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The Effects of Chronic Alcohol Consumption on the Mouse Endometrium

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ABSTRACT

As a result of alcohol consumption being highly prevalent in today's society, research has been done to investigate the effects of alcohol on the body's physiological systems. Research has indicated that heavy alcohol consumption is detrimental to the normal structure and function of some organs, especially the liver. However, little research has focused on the effects of chronic alcohol consumption on the female reproductive system. To investigate these effects, the uterine tissues of mice fed an ethanol diet (the NIAAA model also known as the Lieber-DeCarli diet) and mice fed a control diet were compared. The NIAAA model was chosen for this research because it simulates the drinking pattern that is known to cause liver disease in alcoholic hepatitis patients. This is achieved by incorporating both chronic and binge drinking patterns of alcohol consumption. In this study, the mucin layer that lines the endometrial surface of the uterus was analyzed in mice separated into ethanol and control fed groups. The ethanol fed mice were put on the Lieber-DeCarli 5% (v/v) ethanol diet *ad libitum* for 10-days followed by a single high dose of ethanol (5g/kg) on the 11^{th} day. The control fed mice were placed on an ethanol free isocaloric diet (supplemented with maltose dextrin to match the calories of ethanol). After the 11^{th} day, the mice were sacrificed, and uterine tissues were harvested. The tissues were then embedded in paraffin, sectioned, stained via the Hematoxylin and Eosin (H&E) technique, and examined under a microscope. The thickness of the uterine mucin layer was then measured for each animal and the average thicknesses were calculated. A one-way ANOVA test was employed to compare the mucin thickness between the two groups of animals. The test revealed no statistically significant difference between the thicknesses of the uterine mucin layer in the control and ethanol fed animals (P-value: 0.774).

INTRODUCTION

The consumption of alcohol (ethanol) is a relatively common practice throughout the world, and it has been for many years. In Western civilization, alcohol was discovered after the naturally fermented juices of spoiled fruit were unintentionally ingested by early humans. After the effects of ingesting alcohol were noticed, alcohol consumption became much more prevalent. As a result, the production of alcohol increased, and alcohol was treated as an important commodity that was traded amongst societies and even used in some cultural ceremonies (Hames, 2014). Alcohol consumption also increased in some societies due to unreliable and often contaminated water sources. In these occasions, alcoholic drinks were frequently poured into the contaminated water to help kill the pathogens in the water supply (Vallee, 1998).

Studies have shown that alcohol can have a variety of negative effects on the human body when consumed in chronic and excessive amounts. Some effects of excessive alcohol consumption include:

- alcohol-related brain damage (Zahr, et al., 2011);
- increased risk of cardiovascular conditions like hypertension, coronary heart disease, stroke, and cardiomyopathy (Piano, 2017);
- alcoholic pancreatitis (Herreros-Villanueva, et al., 2013);
- increased risk for various types of cancer (Bagnardi, et al., 2001);
- immune deficiency (Sarkar, 2015);
- depletion of vital nutrients like folate, which is important for the formation of the neural tube in early pregnancy (Medici and Halsted, 2013).

Further research has also indicated that the consumption of alcohol can alter the hormone balance within the body, leading to a variety of endocrine disturbances that may in turn result in

potential cardiovascular, reproductive, and immune abnormalities (Emanuele, et al., 1997). Most notably, alcohol has damaging effects to the liver and can lead to alcoholic liver disease (ALD). Alcoholic liver disease includes liver conditions like fatty liver, alcoholic hepatitis, and liver cirrhosis. Research has indicated that individuals who continue to consume increasing amounts of alcohol on a chronic basis tend to progress from fatty liver to hepatitis and then to cirrhosis, but these disorders can also occur at the same time (Mann, et al., 2003).

The effects of chronic alcohol consumption on certain aspects of the female reproductive system – specifically, the mucin layer of the uterine endometrium – have not yet been studied due to ethical reasons. For this reason, this study will examine the effect of alcohol consumption on the thickness of the mucin layer that lines the epithelium of the mouse endometrium, as this may indicate potential complications for reproduction that can be explored with further research.

Background of Mucins in Reproduction

Human reproduction generally requires the successful interaction between sperm and egg. But reproduction is a complex and multi-faceted process that requires many biological, hormonal, and structural changes to occur within the organs of the female reproductive system prior to the fertilization of an egg by sperm. As a result, when doing research on any aspect of the female reproductive system, care should be taken to ensure that one has at least a general understanding of the female anatomy and the changes required for successful reproduction.

The uterus is an essential organ of the female reproductive system because it is the site of attachment for an embryo during pregnancy. There are numerous functional components within the uterus that also contribute to the successful implantation and development of the embryo in the endometrium. One of these contributing components is the mucin layer that lines the epithelium of the endometrium within the uterus. The mucin layer has many functions,

including: lubricating the epithelial cells of the uterus to prevent desiccation, aiding in the transport of sperm, creating a physiologically sound environment for the embryo to develop during pregnancy, and protecting the female reproductive tract from enzymatic and microbial attack (Carson, et al., 1998). The thick layer of mucins that line the epithelium also function to act as a permeable gel layer that allows the exchange of gases and nutrients between the internal environment and the epithelium underneath the mucin layer (Bansil and Turner, 2006). Mucins also play roles in immune responses, the renewal and differentiation of cells within the epithelium, cell adhesion, and cell signaling (Martínez-Sáez, et al., 2017).

Mucins are secreted by goblet cells and other mucus producing epithelial cells within the epithelium of organs exposed to external environments. These cells secrete mucins that aggregate together on the apical surface of an organ's epithelial tissue and form a mucin layer. Some mucins are not secreted, and instead, they are anchored to the membrane of the tissue they occupy. These are often referred to as cell-bound or transmembrane mucins (Bansil and Turner, 2006; Dhar and McAuley, 2019). In general, mucins are large glycoproteins. There are various types of mucins throughout the body, but they all share a primary structure. This primary structure consists of a protein backbone enriched with a variable number of proline, serine, and threonine amino acids. O-linked oligosaccharide chains bond to the hydroxyl side chains of serine and threonine within the protein backbone and branch outward from the protein core. This results in the formation of highly glycosylated regions that form a "bottle-brush" configuration around the protein core of the mucin (Gevers, 1987).

These highly glycosylated regions help protect the uterine tissue below the mucin layer from both enzymatic degradation and pathogens. The bulky glycosylated regions block proteases from accessing the peptide backbone of the mucin. In turn, they prevent the underlying tissue

from being accessed by enzymes (Martínez-Sáez, et al., 2017). Mucins also protect cell tissue from microbial attack in several ways. In the lungs the thick gelatinous mucin layer traps any inhaled microbial organisms. The beating of cilia then moves the entrapped organisms so they can be expectorated or swallowed into the GI tract (Thai, et al., 2008). During this time, an immunological response by the body may also be activated to help neutralize the pathogen. Microbial organisms can also get trapped by mucins through a different method. Bacteria have receptors called adhesins that recognize and bind to oligosaccharide chains on the surface of mucins. Since these carbohydrate chains are highly variable for each type of mucin, this permits the mucins to trap a multitude of foreign organisms that may be attempting to infect the host (Lagow, et al., 1999). Therefore, one can understand the importance of mucins in protecting host tissue from attack by both enzymes and microbial organisms.

