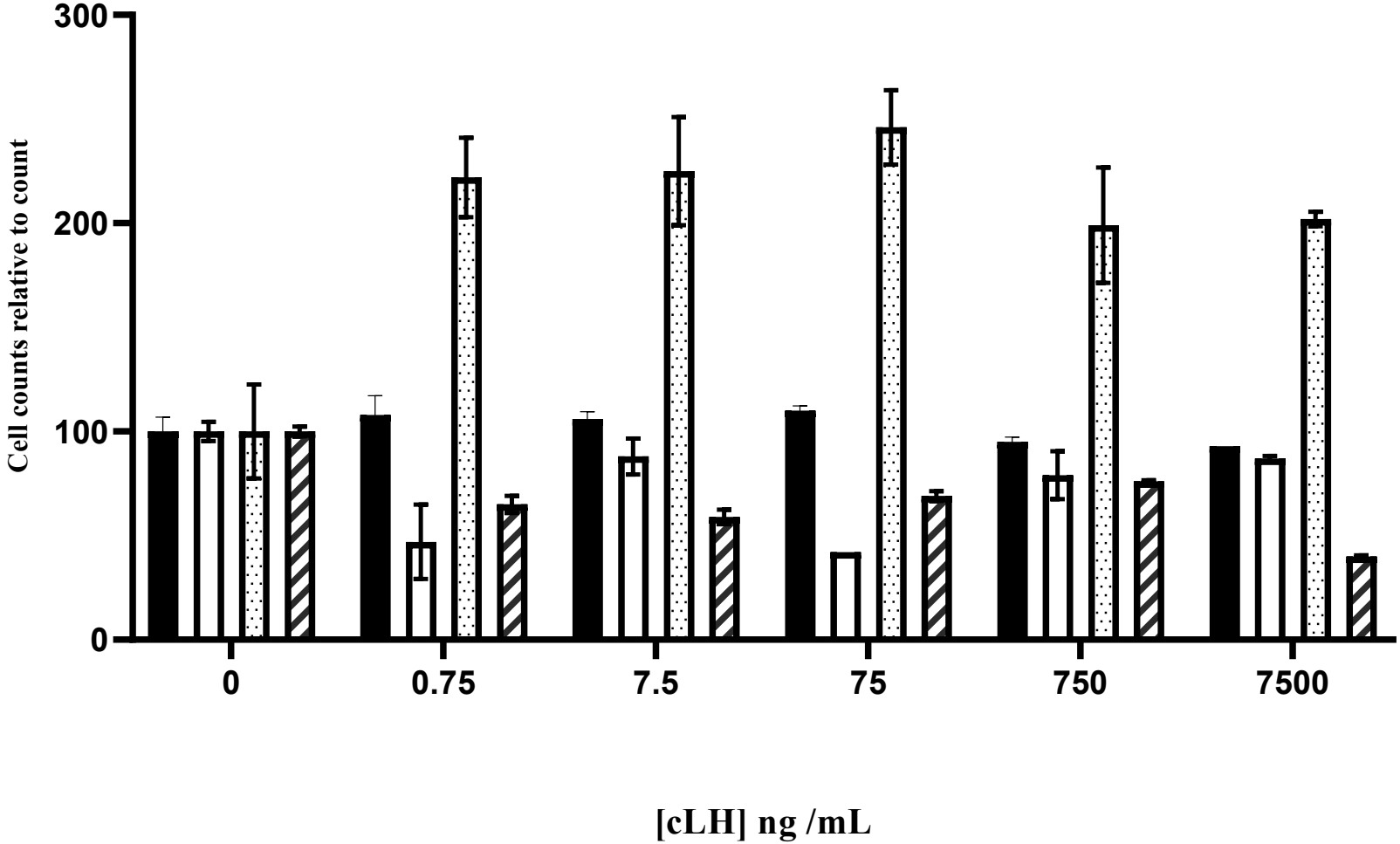


Figure 5.10. No significant effect of cLH concentration, but there was significant effect cLH exposure time and the interaction between cLH concentration X exposure time on cell count for DHSA ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

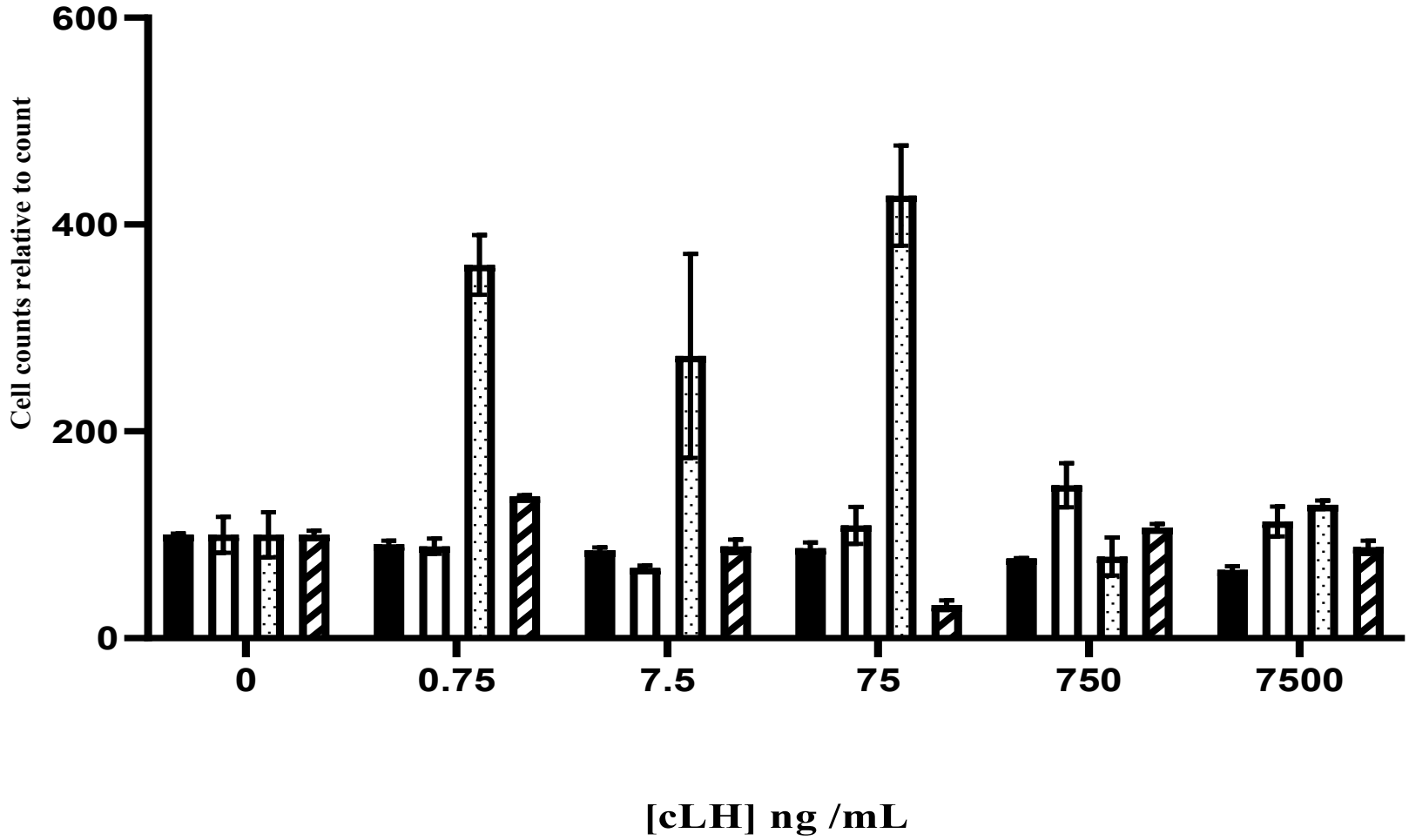


Figure 5.11. Significant effect of cLH concentration, exposure time, and the interaction between cLH concentration X exposure time on cell count for GRACE-HSA ($p<0.005$). Exposure time: 24-hours (black bar), 48-hours (white bar), 72-hours (dotted bar), and 96-hours (striped bar).

