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Effects of Cottonseed Meal Containing Gossypol on Testis Physiology in Boars

Kristin Ryan

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University of Arkansas

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Abstract

Feral hogs are an invasive species found in 35 U.S. states without an effective control method to limit their population growth. According to a 2019 survey of 467 landowners in Arkansas, feral hogs caused an estimated $12 million in damages (Cook, 2019). While there are current control methods such as hunting and trapping, 65% of the feral hog population must be eliminated in a specific area to prohibit population growth (Cook, 2019). It is theorized that gossypol, a phenolic compound known to negatively impact reproductive function in other species, could cause sterility in boars. In this preliminary research study, 21 domestic boars were randomly divided into three test groups to study the effects of ingested gossypol from cottonseed meal (CSM). This experiment is part of a larger research study on the effects of gossypol from CSM on the reproductive function of swine. Testis tissue was harvested from each boar and frozen for subsequent histological analysis. A Cryostat Microm HM550 (Thermo Fisher Scientific, 2010) was used to cut the tissue into 8 um thick slices and the tissue was stained using a Hematoxylin and Eosin staining procedure. The tissue samples were imaged using a Nikon Eclipse Ts2R. The cross-sectional diameters of 100 seminiferous tubules from each test subject were measured using ImageJ (Bethesda, MD). Seminiferous tubule diameters were analyzed using MIXED procedures of SAS (SAS, Inc., Cary, NC) with treatment as a fixed effect. No differences were found between the seminiferous tubule diameters of boars fed CSM compared to those not fed CSM. The results of the study do not support the hypothesis that gossypol from CSM has a negative effect on the reproductive function of boars under the set conditions. Future research studies are needed to determine an effective population control method for feral hogs.

Introduction

Background and Need

Feral hogs are declared an invasive species in the United States with an estimated population of over 6 million and affect 35 different states, including Arkansas (U.S. Department of Agriculture [USDA], 2020). Feral hogs pose a threat to humans, livestock, and property through the spread of disease and damages. A study of 467 Arkansas landowners found that 70% of the surveyed landowners had active feral hogs on their property and researchers estimated that the hogs caused approximately $12 million in damages to these properties (Cook, 2019). In fact, feral hogs are found in every county across the state of Arkansas (Arkansas Department of Agriculture [ADA], 2020).

Feral hogs originated in Eurasia and Africa but were brought to North America in the 1500s as a food source for European explorers (USDA, 2020). Feral hogs operate with a herd mentality and can be a potential danger to both humans and animals. Researchers have documented feral hogs learn how to hide from humans and can even become nocturnal to avoid human interaction (Ellis et al., 2020). Different methods of population control, such as hunting and trapping, are used to control the population but are ineffective. A more efficient method of population control is required to limit the feral hog population.

In the 1950s, rural villages in China experienced a steep decline in childbirth rates. It was discovered that the cottonseed oil they used for cooking contained gossypol and had caused infertility in the men of the villages. When the rural villagers changed to different cooking oil, the birth rates returned to normal over time (Liu et al., 1985). Over the past 50 years, gossypol has been found to inhibit the process of spermatogenesis in animals such as bulls, rats, and mice but is dependent upon both dosage and time (Gadelha et al., 2014). It was hypothesized that

CSM containing gossypol could be used as a negative influence on swine reproduction. As of 2017, swine diets are advised not to contain more than 0.01% gossypol due to the toxic effects caused in monogastric animals (Morgan, 2014). It is of note that ruminant animals are not adversely affected by gossypol due to the presence of Rumen Bacillus Subtilis (RBS), a ruminal bacterium that effectively breaks down gossypol (Zhang et al., 2018). Since pigs lack the necessary bacteria to degrade gossypol in their foregut, they are more susceptible to its toxic effects, than ruminant animals.

In reproductive studies, males are more often targeted than females because they have the potential to produce more offspring in their lifetime. Boars are known to have large seminiferous tubules compared to other species such as bulls. In a study using Brahman bulls, it was determined that a diet containing gossypol resulted in a decreased number of layers in the germinal epithelium within the seminiferous tubules compared to those that did not ingest gossypol (Chase et al., 1994). A diminished germinal epithelium could negatively affect spermatogenesis.