Thus far, 21 mucin genes have been identified and expressed throughout the human body. Fifteen of these genes are expressed in the gastrointestinal tract alone (Kasprzak and Adamek, 2019). However, research has indicated that eight mucin genes are expressed by the epithelia lining the mucosal surfaces of the female reproductive tract. These mucin genes are designated as MUC1-MUC7, with MUC5 containing two different subsets referred to as MUC5AC and MUC5B (Gipson, et al., 1997). MUC1 is a transmembrane mucin that lines most of the epithelial surfaces throughout the body, including those of the mammary gland, prostate, testis, portions of the digestive tract, lungs, and even the eye (Brayman, et al., 2004). MUC1 is thought to be highly significant in female reproduction because it is expressed throughout the reproductive tract. The endometrium has been shown to express high amounts of MUC1 at the apical surface of the epithelium with lower amounts of MUC6 (Gipson, et al., 1997).

MUC1 is thought to play an especially important role in embryo attachment and implantation. It is believed to act as a barrier that prevents premature embryo implantation into the endometrium at inappropriate times during the menstrual cycle. The precise functional mechanism of MUC1 is not fully understood, but research has indicated that one function of MUC1 is the inhibition of cell-cell adhesion via steric hindrance (Meseguer, et al., 2001). Animal studies on mice, pigs, and baboons have indicated that MUC1 expression is controlled by ovarian steroid hormones and downregulated just before implantation (Surveyor, et al., 1995; Bowen, et al., 1996; Hild-Petito, et al., 1996). This loss of MUC1 is thought to transition the endometrium from a pre-receptive state to a more receptive state for embryo implantation.

In mice, MUC1 is high during the proestrus and estrus stages, but decreases during metestrus and diestrus (Surveyor, et al., 1995). The ovarian steroid hormones thought to be involved in this process are estrogen and progesterone. Estrogen is believed to stimulate a high expression of MUC1, and progesterone is thought to act as the antagonist for estrogen that halts the stimulation of MUC1 expression. In this manner, estrogen increases MUC1 levels in the uterus during the pre-receptive phase, forming a barrier that prevents premature implantation of the embryo into the endometrium. As an antagonist, progesterone is thought to decrease the presence of MUC1 in mice just before implantation. This is believed to help the embryo successfully implant into the endometrium (Thathiah and Carson, 2002).

However, the regulation of MUC1 is different in humans than it is in mice. For ethical reasons, few studies have been able to investigate the regulation of MUC1 at human embryo implantation sites. Nonetheless, *in vitro* research has shown that MUC1 expression in humans is actually at its highest during the receptive uterine state (i.e. the midluteal phase). Additionally, MUC1 is believed to be regulated via a complex signaling pathway involving steroid hormones,

maternal epithelial cells of the endometrium, and embryonic cells of the blastocyst. In humans, progesterone levels are high during the receptive state due to the influence of maternal steroid hormones. This indicates that MUC1 expression is upregulated by progesterone in humans, rather than downregulated by progesterone as in mice. MUC1 is also thought to be regulated in a paracrine fashion by the human blastocyst. Although this process is not fully understood yet, the human blastocyst is thought to play a role in signaling to the endometrial epithelial cells that the inhibitory MUC1 barrier must be removed prior to implantation. MUC1 is present in pre-adhesion blastocysts at the outer trophectodermal surface of the cell (beneath the zona pellucida) as well as in the inner cell mass. In embryos that have hatched and attached to the endometrial epithelium, though, MUC1 is not found. Moreover, staining of the epithelial cells surrounding an implanted human embryo shows a lack of MUC1 in the cells below and adjacent to the embryo. This suggests that the human blastocyst downregulates and cleaves MUC1 at implantation sites. Thus, human blastocysts appear to work in conjunction with steroid hormones to allow for proper embryo implantation into the endometrium (Meseguer, et al., 2001).

MUC1 levels in the endometrium have been investigated in fertile women, women suffering from recurrent implantation failure, and women suffering from recurrent miscarriage. The results indicated that women suffering from recurrent implantation failure (RIF) had decreased MUC1 levels in the endometrial epithelia in comparison to fertile women and women suffering from recurrent miscarriage. The implication of this research is that decreased MUC1 is an independent marker for the impaired receptivity of the endometrium in women with RIF during the normal implantation window. The implantation window in female women is defined as the short period of time during the mid-luteal phase around 7 days after the surge in luteinizing hormone. At this time, the endometrium is believed to be receptive for the embryo to

implant. Although many molecules have been investigated as potential markers for endometrial receptivity, this research suggests that the lower levels of MUC1 in women suffering from RIF may have played a role in their lack of reproductive success (Wu, et al., 2018). Nonetheless, direct proof of MUC1 inhibiting embryo implantation and leading to miscarriage in humans has yet to be established. Thus, MUC1's precise role in embryo implantation requires further investigation.

MUC1 has also been investigated in regard to its role in reproductive tract infections. Specifically, research has been performed on wild-type mice (with MUC1 gene) and MUC1 null mice (without the MUC1 gene). One study investigated the rates of infection, inflammation, and reproductive success among MUC1 null mice and wild-type mice under various conditions. To investigate the role of the MUC1 gene in reproductive tract infections and inflammation in mice, MUC1 null mice and wild-type mice were housed in "normal" housing. In this instance, normal housing involved placing the mice under conditions in which pathogens were naturally occurring. The rates of infection and inflammation were then measured among both groups of mice. In the study, MUC1 null mice in normal housing struggled with chronic infection and inflammation of the lower reproductive tract. In contrast, the wild-type mice had lower rates of infection and inflammation (DeSouza, et al., 1999). These results suggest that MUC1 does indeed play a critical role in acting as a barrier to infection when exposed to microbial organisms in the environment. Additionally, the litter sizes of MUC1 null mice in normal housing, wild-type mice in normal housing, and MUC1 null mice in pathogen free-housing were also compared in this study to investigate whether MUC1 had an influence on the reproductive success of the mice. MUC1 null mice in normal housing had reduced litter sizes in comparison to both the wild-type mice and the MUC1 null mice in the pathogen-free housing. On average, the wild-type

mice had the largest litter sizes, followed by MUC1 null mice in pathogen free-housing, and then MUC1 mull nice in normal housing. These results may be explained by the loss of the MUC1 barrier among MUC1 null mice, which is believed to protect against pathogens that may cause inflammation. In turn, higher rates of infection and inflammation among the MUC1 null mice – especially the mice in normal housing – could have impacted the reproductive success of these mice. A proposed mechanism behind this susceptibility to infection and inflammation among MUC1 null mice in normal housing with pathogens present is that the loss of the mucin barrier impacted the normal endogenous flora of the mice and allowed certain bacterial species to become opportunistic (DeSouza, et al., 1999).

This is particularly significant because the natural mating process introduces exogenous sources of microbial organisms into the mouse uterus that may lead to a reproductive tract infection if not naturally cleared from the uterus (Parr and Parr, 1985). Research on commonly occurring human reproductive tract infections like *Chlamydia trachomatis* and *Neisseria gonorrhoeae* has indicated that reproductive infections can be linked to infertility in some cases (Tsevat, et al., 2017). This is also in line with the results mentioned in the above study that was performed on MUC1 null mice because the mice that were exposed to pathogens had higher rates of both infection and infertility (DeSouza, et al., 1999).

