The Effects of Chronic Alcohol Consumption on Ovarian Function/ Morphology

Destiny Roberts
East Tennessee State University

Follow this and additional works at: https://dc.etsu.edu/honors
Part of the Other Chemicals and Drugs Commons

Recommended Citation

This Honors Thesis - Withheld is brought to you for free and open access by the Student Works at Digital Commons @ East Tennessee State University. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of Digital Commons @ East Tennessee State University. For more information, please contact digilib@etsu.edu.
The Effects of Chronic Alcohol Consumption on Ovarian Function/ Morphology

Thesis submitted in partial fulfillment of Honors

By

Destiny Roberts

HID Human Health Program

The Honors College

East Tennessee State University

March 28, 2017
# TABLE OF CONTENTS

Abstract .......................................................................................................................... 3

Introduction .................................................................................................................... 4

Materials and Methods .................................................................................................. 7

Results ............................................................................................................................. 10

- Stage of estrous as determined by vaginal wash ...................................................... 10
- Body weights of control and ethanol ........................................................................ 14
- Analysis of follicles and ovarian morphology ......................................................... 14

Discussion ...................................................................................................................... 18

Acknowledgements ....................................................................................................... 21

References ..................................................................................................................... 22
ABSTRACT

Chronic alcohol (ethanol) consumption has been known to affect the major organs of the body and particularly the liver. However, the effects of chronic ethanol consumption on the female reproductive system remain relatively unstudied. A convenient way to study these effects is by analyzing laboratory mice that have been fed an ethanol diet for an extended period of time and comparing them to control mice. In this study, female mice were separated into control and ethanol fed groups. The mice were placed on their specified diets and observed over the course of six weeks. The mice were fed and weighed daily throughout the duration of the experiment. Once a week, vaginal washes were performed on both groups of mice to determine the stage of the estrous cycle for each mouse. At the end of the six weeks, the mice were sacrificed and the ovaries were harvested and fixed in 4% paraformaldehyde. The ovaries were then paraffin embedded and sectioned. Glass microscope slides were then stained using Hematoxylin and Eosin staining procedures for evaluation using standard light microscopy. The tissue’s morphology, follicle development, presence of corpora lutea, and overall appearance were analyzed. Due to the premature deaths of several mice in first group of ethanol fed mice, the experiment was repeated with three more groups of mice to obtain a better representation of data. The data from the control group was compared to that of the ethanol fed group. The mice that received the ethanol fed diet ceased to cycle and arrested in the diestrous phase of the estrous cycle. Our data indicates that the ovarian follicles within the ethanol fed mice show signs of degeneration in the 4b, 5a, 5b, 6, and 7 levels of development. There are also no notable corpora lutea present within the ovaries of the ethanol fed mice. Our findings indicate that chronic alcohol consumption has deleterious effects on ovarian morphology in mice.
INTRODUCTION

Past studies have shown that chronic alcohol consumption has negative effects on various organ systems. In women, alcoholism has been associated with fertility issues, menstrual abnormalities, and changes in secondary sex characteristics (1). The mechanisms of these effects are not fully understood. One study followed social and heavy drinkers, in which social drinkers were defined as having 3 drinks per day and heavy drinkers were having 7 drinks per day. The results from that study indicated that social drinkers had anovulatory cycles and heavy drinkers had persistent hyperprolactemia (2). Many of the effects of chronic alcohol consumption in women are due to an abnormality of hormone levels. Moderate alcohol consumption has been linked to decreased oxidation of oestradiol to oestrone and decreased progesterone levels. The former reaction results in the high accumulation of NADH. High levels of NADH are known to cause lactate accumulation and inhibit fatty acid oxidation (3). Low progesterone levels can cause women to have weight gain, anovulatory cycles, inability to carry a pregnancy to term, etc.

Studies of the effects of chronic alcohol consumption on the female reproductive system remain relatively limited. While the effects of chronic alcohol consumption cannot be ethically measured in humans, they can be analyzed in animal models. Currently, the effects of chronic alcohol consumption on the reproductive system is more frequently studied in male rodents. In a study with male rats, the rats that received the ethanol based diet had lower fertility and showed an increase in testicular oxidative injury. It is believed that this oxidative injury accelerates apoptosis in male germ cells (4). It is suggested that oxidative injury may also occur in the ovarian tissue of female mice due to chronic alcohol consumption. Furthermore, these ovarian tissues should display some signs of degeneration and accelerated apoptosis. Contrary to the male reproductive system, females are born with a finite amount of germ cells present within the
ovaries. Any damage caused by the chronic consumption of alcohol to ovarian tissue should be considered significant.