As a model for reproductive control in feral hogs, domestic hogs were used to study the effects of CSM containing gossypol on testis physiology in swine. This experiment was part of a larger research study also evaluating plasma gossypol, semen quality, and growth performance. Average daily gain (ADG) decreased as gossypol content increased in the feed via cottonseed meal in both treatment groups during phase 3 of the feeding trial, resulting in a decrease in weight gain (Mudarra et al., 2021). Plasma gossypol concentrations increased as gossypol percentage in the feed increased in treatment groups during phases 1-3 of the feeding trial (Mudarra et al., 2021). There was no effect of gossypol from CSM on sperm concentration (P = 0.72), percent motility (P = 0.17), or percent progressive motility (P = 0.87; Benefield et al., 2021a). It should be noted that semen was not successfully collected from every boar in the study. There was no difference in the number of boars that were able to be successfully collected compared to those that were not (Benefield et al., 2021b). The following paper reviews the findings of the impact of gossypol on the seminiferous tubule diameter in boars fed gossypol from CSM.

Problem Statement

Arkansas landowners across the state have experienced millions of dollars in damages due to the feral hog population (Cook, 2019). Feral hogs cause property damage, pose a physical threat to humans and animals, and can spread numerous diseases and parasites. Although other methods of control are in place to control the population, survey results in the state of Arkansas suggest those methods are largely ineffective (Benefield et al., 2021a). An affordable, effective, and humane solution is required to reduce the population of feral hogs in the state of Arkansas.

Purpose of the Study

The purpose of this study was to collect data on how cottonseed meal containing gossypol affected the reproductive physiology in boars.

Research Questions and Hypothesis

The following questions guided this study:

- To what degree did gossypol impact the size of the seminiferous tubules in boars?
- Is there a difference in seminiferous tubule diameter between the treatment groups and the control?

- Hypothesis: Boars fed a diet of cottonseed meal containing increasing concentrations of gossypol will have smaller seminiferous tubules than boars that received no gossypol in their diet.

Literature Review

Feral hogs are a well-known invasive species in the United States and require an effective control method to minimize the rapidly growing population. This review of literature will explore published studies related to three major concepts – feral hogs, gossypol, and reproductive physiology. By establishing this background, a proper investigation into the impact of gossypol on the reproductive physiology of boars can be explored.

Feral Hogs

An Overview of the Feral Hog Population

While it is widely accepted that the feral hog population in the U.S. is large and continuously growing, the exact size of this population is unknown to researchers. One study estimated the U.S. population of feral hogs to be approximately 6 million in 2018, but it also indicates that the population has continuously grown since then (Ellis et al., 2020). Feral hogs are present in a minimum of 35 U.S. states and have few natural predators aside from humans (USDA, 2020). Other predators include wolves, bears, alligators, and even other feral hogs as they have been reported to attack immature hogs both inside and out of their familial groups (Cooperative Extension, 2019).

In the state of Arkansas, feral hogs are present in every county and are estimated to have a population of over 200,000 (ADA, 2020). A study found that of 467 surveyed landowners in Arkansas, 70% of them had active feral hogs on their property (Cook, 2019). An active feral hog presence can be determined by significant rooting of the land, tracks, and scat. It is difficult to effectively control a species with a high reproductive rate. Feral swine reach sexual maturity at 6-8 months of age and sows can yield one to two litters per year with four to twelve piglets in each litter (ADA, 2020). It is because of their high rate of fecundity that the feral hog population is continuously outgrowing control methods in place.

Various Damages due to Feral Hogs

One of the main motivating factors for controlling the feral hog population is to mitigate the economic losses they cause due to various damages to property. Feral hogs are known to damage and consume crops, contaminate food and water for humans and livestock, and can cause property damage to fences, lawns, vehicles, etc. The USDA estimated that feral hogs cause $1.5 billion annually in damages to crops, property, livestock, and control costs (USDA, 2020). In the state of Arkansas, researchers conclude that feral hogs cause $19 million of damage annually (ADA, 2020).

Feral hogs are classified as invasive species due to their effect on native plants and animals. Researchers have documented that feral hogs threaten the biodiversity of native plant and animal species in the Big Thicket National Preserve (Weeks & Packard, 2009). Feral hogs trample different plants, root and wallow which disrupts plant health and soil, and compete with native species for limited natural resources. Feral hogs are opportunistic feeders and in Arkansas, they are known to eat ground-nesting birds, fawns, and young domestic livestock (ADA, 2021).