As stated earlier, mucins like MUC1 are under the control of ovarian steroid hormones (Surveyor, et al., 1995). This means that the mucin layer is dynamic, undergoing changes during the menstrual cycle that allow for the transport of gametes at the correct time, while preventing gamete transport at other times. Specifically, the layer undergoes changes in the viscosity of the mucus just before ovulation. Prior to ovulation, during the luteal phase, the mucin layer contains very thick mucus that prevents sperm from penetrating the uterus and fertilizing the egg.

However, the viscosity of the mucus within the mucin layer changes just before ovulation from a very viscous consistency to a more thin, watery consistency. In turn, this allows for sperm to enter the vagina, penetrate the uterus, and travel up the uterine tube where fertilization can occur (Gipson, et al., 1997; Carlstedt, et al., 1983).

Although the study of reproductive mucins is a relatively new field of research, the research available suggests that mucins play a critical role in changing the environment of the uterus from a non-receptive state to a receptive state for sperm fertilization and subsequent embryo implantation. This begets the question: if alcohol consumption does indeed alter the mucin layer in some way, how will this impact the ability of sperm to enter the uterine tube and fertilize an egg? Additionally, how would changes in the mucin layer due to alcohol consumption affect the implantation of an embryo into the endometrium? Lastly, if alcohol consumption did affect the mucin layer, would this place the affected individual at greater risk for reproductive tract infections? These questions illustrate the significance of understanding how any changes to the mucin layer by alcohol may impact female reproduction.

Background of Reproductive Anatomy

It is important to understand the basic anatomy of the uterus before engaging in any research on female reproduction. In the human the uterus is a pear-shaped organ that contains 3 layers of tissue: the perimetrium, the myometrium, and the endometrium. The perimetrium is the outermost protective layer. The myometrium is the middle, smooth muscle layer that contracts the uterus and helps expel the fetus into the vagina during childbirth. The endometrium is the innermost glandular and mucosal layer (Kruk, 2004). In response to hormones secreted by the ovaries, the human endometrium mucosa functionally changes during the menstrual cycle to

prepare for the implantation of a fertilized ovum. Thus, the endometrial portion of the uterus is a significant aspect of the female reproductive system that must not be overlooked during research.

Before embedding in the endometrium the egg must first be transported to the uterus via the uterine tube. The uterine tube is also known as the oviduct, or fallopian tube. The uterine tube has various functions, one of which is to transport the ovulated secondary oocyte from the ovary to the uterus. In the human, the portion of the uterine tube closest to the ovary is called the oviductal infundibulum, and it is the funnel-shaped portion of the uterine tube that receives the ovulated secondary oocyte. This portion of the uterine tube contains fimbriae that sweep the secondary oocyte from the ovary into the uterine tube (Jones and Lopez, 2014). From there, the muscular layers within the wall of the uterine tube undergo peristaltic contractions to move the secondary oocyte down to the uterus. The uterine tube contains a mucosal lining and branching folds of epithelial tissue that consist of a single layer of both ciliated and nonciliated columnar cells. The nonciliated columnar cells of the uterine tube, also known as peg cells, produce a mucin secretion that helps propel the secondary oocyte towards the uterus via the beating ciliated cells. This mucin secretion is also thought to provide nutritional value for the secondary oocyte during transport (Kruk, 2004). Furthermore, these mucins help sustain an environment conducive to male gamete maturation, interaction between male and female gametes, and early embryonic development (Gandolfi, et al., 1989).

Before discussing how the endometrium changes throughout the menstrual cycle to prepare for embryo implantation, it is important to understand sexual development in women. Women are considered fertile after the first menstrual period, or menarche. This period of fertility ends with the stop of menstruation, or menopause. During the fertile period, menstrual cycles occur every 28 to 35 days except during pregnancy (Bischof, 2019). At birth, a single

human ovary has around 400,000 primordial follicles that contain primary oocytes. These primary oocytes remain arrested in the prophase stage of meiosis I and await further division until sexual maturity. At sexual maturity, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are produced by the pituitary gland. These hormones induce the development of these primordial follicles within the ovaries. Follicle-stimulating hormone and luteinizing hormone are termed gonadotropins because they stimulate the female gonads – or ovaries – to undergo oogensis. During each cycle, approximately 20 primordial follicles begin maturation, but only one follicle usually matures fully. The remaining follicles are believed to contribute to the endocrine capacities of the ovary, which will be discussed in detail later. Once activated, the follicle completes its first meiotic division and matures into a secondary follicle. A second division then occurs and results in the formation of a Graafian follicle, which contains a secondary oocyte. This secondary oocyte remains arrested in the second stage of meiotic division until it is ovulated and fertilized by sperm (Peckham, et al., 2003).

The hormonal induction of ovulation will be discussed later, but it is important to understand certain consequences of ovulation prior to discussing the mechanism of fertilization. Ovulation occurs when the Graafian follicle ruptures inside the ovary and releases the secondary oocyte into the uterine tube. The portion of the follicle that remains in the ovary following ovulation eventually becomes the corpus luteum. The corpus luteum is a transient endocrine organ that secretes estrogen and progesterone, but progesterone especially. These hormones are essential for further development of the endometrium to prepare for embryo implantation. The fate of the corpus luteum depends on whether fertilization occurs. If fertilization occurs, the developing embryo will secrete human chorionic gonadotropin (HCG). This signals to the corpus luteum that progesterone secretion is still necessary. Progesterone is essential for the

maintenance of the endometrium, which allows for the implantation and development of the embryo. The corpus luteum secretes progesterone for about 12 weeks until the placenta forms. The placenta will then take over, maintaining the pregnancy until birth. After around 12 weeks, the corpus luteum degenerates. However, if fertilization does not take place, the corpus luteum will stop secreting progesterone after about 10-14 days post-ovulation and degenerate into the corpus albicans. The corpus albicans is mainly composed of scar tissue, and it does not secrete hormones. Without this progesterone secretion to maintain the endometrium, the human endometrial lining will be shed in a process referred to as menstruation (Oliver and Pillarisetty, 2019). This will also be discussed later.

In the human, the secondary oocyte is normally fertilized in the ampulla of the uterine tube, which is medial to the infundibulum portion of the uterine tube. The secondary oocyte is surrounded by two protective layers. The first layer surrounding the oocyte is an outer layer of follicular cells called the corona radiata. The second layer, referred to as the zona pellucida, is an underlying glycoprotein membrane that surrounds the secondary oocyte's plasma membrane. In order to fuse with and fertilize the oocyte, sperm must first penetrate the corona radiata and then cross through the zona pellucida. Once the sperm has penetrated the corona radiata, it binds to receptors in the zona pellucida. This initial contact with the zona pellucida induces the sperm to undergo a process referred to as the acrosomal reaction. In this reaction, the sperm releases the contents of its acrosome, which is essentially an enzyme-filled "cap" at the top of sperm. This releases hydrolytic enzymes that digest the zona pellucida and help the sperm tunnel through to reach the plasma membrane of the oocyte. The sperm will then bind to receptors on the secondary oocyte's plasma membrane and fuse with the membrane, releasing its nucleus and other organelles into the cytoplasm of the oocyte. To prevent fertilization by multiple sperm, the

oocyte releases a calcium ion signal when the first sperm fuses with the oocyte. This allows the oocyte to undergo the cortical reaction, which releases the enzymatic contents of cortical granules and hardens the zona pellucida to prevent fertilization by additional sperm. This calcium ion signal also triggers further development of the zygote. This happens 1 day after fertilization by sperm (Alberts, et al., 2002). However, if the secondary oocyte is not penetrated by sperm, this process does not occur. Instead, the secondary oocyte will degenerate, and implantation will not occur. Since implantation does not occur, there will be no formation of a placenta, and therefore no production of HCG. As previously discussed, this will result in the degeneration of the corpus luteum, leading to a drop in progesterone levels. As a result, menstruation will be triggered. This will be discussed in greater detail later.