respectively. At 48-hours, canine splenic hemangiosarcoma cell lines had significantly different cell counts in response to hCG concentration ($p=0.0128$) and a trend towards a difference to cLH ($p=0.0615$). However, there was no effect of hCG or cLH concentration on cell count at 96-hours ($p=0.8867$ and $p=0.1548$, respectively).

5.5. Discussion

This is first study to show LHR expression in isolated canine hemangiosarcoma cells. In the present study, the splenic hemangiosarcoma cells from the female spayed dog (EFS) had higher LHR expression compared the neutered male dog (DHSA) and the intact male dog (DAL-4) ($p=0.0142$). This is supported by previous research that has shown that spayed female dogs have up to 10 times the risk for developing splenic hemangiosarcoma than do intact female dogs (Prymak and Mckee, 1988; Torres de la Riva et al., 2013; Zink et al., 2014). Previous research from our laboratory has shown that the percentage of circulating T- lymphocytes that expressed LHR was significantly higher in gonadectomized dogs (16.6 %) than in sexually intact groups (10.5 %) (Ettinger et al., 2018). Together, these findings illustrate that gonad removal with spaying and neutering alters LHR expression in normal and neoplastic canine cells.

Although the response to LHR activation in the present study varies by cell line and exposure time, it is possible that LHR activation in spayed and neutered animals is involved in the etiopathogenesis of canine splenic hemangiosarcoma. Treatment of human leiomyoma cells with hCG increases cell proliferation (Horiuchi et al., 2000). In addition, the proliferation of gastric cancer cells was significantly higher after hCG

treatment compared with the controls (Zhao et al., 2018). In vivo studies are needed to determine if downregulation of LH concentrations in spayed/ neutered dogs with a gonadotropin-releasing hormone agonist may prolong survival time in dogs with splenic hemangiosarcoma.

5.6. References

American Kennel Club. (2020). Most popular breeds. Accessed on July 24, 2020 from https://www.akc.org/most-popular-breeds/

Beijerink NJ, Buijtels JJ, Okkens AC, et al. (2007). Basal and GnRH-induced secretion of FSH and LH in anestrous versus ovariectomized bitches. Theriogenology, 67(5), 1039–1045.

Brown NO, Patnaik AK, MacEwen EG. (1985). Canine hemangiosarcoma: retrospective analysis of 104 cases. J Am Vet Medic Assoc, 186(1), 56–58.

Chen S. (2010). Advanced diagnostic approaches and current medical management of insulinomas and adrenocortical disease in ferrets (Mustela putorius furo). Vet Clin North Am. Exotic Anim Pract, 13(3), 439–452.

Concannon PW. (2011). Reproductive cycles of the domestic bitch. Anim Reprod Sci, 124(3-4), 200–210.

Clifford CA, Mackin AJ, Henry CJ. (2000). Treatment of canine hemangiosarcoma: 2000 and beyond. J Vet Intern Med, 14(5), 479–485.

Ettinger AM, Gust SK, Kutzler MA. (2019). Luteinizing hormone receptor expression by nonneoplastic and neoplastic canine lymphocytes. Am J Vet Res, 80(6), 572–577.

Flint C, Gust S, Kutzler M. (2019). Luteinizing hormone receptor mediated proliferation of isolated canine T lymphoma cells. Clin Theriogenology. 2019; 11: 477.

Galac S, Kars VJ, Klarenbeek S, et al. (2010). Expression of receptors for luteinizing hormone, gastric-inhibitory polypeptide, and vasopressin in normal adrenal glands and cortisol-secreting adrenocortical tumors in dogs. Dom Anim Endocrinol, 39(1), 63–75.

Gullaba JM, Neumann PR, Kutzler MA. (2014). Role of IL-8 and TIMP-2 on term canine trophoblast migration, invasion, and proliferation. J Vet Sci Res, 2(1): 011

Hammer AS, Couto CG, Filppi J, et al. (1991). Efficacy and toxicity of VAC chemotherapy (vincristine, doxorubicin, and cyclophosphamide) in dogs with hemangiosarcoma. J Vet Intern Med, 5(3), 160–166.

Horiuchi A, Nikaido T, Yoshizawa T, et al. (2000). HCG promotes proliferation of uterine leiomyomal cells more strongly than that of myometrial smooth muscle cells in vitro. Mol Hum Reprod, 6(6), 523–528.

Kiefel CA, Kutzler MA. (2020). Assessment of luteinizing hormone receptor expression in structural support tissues of canine hip and femorotibial joints. Am J Vet Res, 81(7), 565–571.

Kim SE, Liptak JM, Gall T, et al. (2007). Epirubicin in the adjuvant treatment of splenic hemangiosarcoma in dogs: 59 cases (1997-2004). J Am Vet Med Assoc, 231(10), 1550–1557.

Klotz L, Boccon-Gibod L, Shore ND, et al. (2008). The efficacy and safety of degarelix: a 12-month, comparative, randomized, open-label, parallel-group phase III study in patients with prostate cancer. BJU Internat, 102(11), 1531–1538.

Kutzler M, Louey S, Jonker S, et al. (2007). Endothelin-1 (ET-1) regulates coronary blood flow in fetal sheep. Abstract presented at the 2007 Society for Gynecologic Investigation meeting (Reno, Nevada).

Li W, Kutzler M. (2020). Luteinizing hormone receptor gene expression in canine neoplastic lymphoma cells is upregulated by treatment with human chorionic gonadotropin. Abstract presented at the 2020 Society for the Study of Reproduction virtual meeting.

Ng CY, Mills JN. (1985). Clinical and haematological features of haemangiosarcoma in dogs. Aust Vet J, 62(1), 1–4.

Ponglowhapan S, Church DB, Scaramuzzi RJ, et al. (2007). Luteinizing hormone and follicle-stimulating hormone receptors and their transcribed genes (mRNA) are present in the lower urinary tract of intact male and female dogs. Theriogenology, 67(2), 353-366.

Ponglowhapan S, Church DB, Khalid M. (2012). Expression of luteinizing hormone and follicle-stimulating hormone receptor in the dog prostate. Theriogenology, 78(4), 777–783.