Another way in which feral hogs pose a threat is by serving as a host for diseases and parasites. Feral hogs can transmit over 45 different diseases and parasites such as Pseudorabies Virus (PRV), Swine Brucellosis (SB), and Swine Influenza. Several of these, such as Swine

Brucellosis and Swine Influenza, are zoonotic diseases meaning they can infect humans through an animal host (ADA, 2021). The transmission of many of these diseases could prove economically disastrous if they spread to livestock. Although not all of these diseases and parasites are found in Arkansas, their effects are felt in other parts of the country.

Population Management Strategies

Although it is clear there is an overpopulation of feral hogs, the current control methods in place are not effective enough to stop the continuous population growth. The U.S. Congress allocated $20 million in 2014 to create the APHIS National Feral Swine Damage Management Program (USDA, 2020). This program serves to control the damages of feral swine by educating the public, monitoring the current population, and developing strategies and tools to effectively minimize the damages caused by feral hogs. It is not only very expensive to control the feral hog population but it is also difficult to do so effectively. According to the Arkansas Game and Fish Commission (AGFC), 65% of the hogs in a certain area must be eliminated to stop the population from continuing to grow (Cook, 2019).

Current management strategies include hunting, trapping, fencing, harassment, contraception, and several others. A study by Texas A&M University stated that public hunts were not an adequate control method in research conducted at the Big Thicket National Preserve even with the extended hunting season for feral hogs (Chavarria et al., 2004). One potential control method is the use of fertility control via oral or injectable contraceptives. The use of contraceptives is a publicly acceptable control method and could be useful in locations where fatal control methods are not publicly acceptable or legal (Massei et al., 2011). Another method is to use toxicants to control the feral hog population, but this strategy is limited since it must not also harm other species in the area.

Gossypol

Gossypol Overview

Gossypol is a polyphenolic compound found in the cotton plant that can be toxic in high concentrations to species including humans, cows, and mice (Lim et al., 2019). These toxic effects can range from shortness of breath to fatality depending on the amount of gossypol and the species of animal. There are two forms of gossypol – bound gossypol which is the non-toxic form that binds to proteins and free gossypol which is the toxic form. Free gossypol is concentrated in whole cottonseeds and is also found in CSM after the extraction of cottonseed oil (Morgan, 2017). The gossypol in CSM must be measured to find the exact amount of free gossypol as it contains both forms.

Effects of Gossypol in Ruminants and Monogastrics

While the toxic effects of gossypol can affect both ruminants and monogastrics, the reproductive system of monogastrics appears to be more sensitive to gossypol (Randel et al., 1992). Similarly, gossypol is known to have more severe effects on monogastric animals than ruminant animals (Zhang et al., 2007). This is theorized to be mainly attributed to the presence of bacteria in ruminant animals that degrades gossypol before it is absorbed by the system. Researchers found the presence of Rumen Bacillus Subtilis (RBS) in cows which is a ruminal bacterium that degrades gossypol and diminishes its toxicity (Zhang et al., 2018). In monogastric animals, ingestion of gossypol results in the diminished ability of red blood cells to carry oxygen throughout the body causing various symptoms such as edema, shortness of breath, and paralysis depending on the species (Alford et al., 1996). Swine are monogastric animals and therefore do not have the necessary bacteria to degrade gossypol before it is absorbed in the body. In swine, gossypol toxicity presents as labored breathing, anorexia, edema, and even death (Randel et al., 1992).

Reproductive Physiology

Spermatogenesis

The seminiferous tubules are a series of long, convoluted tubules within the tubular compartment of the parenchyma in the testes. They are composed of a basement membrane and a germinal epithelium that contains Sertoli cells and developing germ cells (Senger, 2012). The seminiferous tubules have a circular cross-section, and the size of the tubules is highly dependent on the species. The process of spermatogenesis occurs within the seminiferous tubules of sexually mature animals (Costa et al., 2013; Jedlinska et al., 1995). Spermatogenesis occurs in multiple stages of development progressing down the length of the tubule so that each location along the tubule may contain gametes of different maturities (Sertoli & Geyer, 2017). Spermatogenesis is divided into three phases – the proliferation phase which contains all mitotic divisions, the meiotic phase which contains all meiotic divisions, and the differentiation phase in which spermatogonia undergo morphological changes into fully mature spermatozoa (Senger, 2012). Sertoli cells govern spermatogenesis by supporting developing germ cells in a species-specific manner. Increased Sertoli cell numbers have been associated with increased spermatogenesis (Senger, 2012). Additionally, Sertoli cells protect the developing germ cells from toxicants through the blood-testis barrier (Monsees et al., 2000).