If fertilization by sperm does occur, a zygote is formed. This zygote begins as a single cell, but it undergoes several rounds of cell division in the uterine tube via a process referred to as embryogenesis. During this process, the zygote undergoes cleavage prior to entering the uterus. As the cell divides, the overall cell size is not increased, but the number of cells within zygote is increased via the redistribution of cytoplasm. This results in the formation of a compact cluster of cells that are still within the zona pellucida. A morula is formed once cleavage has produced a compact cluster of 16 cells. This process of forming a morula usually occurs around 3-4 days after fertilization. This morula enters the uterine cavity and undergoes further cleavage to form a blastocyst, which is a cluster of 32 or more cells. This usually occurs 5 days after fertilization (Li and Winuthayanon, 2017). Once the blastocyst reaches the uterus, it makes contact with the uterine wall and implants into the endometrial surface of the uterus. This implantation process has 3 main stages. The first stage includes the initial contact of the blastocyst with the uterine epithelium about 2-4 days after the morula enters the uterine cavity.

This is referred to as apposition. During apposition, the blastocyst differentiates into an inner cell mass (which forms the embryo) and a trophoblast (which forms the placenta). The second stage of the implantation process is often referred to as adhesion. Adhesion occurs when the trophoblast cells of the blastocyst attach to the receptive endometrial epithelium. This process is mediated by cell adhesion molecules like integrins, cadherins, selectins, and immunoglobulins (Kim and Kim, 2017). The third stage of implantation occurs after the blastocyst has adhered to the uterine wall. During this stage, the trophoblast secretes enzymes that digest portions of the endometrial tissue. This permits fetal trophoblast cells to invade into the maternal decidual tissue of the endometrium (Wolter, 2013).

As a result, the fetus is able to access the maternal circulation system and remodel the maternal arteries to create adequate blood flow between the fetus and the mother. This results in the eventual formation of the placenta (Kim and Kim, 2017). The placenta functions to ensure there is an adequate exchange of nutrients between the maternal and fetal circulatory systems to support normal growth and development of the fetus. The placenta also functions to metabolize substances and release metabolic products into the maternal and fetal circulations (Gude, et al., 2004). Thus, the ability of the blastocyst to implant into the endometrium is essential because it allows for the formation of the placenta and further development of the fetus during pregnancy. One can argue that mucins, especially MUC1, are also important for this process because they line the endometrium and are believed to play a role in embryo implantation into the endometrium. Without blastocyst implantation into the endometrium, the maternal blood vessel network cannot be invaded, and the placenta cannot be formed. This would result in the endometrium breaking down and being shed, along with the blastocyst, as part of the normal human menstrual cycle (Wolter, 2013).

To achieve successful embryo implantation, the endometrium must undergo structural and functional remodeling throughout the menstrual cycle. In the human, these monthly cyclic changes begin with puberty around 11 to 15 years old and continue until menopause around 45 to 50 years old. These cyclic changes are achieved through the action of hormones secreted by the ovaries (Fawcett and Bloom, 1986). In general, the endometrium lines the lumen of the uterus and consists of a highly vascularized and glandular mucosal layer composed of simple columnar epithelium. This epithelium is composed of both ciliated and secretory cells. The endometrium is divided into two layers referred to as the stratum functionalis and the stratum basalis. The stratum functionalis is superficial to the underlying stratum basalis and it is often referred to as the "functional" layer of the endometrium. During the menstrual cycle, the stratum functionalis expands and vascularizes. If an embryo does not implant into the endometrium, the stratum functionalis is then sloughed off during menstruation. Thus, the stratum functionalis is regenerated every cycle. The stratum basalis, however, is not shed during the menstrual cycle. Instead, the basal layer remains, serving as a source of cells for the regrowth of the superficial functional layer after menstruation (Jenkins and Tortora, 2016).

As the normal menstrual cycle progresses, the endometrium undergoes morphological changes in the stratum functionalis layer. These changes in endometrial growth are divided into three phases: a follicular (or proliferative) phase, followed by a luteal (or secretory) phase, and lastly a menstrual phase. In general, the follicular phase consists of follicle maturation, selection of the dominant follicle, ovulation of the secondary oocyte, and the secretion of estrogen. During this phase, there is a significant increase in endometrial thickness due to the growth of the epithelial cells in the stratum functionalis. Moreover, glands and spiral arteries also elongate in this stage to prepare for further endometrial development. The changes in this phase are largely

driven by FSH and estrogen. The luteal phase occurs after ovulation and further prepares the endometrium for embryo implantation. The endometrium slightly thickens during this phase and reaches its maximal thickness. The uterine glands and spiral arteries continue to grow and begin to extend into the stratum functionalis. At this point in the cycle, the uterine glands contain carbohydrate rich secretions. The changes in the luteal phase are largely driven by LH and progesterone. Progesterone is secreted by the corpus luteum within the ovary, and this aids in the maintenance of the endometrium for embryo implantation and subsequent development. Without fertilization, the menstrual phase of the menstrual cycle occurs. During this final phase, the spiral arteries in the stratum functionalis layer of the endometrium contract and restrict blood supply to the functional layer. This causes the stratum functionalis to degenerate. Eventually, the arteries rupture and slough off the degenerated functional layer, resulting in bloody discharge from the vagina (Peckham, et al., 2003; Fawcett and Bloom, 1986; Oliver and Pillarisetty, 2019).

These cyclical changes are controlled via hormones. The secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus induces the anterior pituitary gland to secrete FSH and LH. This initiates the follicular phase of endometrial development, which begins at the end of menstrual flow from the previous cycle. This phase is considered the first 14 days of the menstrual cycle. Follicle-stimulating hormone stimulates the maturation of ovarian follicles and triggers the production of estrogen and progesterone by these follicles. Estrogen stimulates the growth of the endometrium during this phase, but it also exerts negative feedback on GnRH, FSH, and LH secretion. As the follicles continue to develop, estrogen levels increase to a certain threshold. Once this threshold is reached around the 14^{th} day of the cycle, the effect of estrogen changes. Instead of suppressing gonadotropin secretion by the pituitary, these higher levels of

estrogen actually induce FSH and LH secretion. As a result, FSH and LH levels start to rise rapidly, but LH levels rise significantly more than FSH levels.