Studies into the effects of chronic alcohol consumption on the female reproductive system have generally focused on young, prepubescent rodents or pregnant rodents. Relatively few studies focus on changes in ovarian function or morphology in adult female mice due to chronic ethanol consumption. A study involving adult female rats discovered that the groups which received the ethanol diet had lower progesterone levels and an overall decreased ovarian weight, compared to that of the control group. Lower progesterone levels generally result from the absence or decreased production of corpora lutea. The corpus luteum secretes progesterone, which helps maintain the endometrium. Since there were fewer corpora lutea present, the resulting progesterone levels would be less than normal. Corpora lutea were absent from 41 out of 50 of the subjects. The ovaries of the ethanol fed rats also exhibited few large developed follicles (1). Another study, exposed 20-day old ethanol-fed female rats to 5% ethanol for 25 to 30 days. At the conclusion of this study only a single generation of corpora lutea were present in the ovarian tissues (5). With formation beginning in metestrous, newly formed corpora lutea are present the length of two to four complete estrous cycles. Since mice and rats are able to complete the estrous cycle in four days, there should be at least six generations of corpora lutea within 30 days. Organelle damage also occurs in the ovarian tissue of ethanol fed rats. Rats that chronically consumed an ethanol based diet showed alterations at the granulosa cell level in ovarian tissues. The alterations present within these cells were dilation of the rough endoplasmic reticulum, detachment of ribosomes, and swollen mitochondria (6).

Progesterone is not the only hormone effected by ethanol. In a study using cultured rat granulosa cells, the effects of follicle stimulating hormone (FSH) on the granulosa cells of
ethanol fed rats were measured. It was discovered that ethanol did not prevent FSH treated granulosa cells from increasing cAMP levels. However, ethanol did suppress the secretion of prostaglandin estradiol from those cells. Ethanol was further able to block cyclooxygenase-2 in those cells. Their results indicated that ethanol is able to inhibit FSH-induced ovarian steroidogenic acute regulatory (StAR) protein which effectively suppresses estradiol production (7). Estradiol acts primarily as a growth hormone for reproductive organs and promotes the development of secondary sex characteristics.

Studies into ethanol consumption in females have also focused on the number of antral follicles present. Antral follicles are a measure of the future egg supply and can be used to determine fertility. In a study that discussed ultrastructural differences in the antral follicles of ethanol fed rats, it was noted that there was an increase in the diameter of antral follicles, as well as the increased presence of vacuoles and lipids within the ovarian tissue. The study also noted that the longer the timeframe the subject ingested the ethanol based diet, the more overall ovarian damage occurred (8). Collectively, the previous studies signify that morphological changes occurred due to chronic ethanol consumption.

Few studies have been conducted on the morphological changes that occur in ovarian tissues due to chronic alcohol (ethanol) consumption. While the effects cannot be ethically measured in humans, they can be analyzed in animal models. In this study, the effects of chronic alcohol consumption on ovarian tissue morphology were analyzed. The tissues were analyzed based on overall appearance of follicles, type of follicles present, and the presence of corpora lutea.
MATERIALS AND METHODS

Animals. 28 wildtype C57BL/6 mice were utilized in this study. The first group of female mice had 6 ethanol fed mice and 6 control mice. The second group of mice had 5 ethanol fed mice and 2 control mice. The third group of mice had 5 ethanol fed mice. The fourth and final group of mice had 4 ethanol fed mice.

Diets and Feeding. The control mice were placed on a Lieber-DeCarli liquid diet, Bio-Serv; Cat#F1259-SP. The ethanol mice were placed on a Lieber-DeCarli, Bio-Serv; Cat#F1258-SP with 5% ethanol. The mice and the amount of food available were weighed daily to track weight gain/loss and total food/alcohol consumption.