Reproductive Physiology of Boars

Several studies on small groups of sexually mature wild boars determined the seminiferous tubule diameter to be between approximately 180 to 300 μm (Almeida et al., 2006;

Costa & Silva, 2006). The seminiferous tubules are a key component of reproductive physiology to study possible effects of reproductive impairment because it is the primary location of spermatogenesis. In boars, spermatogenesis is comprised of 4.5 cycles with each cycle lasting approximately 8.3 days meaning that spermatogenesis requires approximately 39 days total (Senger, 2012).

Reproductive Effects of Gossypol in Various Species

While there is minimal research published on the effects of gossypol in boars, there are several studies of its effects on the male reproductive system in other species. Research indicates that gossypol affects the reproductive system in mammals, particularly in monogastric animals (Morgan, 2017). In a study on the effects of gossypol on rat testis morphology, it was determined that the germinal epithelium experienced damage as a result of gossypol. The damage was described as a reduction in the number of layers to severe damage in each treatment group, depending on the dose of gossypol ingested by the rats (Shepu et al., 1980). In mice testis induced with gossypol, it was determined that gossypol had a preventative effect on the growth of both Sertoli and Leydig cells (Lim et al., 2019). A study concerning the seminiferous tubules in rats found that after four weeks of daily treatment of gossypol, there was severe damage to the germinal epithelium and there was a clear reduction in the number of developing spermatids and spermatocytes within the seminiferous tubules (Shepu et al., 1980).

Human males have also experienced a negative effect on reproductive processes as a result of gossypol ingestion. When rural villages across China experienced a decline in birth rates in the 1950s, it was discovered that the cottonseed oil they used for cooking contained gossypol which was later found to cause infertility (Tso & Lee, 1981). In a study on the use of gossypol as a potential contraceptive pill in human males, 60% of the 134 men in the study experienced infertility within 16 weeks. Additionally, 51% of the men in the study experienced a reversal in results within one year of stopping treatment (Coutinho, 2002).

There have been numerous studies on the effects of gossypol on the reproductive physiology in cattle. A study using Brahman bulls found that a diet containing gossypol decreased the number of germ cell layers and a thinner germinal epithelium within the seminiferous tubules compared to bulls that did not receive gossypol (Chase et al., 1994). Brahman bulls were also used in a study that found gossypol to impact the morphology of mature sperm and interfere with spermatogenesis within the testes (Chenoweth et al., 1994). One research study found that Nellore bulls fed gossypol from whole cottonseeds every day had lower sperm motility and had fewer spermatozoa with intact acrosomes as opposed to bulls exposed to gossypol every other day (Hatamoto-Zervoudakis et al., 2018). Dietary gossypol has been associated with damage to the germinal epithelium in rams and bulls, which may be the cause of diminished spermatogenesis in both species (Randel et al., 1992).

Based on the current and previous research, it is evident the feral hog population in Arkansas is continuously growing in size without an effective control method in place. Additional research using contraceptives to negatively impact the reproductive function of feral hogs is needed as this could affect or eliminate future generations of hogs. As seen in previous works, gossypol from CSM has been used to negatively impact reproductive function in both humans and animals. By feeding hogs CSM containing gossypol, it is theorized that the gossypol could negatively affect or even omit the reproductive capability of the hogs.

Methodology

The following section will delve into the methodology of the research completed in the experiment. This experiment was part of a larger study consisting of a series of experiments to evaluate the effects of gossypol as a way to inhibit reproduction in swine. By performing the specified procedures included in this section, the results of the study would be reproducible.

Research Design

This is a quantitative, true experimental design in which 21 boars were randomly divided into three treatment groups to study the effects of gossypol from CSM on the seminiferous tubules. A blind experimental approach, in which the researchers are unaware of which treatment group a particular sample belongs to, was necessary during the histology portion of the experiment to avoid potential biases.

In the research study, a group of 21 boars was raised in the University of Arkansas Swine Research Unit where they were randomly divided into three treatment groups. The boars were put through a 56-day feeding trial consisting of 4 phases each lasting 14 days. All treatment groups were fed a nutrient adequate control diet for the duration of the trial. In phases 1-3, group 1 received no CSM (0% gossypol), and groups 2 and 3 received CSM in addition to the nutrient adequate control diet to achieve a diet containing 0.02% and 0.04% gossypol respectively. In phase 4, all treatment groups received a nutrient adequate control diet with no CSM. The boars were then fed a common finisher diet before semen collection and until the date of harvest.