This preovulatory LH surge triggers ovulation. Ovulation occurs when the most mature follicle ruptures and releases its secondary oocyte. This signals the start of the luteal phase, which usually occurs the second 14 days of the cycle (approximately days 15 through 28). The ovarian follicles that did not reach full maturation degenerate and no longer produce estrogen, which slightly decreases estrogen levels. After ovulation, LH stimulates the formation of the corpus luteum. The corpus luteum secretes progesterone and estrogen to prepare the lining of the endometrium for implantation if fertilization occurs. Progesterone is produced in greater amounts than estrogen, so blood levels of progesterone rise more than estrogen levels following ovulation. Progesterone is essential to sustain a successful pregnancy, so it is considered the key hormone for this phase. High progesterone levels exhibit negative feedback on the pituitary to suppress the further release of FSH and LH. The inhibition of FSH and LH prevents any additional follicles from developing while progesterone levels are elevated (Molnar and Gair, 2015).

For cycles in which fertilization does not occur, the corpus luteum will degenerate and form the corpus albicans. Since the corpus albicans does not secrete any hormones, this results in a decline in progesterone and estrogen levels. These decreasing hormone levels signal that menstruation is ready to occur, and the lining of the endometrium begins to degenerate. The decrease in progesterone levels permits the hypothalamus to secrete GnRH, which acts on the anterior pituitary and induces the release of FSH and LH. This signals that the cycle is ready to begin again following menstruation (Molnar and Gair, 2015; Fawcett and Bloom, 1986).

The estrous cycle of mice, however, slightly differs from the human menstrual cycle. In women, a menstrual cycle lasts approximately 28-35 days, but the mouse estrous cycle is 4-6

days in length. Additionally, the mouse estrous cycle has 4 stages in comparison to the 3 stages of the menstrual cycle in women. These stages are proestrus, estrus, metestrus, and diestrus. The proestrus and estrus phases are comparable to the follicular phase in women, while the metestrus and diestrus phases are comparable to the luteal phase in women. The stage of estrous in mice can be identified upon vaginal smear cytology, or the examination of certain cells like leukocytes and squamous epithelial cells in the vagina (Bertolin, 2014).

The proestrus stage entails maturation of follicles in the ovary along with the secretion of estrogens from these follicles. In this stage, the follicles begin growing under the influence of FSH, which increases estradiol levels, and then results in an LH peak. After the surge in LH, multiple mature follicles are ovulated from the ovary, beginning the estrus stage. This differs from the menstrual cycle in women because multiple follicles are released in mice, rather than only one mature follicle being released from the ovary in women. At this point in the cycle, the female mouse will allow mating with a male. The metestrus stage then follows, and it entails the movement of mature secondary oocytes through the uterine tubes towards the uterus. During metestrus, there is a hormonal shift from higher estrogen levels to increasing progesterone levels. This increase in progesterone is due to the ovarian follicles remodeling to form corpora lutea that secrete this hormone. For this reason, metestrus is considered the early luteal phase. If successful fertilization occurs, hormonal changes will continue to prepare the uterus for pregnancy. If successful fertilization does not occur, the metestrus stage is then followed by diestrus. During diestrus, progesterone is the dominant hormonal influence. During metestrus and diestrus, progesterone helps inhibit the secretion of LH. This prevents further ovulation. Without fertilization, the corpus lutea will begin to degenerate in late diestrus and progesterone levels will decrease. This will release the inhibition of LH secretion and allow for the cycle to begin again at

the proestrus stage. The mouse estrous cycle is also different from the human menstrual cycle in that the endometrial lining is not shed through menstruation in mice, but rather resorbed if conception does not occur (Bertolin, 2014; Silver, 1995; Yang, et al., 2009).

In both mice and humans, the endometrium has various glands that extend from the surface of the endometrium nearly into the myometrium (Jenkins and Tortora, 2016). These glands secrete a variety of essential products with different reproductive functions, including amino acids, ions, carbohydrates, lipids, cytokines, enzymes, hormones, growth factors, and other additional proteins (Filant and Spencer, 2014). Some of these secretions act as vital sources of nutrients for a developing embryo, but more recent research has indicated that these secretions also have other important functions. For example, secretions like leukaemia inhibitory factor (LIF) and mucin-1 (MUC-1) are involved in the regulation of embryo implantation into the endometrium (Hempstock, et al., 2004).

Thus, it is clear that the endometrium is an important aspect of reproduction as it is the location of embryo implantation, growth, and development for the duration of pregnancy. For this reason, the aim of this research is to investigate how alcohol impacts the mucin lining of the endometrium. If chronic alcohol consumption has an effect on the endometrium mucin layer, this may affect overall reproductive health or, potentially, the ability of the embryo to implant into the endometrium to grow and develop during pregnancy.

Alcohol Consumption and Reproduction

Alcohol's impact on the female reproductive system is still somewhat of an unexplored field of research as most studies on the effects of alcohol have focused on the male reproductive system. Nonetheless, there are some noteworthy studies that suggest alcohol has an overall negative impact on the reproductive system and fertility.

In a study performed to examine the effects of alcohol and caffeine on conception, there was a statistically significant negative dose-response relationship between pregnancies and the consumption of alcohol, suggesting that alcohol consumption somehow negatively impacted the success of a conception. Caffeine was found to have had no independently significant impact on the outcome of a reproductive event, but the results suggested that the consumption of caffeine may enhance the negative effect that alcohol has on reproductive success. In explanation, the study results revealed that the women who abstained from alcohol and consumed less than one cup of coffee per day conceived 26.9 pregnancies per 100 menstrual cycles in comparison to the 10.5 pregnancies per 100 cycles in the women who consumed any amount of alcohol and more than 1 cup of coffee per day. The increased negative effect that caffeine had on reproductive success in combination with alcohol use is intriguing and something that could be researched further in the future even though it is not examined in this research. Nonetheless, there was a >50% decrease in the probability of a conception during a cycle in which the participant consumed alcohol, so the results are still significant to the discussion about alcohol's effect on the female reproductive system within this research (Hakim, R., et al., 1998).

A separate study followed couples who discontinued birth control for 6 menstrual cycles (or until a clinical pregnancy was recognized) and detailed the impact of alcohol on pregnancy outcomes. During the study period, both males and females were surveyed about their level of alcohol intake. The study aimed at determining whether the level of alcohol intake impacted the fecundability, the probability of attaining a clinically recognized pregnancy in a menstrual cycle, among non-pregnant couples. An odds ratio was calculated to indicate the odds of conception among couples who consumed alcohol compared to the couples who did not consume alcohol during the cycle. The odds ratio decreased with increasing levels of alcohol intake among

women, especially among women who consumed more than 10 drinks a week, suggesting that alcohol consumption may reduce a woman's fecundability and ability to achieve a successful pregnancy. However, this negative dose response relationship in women was not observed among men, suggesting that alcohol consumption among men does not impact the probability of obtaining a successful pregnancy. In all, this suggests that alcohol consumption (especially in excessive and increasing amounts) negatively impacts female reproduction in some manner (Jensen, T., et al., 1998).