Tissue Harvesting and Preservation. Once the animals had been sacrificed, the ovaries were harvested and placed in 1% paraformaldehyde for 24 hours. The tissues were then placed in 4% paraformaldehyde for another 12 hours. The following day, the tissues were trimmed and placed in 70% ETOH. The tissues remained in the 70% ETOH until the start of the paraffin embedding procedure. To begin the paraffin embedding procedure, the tissues were placed in their respective embedding cages and then submerged in 80% ETOH, 90% ETOH, 95% ETOH, and 100% ETOH for 30 minutes in each solution. The tissues were then placed in a 50:50 mixture of 100% ETOH and Hemo-De for 30 minutes, followed by 100% Hemo-De and a mixture of 50:50 Hemo-De and Paraplast, each for 30 minutes. Next, the tissues were placed in Paraplast 1 for 1 hour, followed by placement into Paraplast 2 for 1 hour. After the tissues remained in the Paraplast 2 for 1 hour, they were embedded in fresh paraplast until they were needed for sectioning.

Sectioning, Staining, and Visualization. 20 tissues from the mice that survived the duration of the study were sectioned at 4 μm using a Microm HM325 microtome. The sections were placed
in a water bath and then mounted to a microscope slide. Three total slides were made of each tissue. The slides were allowed to dry overnight. Once all the needed tissue had been sectioned, the slides were stained using the Hematoxylin and Eosin (H and E) staining procedure. To begin the H and E staining procedure, tissues were deparaffinized and rehydrated. The rehydration procedure is as follows: Hemo-De 1 for 3 minutes, Hemo-De 2 for 3 minutes, 100% ETOH 1 for 3 minutes, 100% ETOH 2 for 3 minutes, 95% ETOH 1 for 3 minutes, 80% ETOH 1 for 3 minutes, 70% ETOH 1 for 3 minutes, followed by 3 separate rounds of placement in distilled water for 2 minutes. After rehydration, the tissues were placed in filtered Hemotoxylin for 3 minutes, rinsed twice with distilled water, placed in tap water for 5 minutes, dipped 8 times in acid alcohol, rinsed for 1 minute with tap water, rinsed for 2 minutes with distilled water, and soaked for 30 seconds in Eosin. After the 30 seconds in Eosin, the tissues were dehydrated and coverslipped. The dehydration procedure is as follows: 70% ETOH 2 for 2 minutes, 80% ETOH 2 for 2 minutes, 95% ETOH 2 for 2 minutes, 100% ETOH 3 for 2 minutes, 100% ETOH 4 for 2 minutes, Hemo-De 3 for 3 minutes, and Hemo-De 4 for 3 minutes. The slides were then ready to be coverslipped. Once they were coverslipped, the tissue was examined and photographed suing a Zeiss Axioskop 40 microscope equipped with a Cannon Powershot A640 camera.

**Vaginal Washes.** To determine the stage of estrous for each mouse, vaginal washes were performed once per week, during the duration of the study and again at the time of sacrifice. Vaginal washes were performed using a micropipettor to flush the vagina with 100 microliters of PBS at a pH of 7.2. Briefly, the pipette tip containing the PBS, was inserted into the vagina, the saline was dispensed and then redrawn back into the pipettor, and repeated. The washes were placed on their individual, labeled microscope slides and stained using trypan blue. The vaginal
wash samples were analyzed to determine the stage of estrous for each animal. To determine the stage of estrous, the appearance of epithelial cells, cornified cells, and the presence of leukocytes were analyzed.
RESULTS

Results 1: Stage of estrous in ethanol and control groups as determined by the results of vaginal washes.

The stages of estrous in Figure 1 were determined as followed: proestrous was indicated by the presence of nucleated, granular epithelial cells, 1A; estrous was indicated by clustered cornified cells that were mostly non-nucleated, 1B; metestrous was indicated by numerous leukocytes and smaller amounts of non-nucleated epithelial cells and cornified cells, 1C; and diestrous was indicated by few epithelial and cornified cells with many leukocytes, 1D.