Prymak C, McKee LJ, Goldschmidt MH, et al. (1988). Epidemiologic, clinical, pathologic, and prognostic characteristics of splenic hemangiosarcoma and splenic hematoma in dogs: 217 cases (1985). J Am Vet Med Assoc, 193(6), 706–712.

Reichler IM, Welle M, Eckrich C, et al. (2008). Spaying-induced coat changes: the role of gonadotropins, GnRH and GnRH treatment on the hair cycle of female dogs. Vet Dermatol, 19(2), 77–87.

Reichler IM. (2010). Urinary incontinence and puppy coat due to spaying in the bitch. An overview of pathophysiology, diagnosis and therapy. Tierarztl Prax Ausg K Kleintiere Heimtiere, 38(3), 57-62.

Shiu KB, Flory, AB, Anderson, CL, et al. (2011). Predictors of outcome in dogs with subcutaneous or intramuscular hemangiosarcoma. J Am Vet Med Assoc, 238(4), 472–479.

Swayzer DV, Gerriets V, Leuprolide. (2019). Accessed on July 15, 2020 at https://www.ncbi.nlm.nih.gov/books/NBK551662/?report=printable

Torres de la Riva G, Hart BL, Farver TB, et al. (2013). Neutering dogs: effects on joint disorders and cancers in golden retrievers. PloS One, 8(2), e55937. https://doi.org/10.1371/journal.pone.0055937

Treggiari E, Borrego JF, Gramer I, et al. (2020). Retrospective comparison of first-line adjuvant anthracycline vs metronomic-based chemotherapy protocols in the treatment of stage I and II canine splenic haemangiosarcoma. Vet Comp Oncol, 18(1), 43–51.

Welle MM, Reichler IM, Barth A, et al. (2006). Immunohistochemical localization and quantitative assessment of GnRH-, FSH-, and LH-receptor mRNA expression in canine skin: a powerful tool to study the pathogenesis of side effects after spaying. Histochem Cell Bio, 126(5), 527–535.

Zhao R, Zhang T, Xi W, et al. (2018). Human chorionic gonadotropin promotes cell proliferation through the activation of c-Met in gastric cancer cells. Oncol Lett, 16(4), 4271–4278.

Zink MC, Farhoody P, Elser SE, et al. (2014). Evaluation of the risk and age of onset of cancer and behavioral disorders in gonadectomized Vizslas. J Am Vet Med Assoc, 244(3), 309–319.

Zwida K, Kutzler M. (2019). Luteinizing hormone receptor is immunoexpressed within the canine thyroid. ClinTheriogenology, 11(1), 23-29.

Zwida KH, Valentine BA, Kutzler MA. (2018). Immunohistochemical localization of LH receptors in canine splenic hemangiosarcoma. J Vet Sci Anim Husb, 6(4): 410.

CHAPTER 6

CONCLUSION AND FUTURE DIRECTIONS

This thesis investigated three hypotheses: (1) that LHCGR would be expressed in lymphoma, mastocytoma, hemangiosarcoma, (2) that differences would exist in LHCGR expression between intact and gonadectomized dogs in these three cancers, and (3) that LHCGR activation would induce cell proliferation in hemangiosarcoma. Building on a previous study that reported LHCGR expression in canine lymphoma (Ettinger et al, 2019), this thesis research demonstrated that LHR expression in lymphoma of spayed and neutered dogs is not related to body weight, sex, immunophenotype or tumor stage. Also building on a previous study that reported higher LHCGR expression in circulating T-lymphocytes in spayed and neutered dogs compared to intact dogs (Ettinger et al, 2019), this thesis research demonstrated that LHCGR expression is increased in mastocytoma tumor cells from gonadectomized dogs compared to intact dogs. This was further supported by this thesis research in isolated canine splenic hemangiosarcoma cell lines that showed that LHCGR expression is significantly higher following spaying. The results from this thesis provide evidence for how gonadectomy may increase the incidence of cancer and other long-term health disorders in dogs.

Future studies should continue this research with in vivo clinical trials aimed at reducing circulating LH concentrations. Previous clinical studies have reduced LH concentrations in gonadectomized dogs using gonadotropin releasing agonist (GnRH agonists) or immunization to restore continence to incontinent spayed dogs (Donovan et al., 2012; Donovan et al., 2014; Reichler., 2006) as well as to restore a normal hair coat in dogs with puppy coat syndrome (Reichler 2010). We hypothesize that reducing LH concentrations in canine patients with

lymphoma will prolong remission times, thereby extending life expectancy for months or years. In addition, reducing LH concentrations in dogs with hypothyroidism may restore normal thyroid function. This could be investigated by using fresh canine thyroid tissues to generate three-dimensional multicellular thyrocyte spheroids for in vitro functional studies. We hypothesize that increasing LHCGR activation will reduce thyrocyte hormone (T4) secretion in response to thyroid stimulating hormone (TSH) in vitro. Last, additional research is needed investigating the long-term effects of elevated LH concentrations on life span. Previous research in this area has focused on only one breed (Rottweiler) (Kengeri et al., 2013; Waters et al., 2009). Expanding this research to other breeds and mixed breed dogs will provide valuable insight to veterinarians counseling their clients when (or if) they should spay or neuter their dogs. This longevity research could also serve as an emerging model of for studying human longevity.

6.1. References

Donovan CE, Gordon JM, Kutzler MA. (2014). Gonadotropin-releasing hormone immunization for the treatment of urethral sphincter mechanism incompetence in ovariectomized bitches. Theriogenology, 81(2),196-202.

Donovan CE, Greer M, Kutzler MA. (2012). Physiologic responses following gonadotropin-releasing hormone immunization in intact male dogs. Reprod Domest Anim, 47, Suppl 6,403-5.

Ettinger AM, Gust SK, Kutzler MA. (2019). Luteinizing hormone receptor expression by nonneoplastic and neoplastic canine lymphocytes. Am J Vet Res, 80(6), 572–577.

Kengeri SS, Maras AH, Suckow, CL, et al. (2013). Exceptional longevity in females Rottweiler dogs is not encumbered by investment in reproduction. AGE, 35, 2503–2513.

Reichler IM. (2010). Urinary incontinence and puppy coat due to spaying in the bitch. An overview of pathophysiology, diagnosis and therapy. Tierarztl Prax Ausg K Kleintiere Heimtiere, 38(3), 57-62.

Reichler IM, Jochle W, Piche CA, et al. (2006). Effect of long-acting GnRH analogue or PPA on plasma LH/FSH, urethral pressure profiles and clinical signs of urinary incontinence due to sphincter mechanism incompetence in bitches. Theriogenology, 66,1227-1236.

Waters DJ., Kengeri SS, Clever B, et al. (2009). Exploring mechanisms of sex differences in longevity: lifetime ovary exposure and exceptional longevity in dogs. Aging cell, *8*(6), 752–755.