Other research on the effects of alcohol consumption on female reproduction has focused on the effect of chronic alcohol exposure on hormone secretion and the cyclicity of estrous cycles. One such study was performed on adult female rats that were either given an ethanol diet, a calorically matched control diet, or an ad libitum diet. Rats placed on each of these diets were further split up into a short chronic group (with alcohol consumption being 2 weeks long) and a long chronic group (with alcohol consumption being 2 months long). The results of this study indicated that the consumption of alcohol notably disrupted the normal estrous cycle of female rats, particularly by lengthening the cycle at the diestrous stage (Emanuele, N., et al., 2001).

Since these rats were group housed without the presence of a male stimulus, it is possible that the lengthening of the cycle at the diestrous stage could potentially be due to the suppression of estrous through what is known as the Lee-Boot effect. When these longer periods of diestrous were first discovered, they were thought to be caused by spontaneous pseudopregnancies in mice (Lee and Boot, 1955) (Lee and Boot, 1956). However, further research performed by Wes Whitten clarified that this suppression of estrous was due to the overcrowding of females in group cages without the presence of a male or male excreta. Similar to the studies performed by Van der Lee and Boot, group housed females had a significant reduction in the incidence of

estrous among group housed females (even after 3 months), but this effect was reversed within 3-4 days after the introduction of a male into the cages (Whitten, 1959). The introduction of a male into a cage results in the accumulation of urine within the cage, and this excreta is believed to act as a chemosignal for normal estrous cyclicity.

Although it is indeed possible that the Lee-Boot effect could have been a confounding variable in the results from the study that indicated chronic ethanol consumption lengthened the diestrous stage of the rat estrous cycle, the results from this experiment are still worth mentioning because the pair fed rats (with the calorically matched diet) and the ad-libitum fed rats for the short chronic study continued to have regular 4 or 5 day cycles even though 40% of the ethanol fed rats had disruptions in their cycles. Somewhat similarly, in the long chronic study, most of the pair fed and ad libitum fed rats cycled regularly (with only 16% of pair fed and 12.5% of ad libitum fed rats cycling abnormally) in contrast to the 83% of ethanol fed animals that had abnormal cycles (Emanuele, N., et al., 2001). These findings illustrate that, even if the Lee-Boot effect slightly impacted the results of the experiment, alcohol still had a significant impact on normal estrous cyclicity.

In a study performed on adult male and female rats, alcohol fed animals had significant gonadal atrophy and reduced sex steroid levels. The gonadal atrophy exhibited in the study was demonstrated by a loss in ovarian and testicular mass. However, the uterus and uterine tubes of the alcohol-fed mice also weighed significantly less than the control fed animals. Histological examination of the uterine tissues indicated estrogen deprivation, with a single layer of cuboidal cells that lacked any sign of secretory activity. This was in direct contrast to the tissues of control fed rats, which all had normal epithelial linings composed of tall columnar cells rich with secretory granules. Sex steroid levels of progesterone and testosterone were also reduced in

females and males, respectively. These results suggest that alcohol is a gonadal toxin that causes endocrine and reproductive failure of the gonads in both male and female rats (Gavaler, et al., 1980). In a separate study performed on adult female rats, the endometrial epithelium of the uterine horns in rats submitted to chronic alcoholism exposure showed intense atrophy compared to control fed rats. (Martinez, et al., 1999). These studies suggest that alcohol is a toxin that can significantly impact the epithelial layer of the rat endometrium along with the secretory ability of glands in the endometrium. Since mucins are secreted from uterine glands and line the epithelial layer of the endometrium, one can infer from these results that the model of chronic alcohol consumption implemented in this research would have a significant impact on the mucin layer of the endometrium.

MATERIALS AND METHODS

All the tissue samples used in this study were obtained from the laboratories of Dr. Jonathan Peterson and Dr. Allan Forsman at East Tennessee State University in Johnson City, Tennessee via a tissue sharing experiment. Nine female wildtype C57BL/6 mice were utilized in this study with 3 mice belonging to the control group (C), and 6 mice belonging to the ethanol fed group (ETOH). All of the mice were group housed in polycarbonate cages with 2-4 mice placed in the same cage together. The cages were set to a 12-hour light-dark photocycle and the mice had *ad libitum* access to food. All animal procedures were conducted in accordance with institutional guidelines, and ethical approval was obtained from the University Committee on Animal Care (Animal Welfare Assurance no. A3203-01).

The mice in the ethanol fed group were placed on the NIAAA model diet, which is also known as the Liber-DeCarli diet. This diet consists of a 5% (v/v) ethanol diet *ad libitum* for 10-

days followed by a single high dose of ethanol (5g/kg) on the 11th day. The control fed mice were placed on ethanol free isocaloric diet that was supplemented with maltose dextrin in order to match the calories of ethanol. The food intake and body weight of the mice were measured daily and the food intake of the control mice was limited to match the food intake of the ethanol mice in order to prevent any potential variability in results. Since the mice were group housed, the food (and ethanol) intake for each day was estimated as the total consumed per day divided by the number of mice per cage. After the 11th day of the study, the mice were sacrificed, and uterine tissues were harvested and preserved in 4% paraformaldehyde. The tissue samples were then trimmed and stored in a 70% ethanol solution until they could be embedded in paraffin.

To prepare the tissue samples for embedding in paraffin, the uterine sections were placed in individually labeled cages and then dehydrated through a series of runs in increasing concentrations of ethanol and CitriSolv. After the dehydration process was completed, the uterine sections were vertically embedded in paraffin so the slides created from each sample would provide a cross-sectional view of the uterine tissue. The steps for the dehydration and embedding process are found in appendix A. The tissues were then sectioned at 4 microns using a Microm HM325 microtome, placed in a glycerin water bath mixture, and mounted onto glass slides. For each tissue sample, three slides, with three sections per slide, were prepared.

The slides were then deparaffinized, rehydrated via the technique outlined in Appendix B, stained via a standard Hematoxylin and Eosin (H&E) staining technique, dehydrated, also via the technique outlined in Appendix B, and cover slipped. The slides were examined and photographed with a Zeiss Axioskop 40 microscope with a Cannon Powershot A640 camera. Measurements of the mucin layer thickness for each animal were made using the Carl Zeiss AxioVision software. For each animal uterine tissue sample, a total of 15 measurements were

made via a randomization grid, with 5 random measurements being made per slide from each of the 3 prepared slides. The average thickness of the mucin layer lining the endometrium of each animal was calculated and statistical analysis was then performed. A one-way ANOVA test was conducted using MiniTab statistical software in order to analyze the effects of chronic ethanol consumption on endometrial mucin layer thickness. To prevent any bias during the collection of data, the information on which treatment group the samples belonged to remained unknown until all data was collected.

RESULTS

Data for the thicknesses of the uterine endometrial layers across treatment groups (C, ETOH) was collected. Photomicrographs of the endometrial tissue are shown in Figures 1-3.