Figure 1. A, example of a slide showing proestrous. Red arrow – a nucleated, epithelial cell. B, example of a slide showing estrous. Green arrow – a cornified cell. C, example of a slide showing metestrous. Blue arrow – cluster of leukocytes. Orange arrow – a cornified cell. D, example of a slide showing diestrous. Yellow arrow – a leukocyte. (400X).
The results shown in Figure 2 also include the stage of estrous from the estrous cycles of mice that died prematurely. Figure 2 displays the progression of the estrous cycles from the ethanol and control mice from all groups throughout the study. The stages of estrous are represented by numbers 1 through 4. 1 refers to proestrous, 2 refers to estrous, 3 refers to metestrous, and 4 refers to diestrous. C and E represent the control and ethanol fed groups, respectively. It is important to note that the first and second ethanol and control groups ended the study on week 6. The remaining third and fourth groups had vaginal washes read on week 7 of the study, and sacrifice occurred on a different day within that week. Due to different groups ending on different weeks, and a difference between the number of mice within each group, there appeared to be missing data present when all the groups were graphed together. This ‘missing’ data has been represented with a *, as shown in Figure 2. Figure 2 includes the vaginal wash results from mice that died prematurely in the study as well.

The mice in the first ethanol group were MS040301, MS040302, MS040303, MS040304, MS040305, and MS040306. The mice in the first control group were MS040307, MS040308, MS040309, MS040310, MS040311, and MS040312. The premature deaths from ethanol group 1 are as listed: MS040301 died before the time of sacrifice after week 6, MS040302 died before the vaginal wash for week 3 was performed, and MS040304 and MS040305 both died before the vaginal wash for week 4 was performed. As shown, some of the control mice from the first group continued to cycle through the duration of the experiment.

The mice in the second ethanol group were F052808, F052809, F052810, F052811, and F052812. The mice in the second control group were F052813 and F052814. The premature deaths from ethanol group 2 are as listed: F052808 and F052810 both died before the vaginal wash for week 6 was performed, F052811 died before the vaginal wash for week 7 was
performed, and F052812 died for the time of sacrifice after week 6 vaginal wash. The mice in the second control group continued to cycle throughout the experiment.

There was one premature death in third ethanol group, F060823, which occurred early on the day of sacrifice. There were no premature deaths from the fourth ethanol group. There was not a designated control group for the third and fourth ethanol groups. In the third ethanol group, many of the mice remained arrested in the diestrous phase of the estrous cycle, with the exception of two mice that continued to cycle up to the time of sacrifice. In the fourth ethanol group, with the exception of one mouse, the majority of the mice remained arrested in the diestrous phase beginning on week 4 of the experiment.

Figure 2. Vaginal wash results from all ethanol fed and control mice throughout the duration of the study. 1, 2, 3, and 4 represent proestrous, estrous, metestrous, and diestrous stages of the estrous cycle, respectively. C and E represent control and ethanol groups, respectively. An asterisk, *, has been used to represent any missing data present within the groups.
Statistical analysis was performed to determine the significance of the vaginal wash results of the control groups compared to that of the ethanol groups. There was not a significant difference between weeks 1, 2, 3, and 4. However, when StatKey was used to analyze the difference between calculated and randomized Chi-square tests, there was significant difference determined between weeks 5, 6, 7, and on sacrifice. It is important to note that since the values present were low, an accurate p-value could not be obtained for this categorical analysis, thus the analysis of comparison of Chi-square values was performed using StatKey.

The significant difference between the control and ethanol groups was determined for the weeks that the mice remained in proestrous, estrous, metestrous and diestrous. T tests were performed and the resulting p-values for the significance between the weeks the groups remained in proestrous, estrous, metestrous, and diestrous are as follows: the p-value for proestrous was 0.40; the p-value for estrous was 0.13; the p-value for metestrous was 0.23; and the p-value for diestrous was 0.39. Since all of the p-values are larger than 0.05, it is determined that there is not a significant difference between the ethanol and control groups in relation to the number of weeks spent in each stage of estrous.

Statistical analysis was also performed to determine if there was significant difference between the control and ethanol groups in relation to how many times each mouse within the ethanol or control group changed to a different stage of estrous. The resulting p-value for this analysis was 0.02. Since the p-value is below 0.05, it is determined that a significant difference is present between the ethanol and control groups, in relation to the number of times they changed to a different stage of estrous.
**Results 2: Body weights of control and ethanol groups.**

The average weight gain for all ethanol groups was approximately 3.8 grams, while the average weight gain for the control group was approximately 3.3 grams. Statistical analysis was performed to determine if a significant difference was present between the body weights of the ethanol and control groups. A p-value of 0.03 resulted, indicating that a significant difference did exist between these two groups.

![Graph showing body weights of ethanol and control groups](image)

**Figure 3.** Body weights of the ethanol and control groups throughout the duration of the experiment. **A,** Body weights are displayed in grams. **B,** Body weight has been normalized to starting weight (9).