APPENDIX A. IMMUNOCYTOCHEMICAL ASSAY PROTOCOL

A.1. Supplies/Equipment

1. Cultured cell lines
2. Hemangiosarcoma medium.
3. 22 mm^2 glass coverslips.
4. 35 mm diameter dishes.
5. Microscope slides
6. PBS
7. 70% ethanol.
8. 50 mM glycine (pH 2.0)
9. 1 % sodium azide.
10. Blocking buffer (PBS with 1% BSA and 0.5% Triton X100).
11. Primary antibodies for canine HSA:
    a. Rabbit Polyclonal LHR Antibody (#NLS1 436)
    b. A universal negative control.
12. Secondary antibody: Goat Polyclonal Goat anti-Rabbit lgG (H+L) Secondary Antibody [FITC]
13. VECTASHIELD(R) HardSet (TM) Antifade Mounting Medium with DAPI”
14. Fluorescence microscope.

A.2. Preparation in advance

1. Sterilize glass coverslips:
    a. Put 30 coverslips into a 10 mL beaker and cover it with foil on it and tape on it then autoclave.
    b. Keep inside the hood to be ready to use.
2. 50 mM glycine (pH 2.0):
    a. Dissolve 0.375 g of glycine in 70 ml of 70 % ethanol, check PH first, then adjust PH to 2.0.
    b. Adjust it by using acid or base based on PH., add a few drops to the solution in the beaker and wait at least 20 seconds before reading the pH on the meter until to get right PH.
    c. Add more ethanol until to reach to total volume 100 ml.
    d. Put the solution in bottle/centrifuge tube at refrigerator.
3. 1% sodium azide: Dissolve 1 g of sodium azide in 100 ml of PBS.

A.3. Steps

1. Label the dishes according to their cell line.
2. Place one coverslip into sterile 35 mm dish. 12 dishes per cell line.
3. Thaw trypsin at room temperature and warm 50 ml of media and HBSS.
4. Remove the media from culture flask by pouring them in waste container
5. Wash the cells with HBSS twice; add 1 ml of HBSS.
6. Add 1 ml of trypsin flask and rock the flask gently to ensure solution cover all the cells.

7. Wait 1-3 min and read under microscope, then rack it or hit the side of the flask against palm of hand and check it again to ensure that > 90 % of cells are detached (floated).
8. Pour all cell suspension from flask with cell line into a labeled 15 ml conical tube and then add equal volume of media to the flask to rinse out any remaining cells, pour media into flask, and repeat step twice to ensure all cells in the tube.
9. Centrifuge cells at 1000 RPM, for 5 min.
10. Remove supernatant down to 2 ml.
11. Mix up cell suspension by pipetting and mix well to avoid clumps.
12. Remove 10 ul of cell suspension into a microcentrifuge tube (990 microliter of formalin).
13. Count the cells on hemocytometer.
14. Fill both sides of the chamber with cell suspension (~5-10 ul) and view under microscope using X 20 magnification.
15. Count the number of cells. Count all the cells on grid. **Dilute cell suspension before adding media to the cells
16. Make suspension of cells and should have at least 6 ml and at least 1.5 M cells so, we can add 2 ml of cell suspension in each dish.
17. Make dilution for 7 ml. (n (6) +1) as extra in case pipetting loses!
18. Prepare cell suspension for “250.000 cells/ ml” that’s concentration. we need 7 ml.
19. Add 2 ml of cell suspension (1M cells/ml) over each coverslip in the dish.

20. Grow the cells at 37°C in a humidified 5% CO2 incubator and wait one day and next day, remove media and add fresh media and 2 days after passaging them until reaching 70-80% confluence.
21. Aspirate the culture medium from each dish and rinse the cells by washing with PBS.
22. Fix the cells onto the coverslips by incubate them in 70% ethanol with 50 mM glycine (pH 2.0) for 30 minutes at -20°C.
23. Rinse the cells again with PBS.
24. Store the cells in PBS with 1% sodium azide at 4°C until staining. "STOP STEP"
25. Dilute the primary antibody/antibodies in diluted antibody.
    a. Mouse Monoclonal VEGF Antibody (VG1) [Alexa Fluor® 594]
    b. Rabbit Polyclonal LHR Antibody (#NLS1 436)
    c. A universal negative control.
26. Add primary antibodies and control to coverslips and incubate them overnight at 4°C in dark humidified chamber.
27. Rinse the cells in PBS.
28. Dilute Goat Polyclonal Goat Anti-Rabbit lgG (H+L) secondary antibody conjugated to FITC at a 1:200 dilution in a dark room and then apply to coverslips for a 2-hour incubation at 20°C.
29. Rinse the cells in PBS.
30. Label a microscope slide for each coverslip.

31. Add drop of VECTASHIELD(R) HardSet (TM) Antifade Mounting Medium with DAPI to each slide.
32. Pick up each coverslip with a forceps and place it on the mounting medium, with the cell-side face down.
33. Visualize the cells using a fluorescence microscope and count 300 hemangiosarcoma cells at 400 X magnification.

APPENDIX B. MTT ASSAY STANDARD CURVE EXPERIMENT

B.1. Establish standard curve

1- Estimate Confluence: 60-65 %

2- Remove 0.5 mL and restart cells in T-25 flask.

3- Centrifuge tube, remove supernatant, add 500 µL protein free media to create concentration.

4- Pipette 100 µL (500,000) onto 4 wells and transfer 100 ul into new tube and add 400 µL of protein free media.

5- Pipette 100 µL (100,000) onto 4 wells and transfer 100 ul into new tube and add 400 µL of protein free media.

6- Pipette 100 µL (20,000) onto 4 wells and transfer 100 ul into new tube and add 400 µL of protein free media.

7- Pipette 100 µL (4,000) onto 4 wells and transfer 100 ul into new tube and add 400 µL of protein free media.

8- Pipette 100 ul (800) into 4 wells.

9- Then add ul MTT reagent to each well and return to incubator for 3.5 hours.

10- Passage and count cells: 1 M/mL (1Mtotal)

11- When the precipitant is clearly visible under microscope, add 100 ul of detergent reagent to all wells, including controls. Swirl gently for 60 sec, do not shake.

12- Return plate to incubator overnight (4-18 hours). Read by plate reader at 750nm on Day 2.