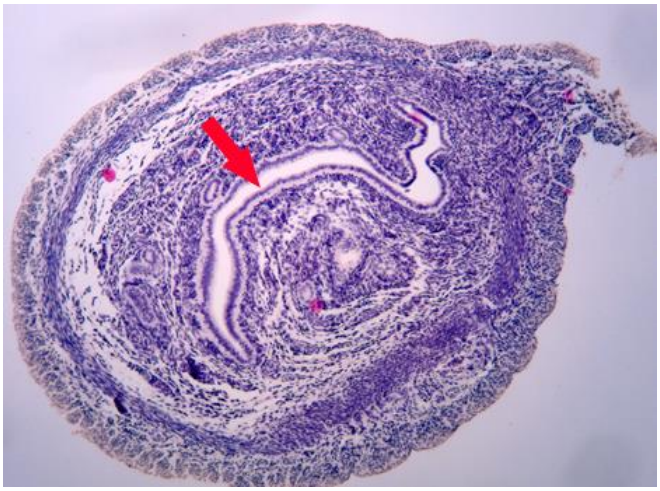

Figure 1. Cross section of the uterus from an ethanol-fed mouse depicting the mucin layer under analysis. Red arrow indicates the mucin layer lining the endometrium (100x).

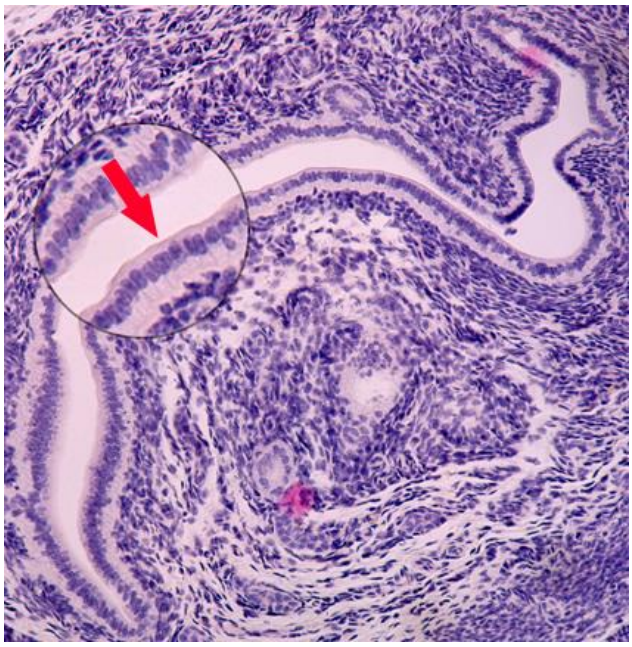

Figure 2. Enhanced view of the cross section of the uterus depicting the mucin layer under analysis. Red arrow indicates the mucin layer lining the endometrium (200x).

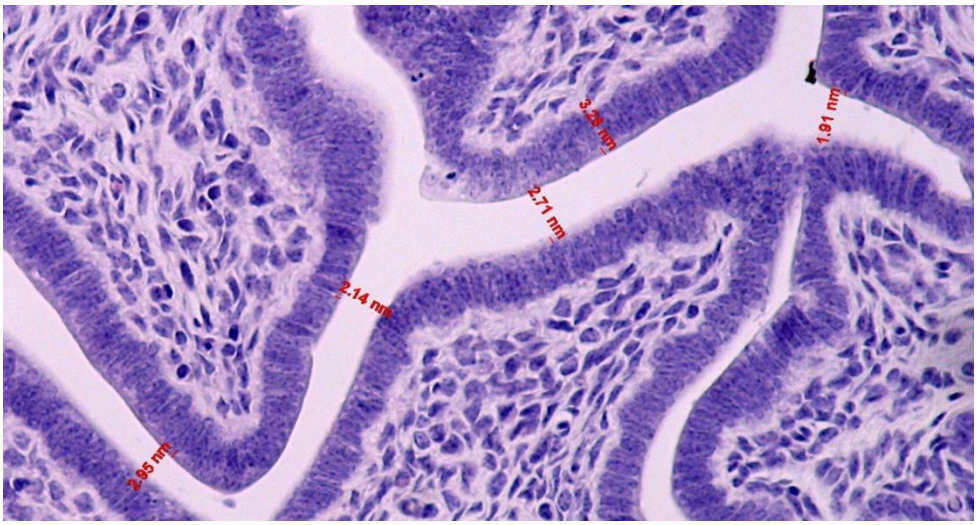

Figure 3. Example of the mucin thickness measurements (in red) made for each of the three slides prepared per animal (200x).

The values collected for analysis were the mean thicknesses for each treatment group. An interval plot comparing the treatment groups is presented below in Figure 4. A table of the average mucin thickness for each mouse can be found in appendix C.

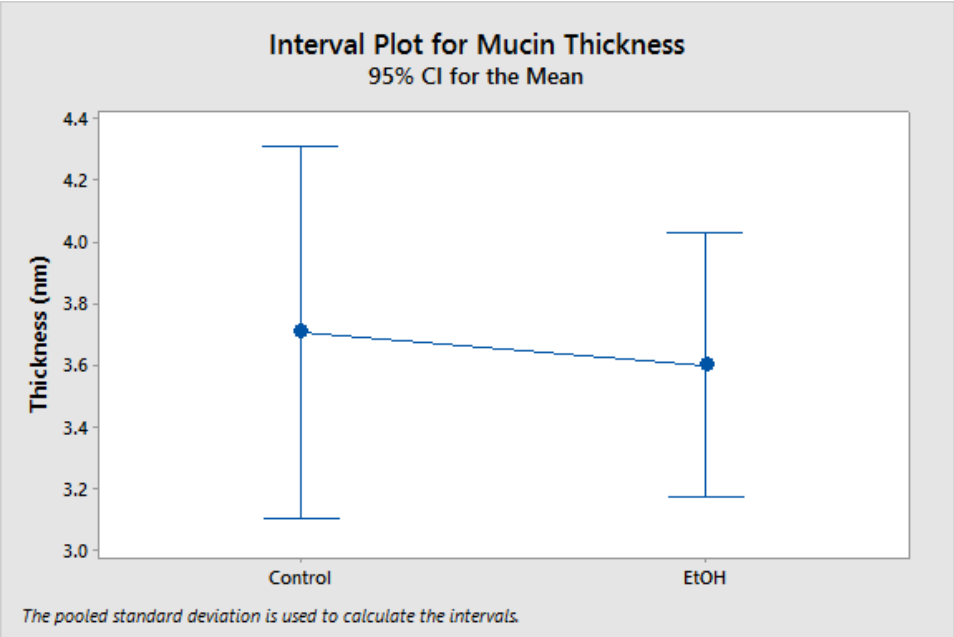

Figure 4. Graph comparing the mean thickness of the mucin layer of the endometrium between treatment groups; [n(control) = 3; n(ETOH) = 6]; P value: Comparing C to ETOH: 0.774.

A one-way ANOVA test was performed to compare the mucin thicknesses between mice in the control group and mice in the experimental group. Analysis of the mucin thickness of the uterine tissue showed no statistically significant difference between the control and ethanol fed groups of mice (P = 0.774). These results, along with the average mucin thickness for each group of animals, are shown below in Figure 5.

Average Mucin Thickness	Control animals: 3.706 ETOH animals: 3.598
P-value (Control-ETOH)	0.774

Figure 5. Results from the one-way ANOVA test performed to compare the mean thicknesses of the control and experimental (ethanol fed) groups.

DISCUSSION

Data analysis by means of a one-way ANOVA indicated that there was a slight decrease in the average mucin thickness of the ethanol fed animals. Nonetheless, these differences in uterine mucin layer thicknesses among control and ethanol fed mice were not enough to be considered statistically significant.