**Results 3: Analysis of follicles and ovarian morphology.**

Figures 4 and 5 display signs of degeneration that were commonly found in all ethanol tissues. Follicles were assigned stages based on Oakberg’s classifications (Figure 4). Degeneration was noted by the premature presence of an antrum. Antra are filled with follicular fluid and form naturally in a mature follicle. Antra signify that a transition has occurred from a primary follicle to a secondary follicle, i.e. antral follicle. Considering all ovarian tissues from the ethanol mice, there were signs of degeneration, i.e. antral formation, within follicles of levels
4b, 5a, 5b, 6, 7, and 8. While natural degeneration, or antral presence, can occur in levels 6, 7, and 8, degeneration should not be seen in earlier levels. As depicted in Figures 5 and 6, there were no notable corpora lutea found within the ethanol tissues. From the 11 ethanol mice that survived the duration of the study, there were an average number of 17 follicles found within the ovarian tissue. Control tissues rarely displayed signs of degeneration (Figures 7-8). Many of the control tissues displayed 1 to 3 corpora lutea throughout the tissue. From the 8 control mice used in the study, there was an average number of 15 follicles found within the ovarian tissues. When statistical analysis was done on the follicular count, a p-value of 0.78 was calculated, indicating that there was not a significant difference between the ethanol and control groups with regard to follicular numbers.

Figure 4. Pictures of follicular levels 1-8 from Oakberg’s “Follicular Growth and Atresia in the Mouse” (10).
Figure 5. Ovarian tissue from an ethanol fed mouse, F071230. Green arrow – degenerating level 7 follicle, Red arrow – degenerating level 5a follicle, Blue arrow – level 5b degenerating follicle. 100X

Figure 6. Signs of follicular degeneration in an ethanol fed mouse, F060824, Green arrow – level 5a follicle, Blue arrow – level 5b follicle. 200X
Figure 7. Examples of tissue from a control mouse, MS040312. Blue arrow - healthy 4b follicle, Yellow arrow - healthy 5a follicle, Red arrow - corpus luteum. 100X.

Figure 8. Example of tissue from a control mouse, MS040307. Green arrow - level 5a follicle. 400X.
DISCUSSION

The four stages of estrous are proestrous, estrous, metestrous, and diestrous (Figure 1). The continuation of the estrous cycle for the mouse was theorized to be an important indicator of the effects of chronic ethanol consumption. A significant difference was noted between the results of the vaginal washes of the control groups versus the results of the ethanol groups for weeks 5, 6, 7, and on sacrifice. Of the 20 ethanol mice used, 17 of them remained in diestrous for 2 or more weeks until the time of premature death or sacrifice (Figure 2); this is 85% of the ethanol subjects. It is important to note that 14 of these females were housed in an environment near male mice. In the 14 ethanol females that were exposed to male mice, 11 of those females ceased to cycle. According to the Lee-Boot effect, females will cease to cycle if they are not exposed to male pheromones and housing females in groups, not exposed to male pheromones, will result in the synchronization of their cycles, resulting in them arresting in diestrous (11).

Since 11 of the ethanol fed females ceased to cycle, it could be proposed that the effects of chronic alcohol consumption were able to suppress the Lee-Boot effect and cause the mice to remain in diestrous. It is important to note that these results were in some measure expected and that anovulatory cycles are even known to occur in women who are regularly heavy drinkers. Additionally, ethanol mice were not the only subjects that ceased to cycle. 4 out of 8 of the control mice, or 50%, remained in diestrous for 2 or more weeks until the time of death. From control mice, there were two females that were part of the later study that had male mice near their environment. Those two control mice continued to cycle until the time of sacrifice.

With the added consideration of the addition of males, and the Lee-Boot effect, along with only 50% of control mice ceasing to cycle, it can be concluded that chronic ethanol consumption impacts continuation of the estrous cycle and results in cessation. There were 9
ethanol mice that died prematurely during this study. While the ovarian tissue within these mice could not be analyzed, the results from their vaginal washes were noted and used in Figure 2. The amount of ethanol to be consumed was modified to prevent future deaths and to allow the mice to adjust to ethanol consumption.