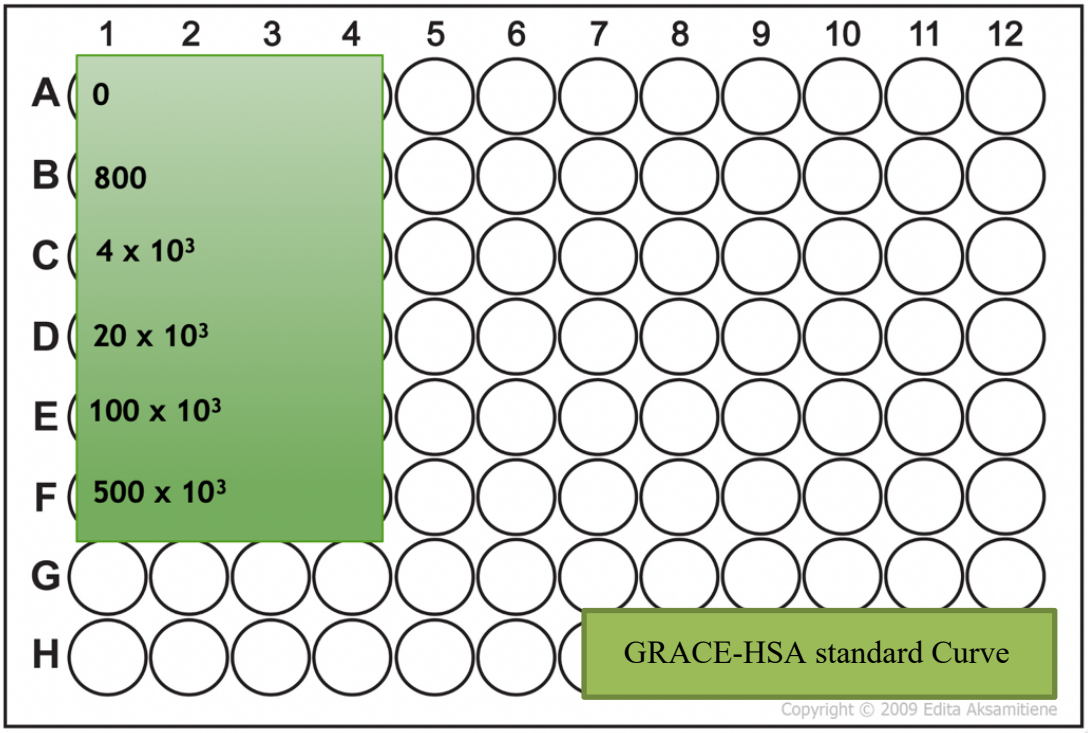


Figure B.1. Standard curve used for one cell line (GRACE-HSA)96 well plate layout

B.2. HCG response experiment: DHSA / EFS / DAL-4/ GRACE

1. Resuspend remaining cells in serum containing culture media for a final dilution of $2x10^6$/mL. (cell suspension was diluted to 2 mL)
2. Aliquot 750 µL of the cell suspension into six Eppendorf tubes and label the tubes

   0, 1, 10, 100, 1,000, and 10,000 IU/mL.
3. Add equal volume (750 µL) following hCG dilutions: 2, 20, 200, 2,000, and 20,000 IU/mL.
4. Prepare four 96-well plates in triplicate.
5. In three separate wells, aliquot 100 µL of culture media with increasing concentrations of hCG (0-20,000 IU/mL).
6. Incubate the cells at 37°C in a humidified 5% CO_2 incubator for 24, 48,72, and 96 hours.

1. After the incubation period, MTT was thawed in warmer beads.
2. Carefully remove media without disturbing cell monolayer and rinse cells two times in media without serum. (use one transfer pipette per 3 triplicates then add 100 µL media serum free and so on.)
3. Each well now has 100 µL media serum free.
4. Then add 10 µL of MTT Reagent to each well, swirl gently for 60 seconds (do not shake) and then return to incubator for 3.5 hours.
5. Add 100 µL of Crystal Dissolving Solution to all wells, including controls. Swirl gently for 60 seconds (do not shake). Return plate to incubator overnight (4-18 hours).
6. *Read by plate reader at 570 nm on Day 2*.

Figure B.2. HCG response experiment for DAL-4, EFS, and DHSA 96 well plate layout at 24-hours, 48-hour, 72-hours, and 96-hours.

APPENDIX C. ABSTRACTS, PRESENTATION AND POSTERS

C.1. 2020 Animal and Rangeland Sciences Poster Symposium

EXPRESSION OF LUTEINIZING HORMONE RECEPTOR (LHR) IN CANINE LYMPHOMA IS NOT AFFECTED BY BODY WEIGHT, SEX, IMMUNOPHENOTYPE, OR TUMOR STAGE

Expression of Luteinizing Hormone Receptor (LHR) in Canine Lymphoma is Not Affected by Body Weight, Sex, Lymphoma Phenotype, and Lymphoma Stage

Khawla Zwida, Alyssa Vedus, Michelle Kutzler
Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR 97331

Introduction

- Canine lymphoma is the most common hematopoietic neoplasm in the dog,[1] accounting for up to 24% of all canine cancers.[2]
- Previous research in our laboratory has shown that luteinizing hormone receptors (LHR) are present in normal and neoplastic canine lymph nodes and lymphocytes.[3]
- In circulating canine lymphocytes, gonad removal (spaying/neutering) increases the percentage of cells positive for LHR compared to intact dogs.[3]
- It is not known if body weight, sex, lymphoma phenotype (B-cell, T-cell) and lymphoma stage affect LHR expression.
- We hypothesized that LHR expression would differ with body weight, breed, sex, and lymphoma phenotype.
- The objective was to determine LHR expression (percentage of LHR positive cells, LHR staining intensity) in neoplastic lymph nodes with known lymphoma phenotypes and stages.

Methods

- Formalin-fixed, paraffin embedded lymphoma tissue samples (n=40) were subjected to routine immunohistochemical technique using a rabbit polyclonal anti-human LHR antibody at a 1:100 dilution.
- The percentage of cells positive for LHR and the staining intensity (scored 0-3) were determined at 400X magnification by a single observer (AV).
- Differences between sex and tumor phenotype were compared using a Welch two-sample t test in the statistical package R.
- Differences in body weight and tumor stage were compared using a simple linear regression (Microsoft Excel).
- Data were expressed as mean ± standard deviation and significance was defined as $p < 0.05$.

Results

- All tumor samples contained cells positive for LHR but the percentage of cells expressing LHR and the LHR immunostaining intensity varied between individual (**Figure 1**).
- There were no significant differences in the percentage of LHR positive cells or staining intensity when compared to body weight, sex, lymphoma phenotype, and lymphoma stage (**Figures 2-5**).

Discussion

- This is the first study to evaluate the influence of body weight, sex, tumor phenotype and tumor stage on LHR expression in canine lymphoma.
- Current research includes investigation of the effects of LHR activation (e.g. cell proliferation) using canine lymphoma cell lines.
- Future research efforts will provide evidence to support using a complementary treatment for canine lymphoma by downregulating LH with a commercially-available canine gonadotropin-releasing hormone (GnRH) agonist.

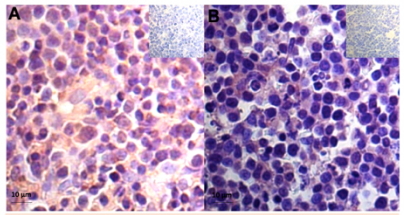


Figure 1. Representative cellular expression and staining intensity for LHR in B-cell (A) and T-cell (B) canine lymphoma. Negative control in upper right inset

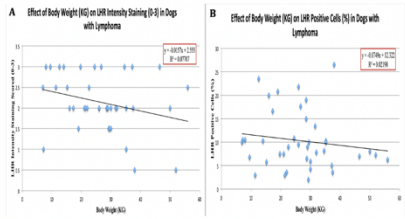


Figure 2. There was no correlation between body weight and LHR staining intensity (A) or the percentage of LHR positive cells (B).