Although the results indicate no statistically significant difference in the mucin thickness of the endometrium between control and ethanol fed mice, further studies should be done to eliminate potential confounders that may have influenced the results of this experiment. In explanation, this experiment was a partial tissue sharing experiment in that the uterine tissues analyzed in this study were harvested from mice that were a part of a larger study on the sex-specific effects of alcohol on circulating levels of CTRP3 (DeGroat, et al., 2018). It was decided that the uterine tissues were not necessary for the focus of the CTRP3 research, and thus, the uterine tissues of the 9 control and ethanol fed mice from that study were harvested and stored until use in this research. Since the procedure in the CTRP3 study was not built specifically to study the effects of alcohol on the uterine mucin layer, there are some weaknesses in the study design that could have potentially impacted the results from this research. These weaknesses include treatment group's small sample size, a lack of collected data on the stage of estrous for each animal during the experiment, and group housing that prevented the exact amount of alcohol consumed by each individual mouse from being determined. This group housing also could have potentially induced the Lee-Boot effect. In explanation, male mice were housed near the female mice, but the bedding from the males was not added to the bedding of the cages housing the female mice. The excreta in this bedding could have counteracted the Lee-Boot effect. Although the male mice were housed nearby, it is unclear whether the presence of male

mice in the vicinity would be sufficient to counteract the Lee-Boot effect. It is also possible that any discrepancies in data could be due to individual variability in mucin thickness among different mice.

This study is missing estrous cycle data for all of the mice included in the study because no vaginal smears were performed to determine the stage of estrous for each mouse throughout the study. The precise relationship between uterine mucin thickness and the hormonal fluctuations that naturally occur during the estrous cycle has yet to be elucidated, but it is possible that the steroid hormones that regulate reproduction may influence the thickness of the uterine mucin layer in certain ways throughout the estrous cycle. This could be of potential significance because certain hormones produced in higher amounts during certain stages of estrous may in turn influence the thickness of the mucin layer. Thus, if not all of the mice included in the study were synchronized together on the same estrous cycle, there could be variability in mucin thickness among the animals that could result in discrepancies during data collection.

However, the mice in this study were group housed with 2-4 per cage without the presence of a male or any male excreta, so one could infer that the Lee-Boot effect took place and most of the mice remained arrested in the diestrus stage of the estrous cycle. Theoretically, then, the animals *should* be in the same stage of estrous, but this information cannot be confirmed since no vaginal smears were performed to determine the precise stage of estrous among all the mice. In the future, this potential confounder could be mitigated by performing vaginal smears on the mice to determine stage of estrous, along with housing the mice in a manner that has been shown to diminish the Lee-Boot effect. This could be done by either

housing the female mice in single cages (rather than in grouped housing) or by adding the presence of a male or male excreta.

Another weakness of this study was that the amount of alcohol consumed per animal in the ethanol fed treatment group was calculated as the total amount consumed per cage per mouse (total/number of mice in the same cage together). This means that the assumption was made that each mouse consumed the same amount of alcohol as the other ethanol fed mice in the cage, and this could have in turn increased variability in results.

Finally, it is possible that the 11-day period of the NIAAA model could have been too short to induce alcohol-related changes in the endometrium. The NIAAA model was chosen because it is a cost and time efficient method that has been shown to resemble the progression of human alcoholic hepatitis. However, the model may not have been long enough to induce alcohol-related changes in the mucin layer thickness of the mice endometrium. In the future, a longer study time could be employed to mitigate this potential confounder.

CONCLUSION

In conclusion, the results of this study indicated that chronic alcohol consumption has no statistically significant impact on the uterine mucin layer thickness of ethanol fed mice. However, there were some limitations in the design of this study that could have attributed to discrepancies in data that may have skewed the results. For more statistically accurate results, future research could improve on the study design by increasing the sample size among both treatment groups and by housing the mice individually. Although this is not always cost effective, this would help mitigate the Lee-Boot effect and allow for the more precise recording of the amount of ethanol consumed per ethanol fed mouse. Nonetheless, the results of this study

are still noteworthy in that they further the advancement of the impacts of alcohol consumption on the female reproductive system.

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APPENDIX A

Detailed Tissue Embedding Process:

Step 1: Paraffin dispensing unit was powered on to heat up paraffin.

Step 2: All the necessary concentrations of ETOH, Citrisolv, and mold release were prepared so that the embedding run could be performed.

Step 3: Labels were made for all of the tissues that were to be embedded.

Step 4: Tissues were placed in embedding cages with the appropriate label.

Step 5: Tissues were then dehydrated using the following procedure:

80% ETOH: 30 minutes
→ Tissues were stored in 70% ETOH prior to embedding
90% ETOH: 30 minutes
95% ETOH: 30 minutes
100% ETOH: 30 minutes
100% ETOH: 30 minutes
50:50 100% ETOH:CitriSolv: 30 minutes
100% CitriSolv: 30 minutes

--In the embedding oven--

50:50 CitriSolv:Paraplast: 30 minutes
Paraplast 1: 1 hour
Paraplast 2: 1 hour

Step 6: While the tissues were being dehydrated, embedding rings were labeled with the appropriate tissue code.

Step 7: Embedding molds were prepared, submerged in mold release, and set aside to dry. Once dry, this procedure was repeated a second time. Once mold released two times, the embedding molds were placed in the heated chamber of the embedding unit to await embedding.

Step 8: With 5 minutes remaining in the paraplast 2 step, the cold plate of the embedding unit was turned on.

Step 9: The tissues were removed one at a time, embedded in paraffin, and properly labeled. The empty embedding cages were immediately placed in CitriSolv to dissolve the excess paraffin.

APPENDIX B

Tissue Staining Procedure:

Step 1 = Deparaffinization and Rehydration Process

Hemo-de 1	3 minutes
Hemo-de 2	3 minutes
100% ETOH 1	3 minutes
100% ETOH 2	3 minutes
95% ETOH 1	3 minutes
80% ETOH 1	3 minutes
70% ETOH 1	3 minutes
distilled H_20	2 minutes
distilled H_20	2 minutes
distilled H_20	2 minutes

Step 2 = H&E Staining Technique

Harris's Hematoxylin (filtered)	3 minutes
Rinse with distilled H_20	x2
Rinse with tap water	5 minutes
Acid alcohol	10 dips
Rinse with tap water	1 minute, x2
Rinse with distilled H_20	2 minutes
Blot off excess water	(as necessary)
Eosin	45 seconds

Step 3 = Dehydration Process

70% ETOH 2	2 minutes
80% ETOH 2	2 minutes
95% ETOH 2	2 minutes
100% ETOH 3	2 minutes
100% ETOH 4	2 minutes
Hemo-de 3	3 minutes
Hemo-de 4	3 minutes

Step 4 = Coverslip slides

APPENDIX C

Average Mucin Layer Thickness by Mouse ID:

Mouse ID	Average Mucin Thickness (nm)
MS111801	2.397
MS111802	3.704
MS111803	5.886
MS111804	5.804
MS111805	1.558
MS111806	2.429
MS111807	2.565
MS111809	4.395
MS111810	4.159

(Control: MS111807, MS111809, MS111810)

(NIAAA: MS111801, MS111802, MS111803, MS111804, MS111805, MS111806)