It had been suggested from previous studies that the presence of corpora lutea in ethanol tissues would be greatly reduced (1,5). Upon examination of the follicles present within the tissue, it was concluded that no notable corpora lutea were present in the tissues from ethanol fed mice. This is very interesting as corpora lutea are formed after a mature ovum is released from the ovarian follicle. Since many of the follicles present within the ovarian tissue from ethanol fed mice had the presence of a premature antrum, it is implied that those follicles never reached maturation and thus could not ovulate and produce a new corpus lutea. A new corpus luteum should develop in the ovary with the completion of the estrous cycle. As multiple sections were taken of each ovary, and thus various depths into the tissue were analyzed, any present corpora lutea should have been visualized.

It was theorized that ethanol would have additional effects on the follicles present within the ovarian tissue. The number of follicles present, along with the marked presence of antra, were analyzed. Antra are a sign of follicle maturation and should only be present in secondary follicles. In studies that analyze follicular development and degeneration, antra are commonly noted as a sign of degeneration if they occur in less mature follicles. For example, in a study performed by Oakberg, et al., it was noted that formation of an antrum within a follicle with less than 7 to 8 layers marked degeneration of that follicle (10). The mice that received the ethanol based diet showed a remarkable presence of degeneration in 4b, 5a, 5b, 6, and 7 follicles as determined by the premature presence of antra (Figures 5-6); it is important to note that
degeneration was not seen in less mature follicles. Additionally, both control and ethanol mice presented follicles as low as level 1 and as developed as level 8. However, since the antra formed in ethanol follicles that had not reached full maturity, it can be assumed that these follicles were in the process of degeneration and thus would have been unable to reach maturity and release the ovum. According to literature, the premature presence of an antum produces pressure on the immature oocyte. This pressure can lead to rupture of the zona pellucida, which can harm the ooplasm. The zona pellucida is a layer that surrounds the oocyte. The harming of the ooplasm results in fragmentation of intracellular structures and eventual cell death (12). The inability to release the ovum would have resulted in the cessation of cycling, along with the inability of that follicle to become a corpus luteum. Again, since there were no corpora lutea visualized within the tissues from the ethanol fed mice, it is concluded the presence of the premature antrum resulted in the follicles inability to release a mature oocyte and form the corpus luteum. Since the corpus luteum is responsible for producing progesterone, and there are known low levels of progesterone in ethanol fed mice from previous studies (3), it could be proposed that the ethanol fed mice in this study would have presented low progesterone levels as well. Overall, the premature presence of antra, in conjunction with no visible corpora lutea being present within the ethanol tissues, would aid in support of possible methodology behind why more ethanol mice remained in the diestrous phase of the estrous cycle.

Ethanol mice had an average number of 17 follicles present within their tissues, while control mice had an average number of 15 follicles present, in addition to corpora lutea. There was a p-value of 0.78 which indicates no significant difference was between the groups. No other morphological differences were noted upon analysis of the tissues.
In conclusion, chronic ethanol consumption had 3 major effects in regards to ovarian function/ morphology: the majority of the ethanol mice ceased cycling, remaining in diestrous, there were no corpora lutea present within the tissues of ethanol fed mice, and there was premature degeneration present in the levels 4b, 5a, 5b, 6, and 7 follicles. While hormonal aspects of the ethanol mice were not analyzed, it could be theorized that they would exhibit low progesterone levels (1), due to the overall absence of corpora lutea in the tissues. Additionally, there was a significant difference between the average weight gain between ethanol and control mice (Figure 3), with a p-value of 0.03. Significant difference was detected between the number of estrous stage changes of the ethanol groups compared to the estrous changes of the control groups, a p-value of 0.02. Significant difference was also detected between the results of the vaginal washes for the control and ethanol groups on weeks 5, 6, 7, and on sacrifice. Despite the premature deaths of 9 female mice, there was still enough data present to study the effects of chronic ethanol consumption and our results should aid in the future research into chronic alcohol consumption’s effects on the female reproductive system.

ACKNOWLEDGEMENTS
Thank you to Dr. Johnathon Peterson for supplying the mice and the materials needed for their care. Special thanks to the rest of the research team, Josh Bacon, Ashley Degroat, Kendra Hagood, and Greta Trogen, for their help and support in caring for the mice during this study.

Additional thanks to the ETSU Dept. of Health Sciences and the ETSU Honors College.
REFERENCES