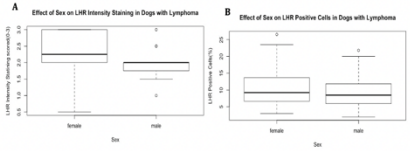


Figure 3. There was no effect of sex on LHR staining intensity (A) or the percentage of LHR positive cells (B).

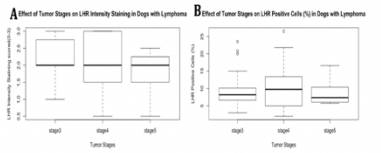


Figure 4. There was no effect of lymphoma stage on LHR staining intensity (A) or the percentage of LHR positive cells (B).

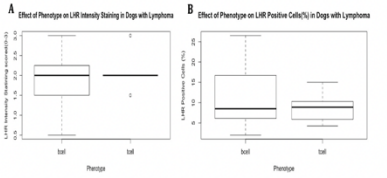


Figure 5. There was no effect of lymphoma phenotype (B-cell and T-cell) on LHR staining intensity (A) or the percentage of LHR positive cells (B).

References

1.MacEwen EG, et al. Evaluation of some prognostic factors for advanced multicentric lymphosarcoma in the dog: 147 cases (1978-1981). J Am Vet Med Assoc. 1987; 190:564-8.
2.Vail DM, et al. Canine lymphoma and lymphoid leukemia. In: Withrow, S.J., MacEwen, E.G. (Eds.), Small Animal Clinical Oncology, 3rd ed, WB Saunders Co, Philadelphia, PA 2001; 558-590.
3. Ettinger A. Luteinizing hormone receptor expression in normal and neoplastic canine lymphocytes. Oregon State University: Undergraduate Thesis 2018.

Acknowledgements

We thank the Canine Comparative Oncology and Genomics Consortium Inc. for allowing access to the tissue samples.

C.2. 2022 Northwest Reproduction and Sciences Symposium Abstract

Canine Splenic Hemangiosarcoma Cells Express Luteinizing Hormone Receptors In Vitro

Khawla Zwida, Michelle Kutzler
Department of Animal and Rangeland Sciences, Oregon State Univ., Corvallis, OR

Hemangiosarcoma is a rapidly growing, highly invasive cancer arising from the lining of blood vessels. More than half of all canine hemangiosarcoma primary tumors arise within the spleen. German shepherds, Golden Retrievers, and Labrador Retrievers are overrepresented compared to other breeds. In addition to breed, gonadectomy (spaying/neutering) increases the likelihood for developing hemangiosarcoma. Spayed female dogs have two to ten times the risk for developing splenic hemangiosarcoma compared to intact female dogs. Following gonadectomy, luteinizing hormone (LH) concentrations are significantly and persistently elevated. Our laboratory has previously demonstrated that formalin-fixed canine splenic hemangiosarcoma tissues express LH receptors (LHR). We hypothesized that isolated canine splenic hemangiosarcoma cells would also express LHR. The objective of this study was to use immunocytochemistry to determine the percentage of cells expressing LHR in each cell line. Immortalized cell lines isolated from four dogs with a primary splenic hemangiosarcoma (DAL-4, DHSA, GRACE-HSA, EFS; Kerafast, Inc, Boston, Massachusetts, USA) were used in this study. Cells were fixed onto coverslips and incubated with either a rabbit polyclonal anti-human LHR antibody (#NLS1436, Novus Biologicals, Littleton, Colorado, USA) or a universal negative control (#NC498H, BioCare Medical, Pacheco, California, USA). Cells were then incubated with horse anti-rabbit IgG conjugated to FITC (#NB 7159, Novus Biologicals, Littleton, Colorado, USA). The coverslips were then inverted and mounted to slides with medium containing DAPI (#H-1500, Vectashield® HardSet™ Antifade Mounting Medium with DAPI., Burlingame, CA, USA). Three randomly selected fields from each cell line were captured using fluorescence microscopy (Leica Microsystems, Germany) at 400X magnification. The mean±SD percentage of LHR positive cells for each cell line was compared by one-way ANOVA (GraphPad Prism 8.4.3) and significant was defined as $p< 0.05$. The percentage of cells positive for LHR was 6.9± 2.5%, 8.5 ± 1.0%, 11.8±3.1%, and 17.2± 4.5% in DAL-4, DHSA, GRACE-HSA, and EFS, respectively. The percentage of LHR positive cells was greater in EFS compare DHSA and DAL-4 ($p=0.0142$). This is first study to show LHR expression in isolated canine hemangiosarcoma cells. Our laboratory is currently examining the effect of LHR activation on hemangiosarcoma cell proliferation with increasing concentrations of LHR agonists. If activation of LHR is shown to induce splenic hemangiosarcoma cell proliferation, future clinical trials could begin using gonadotropin releasing hormone agonists to reduce LH concentrations in gonadectomized dogs to prevent the development or recurrence of hemangiosarcoma.

This research was funded by American Kennel Club Canine Health Foundation.
Keywords: Cancer, dog**,** gonadectomy, immunocytochemistry

C.3. 2022 Society for Theriogenology Abstract

Canine Splenic Hemangiosarcoma Cells Express Luteinizing Hormone Receptors In Vitro

Khawla Zwida[a], Lineth Truijillo[b], Michelle Kutzler[a]
[a]Department of Animal and Rangeland Sciences, [b]Department of Integrated Biology, Oregon State University, Corvallis, OR

Hemangiosarcoma is a rapidly growing, highly invasive cancer arising from the lining of blood vessels. More than half of all canine hemangiosarcoma primary tumors arise within the spleen. German shepherds, Golden Retrievers, and Labrador Retrievers are overrepresented compared to other breeds. In addition to breed, gonadectomy (spaying/neutering) increases the likelihood for developing hemangiosarcoma. Spayed female dogs have two to ten times the risk for developing splenic hemangiosarcoma compared to intact female dogs. Following gonadectomy, luteinizing hormone (LH) concentrations are significantly and persistently elevated. Our laboratory has previously demonstrated that formalin-fixed canine splenic hemangiosarcoma tissues express LH receptors (LHR).[1] We hypothesized that isolated canine splenic hemangiosarcoma cells would also express LHR. The objective of this study was to use immunocytochemistry to determine the percentage of cells expressing LHR in each cell line. Immortalized cell lines isolated from four dogs with a primary splenic hemangiosarcoma (DAL-4, DHSA, GRACE-HSA, EFS; Kerafast, Inc, Boston, Massachusetts, USA) were used in this study. Cells were fixed onto coverslips and incubated with either a rabbit polyclonal anti-human LHR antibody (#NLS1436, Novus Biologicals, Littleton, Colorado, USA) or a universal negative control (#NC498H, BioCare Medical, Pacheco, California, USA). Cells were then incubated with horse anti-rabbit IgG conjugated to FITC (#NB 7159, Novus Biologicals, Littleton, Colorado, USA). The coverslips were then inverted and mounted to slides with medium containing DAPI (#H-1500, Vectashield® HardSet™ Antifade Mounting Medium with DAPI., Burlingame, CA, USA). Three randomly selected fields from each cell line were captured using fluorescence microscopy (Leica Microsystems, Germany) at 400X magnification. The mean±SD percentage of LHR positive cells for each cell line was compared by one-way ANOVA (GraphPad Prism 8.4.3) and significant was defined as $p< 0.05$. The percentage of cells positive for LHR was 6.9± 2.5%, 8.5 ± 1.0%, 11.8±3.1%, and 17.2± 4.5% in DAL-4, DHSA, GRACE-HSA, and EFS, respectively. The percentage of LHR positive cells was greater in EFS compare DHSA and DAL-4 ($p=0.0142$). This is first study to show LHR expression in isolated canine hemangiosarcoma cells. Our laboratory is currently examining the effect of LHR activation on hemangiosarcoma cell proliferation with increasing concentrations of LHR agonists. If activation of LHR is shown to induce splenic hemangiosarcoma cell proliferation, future clinical trials could begin using GnRH agonists to reduce LH concentrations in gonadectomized dogs to prevent the development or recurrence of hemangiosarcoma.

This research was funded by American Kennel Club Canine Health Foundations.
Keywords: Cancer, dog**,** gonadectomy, immunocytochemistry

Zwida KH, Valentine BA, Kutzler MA: Immunohistochemical localization of LH receptors in canine splenic hemangiosarcoma. J Vet Sci Anim Husb 2018; 6(4): 410.

C.4. 2022 Northwest Reproduction and Sciences Symposium Presentation

Oregon State University

Canine Splenic Hemangiosarcoma Cells Express Luteinizing Hormone Receptors in Vitro

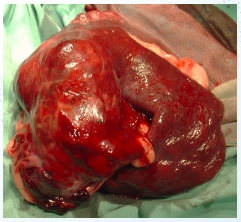
Khawla Zwida
Ph.D. Student.
Department of Animal and Rangeland Sciences

Introduction

- Approximately 6 million new cancer diagnoses are made in dogs each year.
  (The National Cancer Institute)
- 1 in 4 dogs will develop cancer at some point.
  (The Veterinary Cancer Society)
- Half of all canine cancers are not treatable even if caught early.
  (The Veterinary Cancer Society)

Introduction

- **Hemangiosarcoma (HSA)**
  - Rapidly growing, highly invasive cancer arising from the lining of blood vessels.
  - More than half of all hemangiosarcoma primary tumors arise within the spleen.



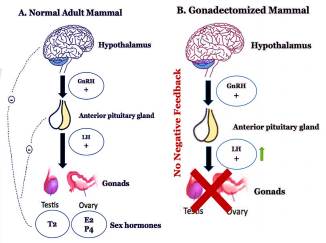
https://commons.wikimedia.org/wiki/File:Splenic_hemangiosarcoma.JPG

Introduction

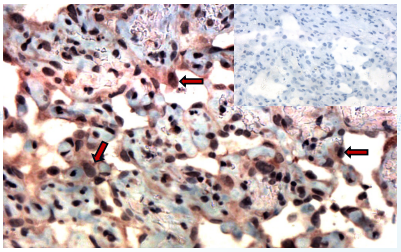
- **Hemangiosarcoma (HSA)**
  - German shepherds, Golden Retrievers, and Labrador Retrievers are overrepresented compared to other breeds.
  - Gonadectomized dogs are reported to have 2X,4X, or 10X the risk for developing splenic hemangiosarcoma compared to intact dogs.
    (Prymak, 1988;de la Rivera et al., 2013;Zink et al., 2014)

Introduction

- Luteinizing hormone (LH) concentrations in gonadectomized dogs are more than 30X the concentrations found in normal adult dogs.
  (Beijerink et al., 2007)

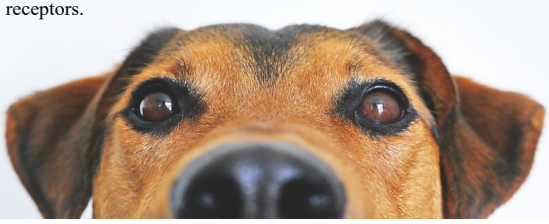


Introduction



Hypothesis

- Isolated canine splenic hemangiosarcoma cells will express LH receptors.



Material and Methods

- Immortalized cell lines isolated from dogs with a primary splenic HSA (Kerafast, Inc. Boston, MA).

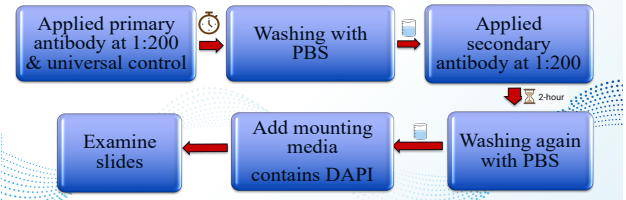
Cell line	Breed	Age	Sex and Status
DAL-4	Dalmatian	7 years	Intact male
EFS	Golden Retriever	9 years	Spayed female
DHSA-1426	Golden Retriever	10 years	Neutered male
GRACE-HSA	Portuguese Water Dog	8 years	Intact female

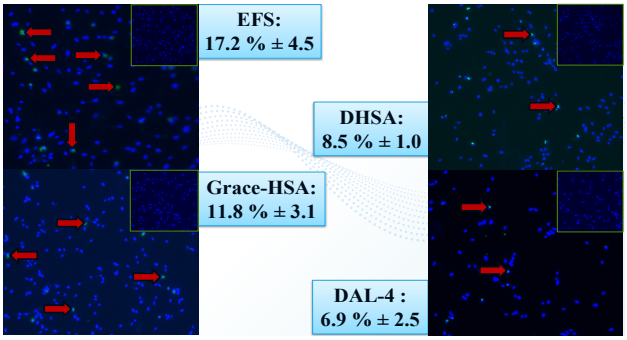
Cell preparation

- Splenic HSA cells were plated on sterile 22 mm^2 coverslips in 35 mm diameter dishes (500,000 cells/dish).
- Cells then were cultured until reaching 70-80% confluence.
- Removing culture media and washing with phosphate-buffered saline (PBS).

https://www.istockphoto.com

Immunocytochemistry Protocol





Conclusion

- LH receptors are expressed in isolated canine splenic hemangiosarcoma cells.
- Gonadectomy increases the numbers of splenic HSA cells expressing LHR.
- This finding is supported by previous research showing that gonadectomized dogs had higher percentage of circulating T-lymphocytes that expressed LH receptors compared to sexually intact dogs (mean ± SD, 16.6 ± 7.8% vs 10.5 ± 2.3%, respectively).

(Ettinger et al., 2019)

Cell fixation

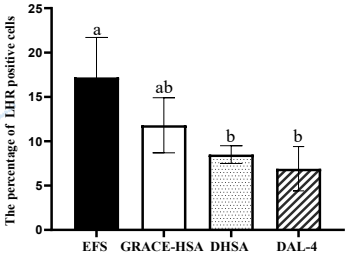
- The cells then fixed onto the coverslips in 70% ethanol with 50 mM glycine (pH 2.0) for 30 minutes at -20°C.
- The coverslips washed again with PBS and then stored at 4°C in PBS with 1% sodium azide until staining.

https://stock.adobe.com

Data Analysis

- Data were expressed as the mean ± SD percentage of LHR positive cells per field for each cell line.
- One-way ANOVA was used to compare the percentage of LHR positive cells for each cell line.
- Significance was defined as $p<0.05$.

Results



Acknowledgements

American Kennel Club
Canine Health Foundation

Supervisor
Dr. Michelle Kutzler

C.5. 2022 Society for Theriogenology Presentation

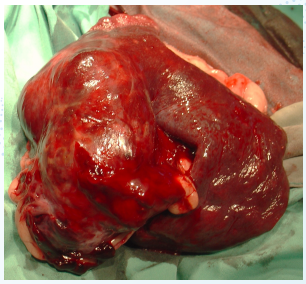
Oregon State University

Canine Splenic Hemangiosarcoma Cells Express Luteinizing Hormone Receptors in Vitro

Khawla Zwida
Ph.D. Student
Department of Animal and Rangeland Sciences

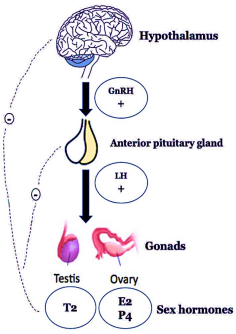
Hemangiosarcoma

- Rapidly growing, highly invasive cancer arising from the lining of blood vessels.
- More than half of all hemangiosarcoma primary tumors arise within the spleen.



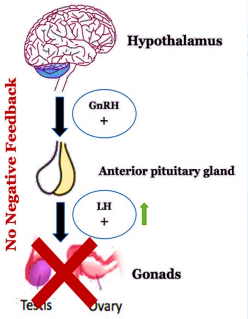
https://commons.wikimedia.org/wiki/File:Splenic_hemangiosarcoma.JPG

Hypothalamic-Pituitary-Gonadal Axis



- Up to 30X higher compared to intact dogs

(Beijerink et al., 2007)



Hypothesis and Objective

- Overall hypothesis: differences in LHR expression in normal and neoplastic tissues explains the increased risk for health problems in spayed and neutered dogs
- Study hypothesis: isolated canine splenic hemangiosarcoma cells will express LH receptors
- Objective: to use immunocytochemistry to determine the percentage of cells expressing LHR in four hemangiosarcoma cell lines

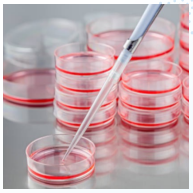
Material and Methods

- Immortalized cell lines isolated from dogs with a primary splenic HSA (Kerafast, Inc. Boston, MA).

Cell line	Breed	Age	Sex and Status
DAL-4	Dalmatian	7 years	Intact male
EFS	Golden Retriever	9 years	Spayed female
DHSA-1426	Golden Retriever	10 years	Neutered male
GRACE-HSA	Portuguese Water Dog	8 years	Intact female

Cell Preparation

- Cells plated on sterile 22 mm^2 coverslips in 35 mm diameter dishes (500,000 cells/dish)
- Cells cultured until 70-80% confluence
- Cells fixed onto the coverslips in 70% ethanol with 50 mM glycine (pH 2.0)



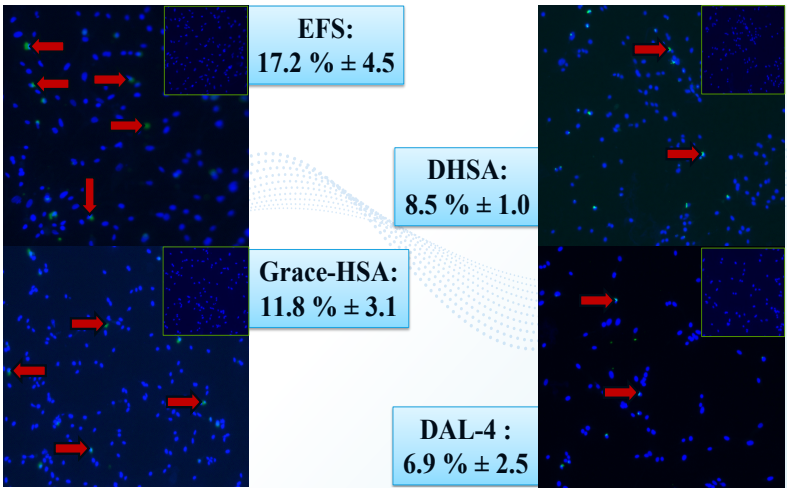
https://www.istockphoto.com

Immunocytochemistry Protocol

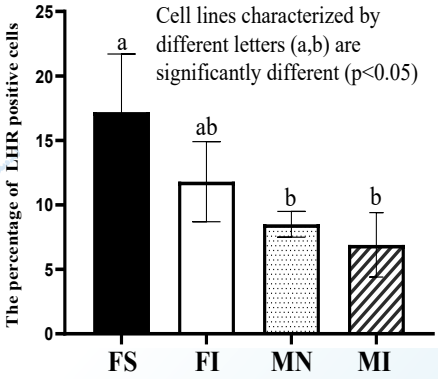
- Primary antibody: #NLS1436, Novus Biologics (1:200)
- Negative control: #NC495H, Biocare Medical
- Secondary antibody: #NB 7159, Novus Biologicals, Life Technologies (1:200)
- Nuclear staining: #H-1500, Vectashield® HardSet™ Antifade Mounting Medium with DAPI

Data Acquisition and Analysis

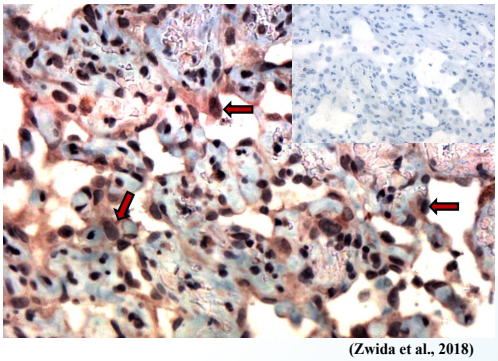
- Three randomly selected fields from each cell line were imaged using fluorescence microscopy at 400X magnification
  - From each field, the total number of cells was counted and the percentage of LHR positive cells was determined
- Data were expressed as the mean ± SD
- One-way ANOVA was used to compare the percentage of LHR positive cells for each cell line
  - Significance was defined as $p<0.05$



Results



Discussion



(Zwida et al., 2018)

- % of circulating T-lymphocytes that expressed LHR
  - Spayed/neutered dogs > > intact dogs

(Ettinger et al., 2019)

Conclusion and Future Research

- LHR expression in neoplastic cells of spayed/neutered dogs with higher LH concentrations => increased susceptibility to certain cancers
- Clinical trials downregulating LH concentrations in spayed/neutered dogs => prolong survival time in dogs with splenic hemangiosarcoma

Acknowledgements

AMERICAN KENNEL CLUB
CANINE HEALTH FOUNDATION
PREVENT TREAT & CURE®

Canadian Bureau for International Education

Any Questions?