Rigor

The following paragraph will explore the rigor of the research experiment. In the histology portion, experimenter bias was minimized by keeping research assistants blind to which treatment group a sample belonged. A potential limitation could be the size of the chosen sample population as there were only 21 boars in the study.

Population and Sampling

Twenty-one boars from the University of Arkansas Swine Research Unit made up the test population for this study. The group of boars was randomly assigned to three treatment groups for the duration of the study. The boars are a crossbreed of PIC (Pig Improvement Company) 29 with PIC 1050. A random assignment method was used so that the chosen sample participants would indicate a similar result within the domestic boar population from the crossbreed of PIC 29 with PIC 1050 (Taherdoost, 2016).

Data Collection

In the Fall of 2020, 21 boars were euthanized and castrated at approximately 266 ± 3 days to obtain tissue from the left testicle. Samples were collected from each testis by cutting a 1 cm x 1 cm cubed section of tissue from the parenchyma of the testis. Each tissue sample was fixed in Optimum Temperature Cutting (OTC) media, and super-cooled using 2-Methylbutane and liquid nitrogen. The testis samples were stored at -80°C until histological analysis began in the Fall of 2021.

The histological analysis consisted of sectioning, staining, and imaging the testis tissue. First, the samples were thawed to -20°C, and 8 μm thick slices were obtained using a Cryostat Microm HM550 (Thermo Fisher Scientific, 2010). Each section of tissue was fixed to a microscope slide and stained using the Vector Laboratories Hematoxylin and Eosin Stain Kit (H-3502; Vector Laboratories, 2021). The Hematoxylin and Eosin staining procedure was optimized for the species and tissue type. Images of the stained seminiferous tubules were captured at 4x magnification using a Nikon Eclipse Ts2R and uploaded to a computer. A completed slide containing a circular cross-section of a seminiferous tubule can be seen in Figure 1.

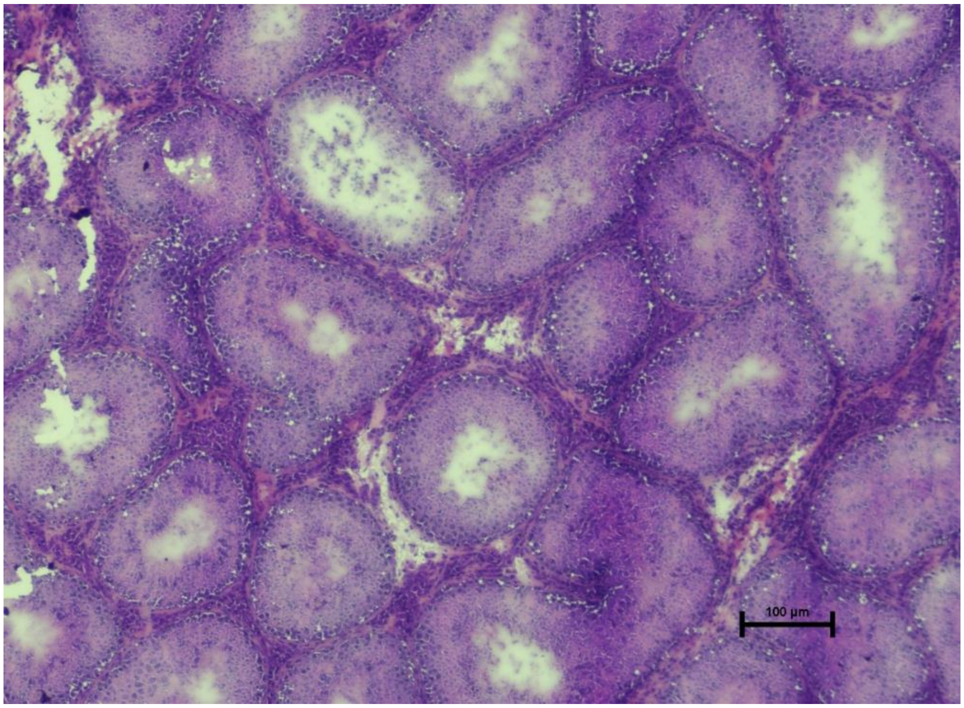

Figure 1. Seminiferous tubule cross-sections.

Data Analysis

Seminiferous tubule diameters were measured using the software, ImageJ (Bethesda, MD). For each of the 21 samples, 100 seminiferous tubule cross-sectional diameters were measured and recorded to ensure a representative subsample. Seminiferous tubule diameters were analyzed using MIXED procedures of SAS (SAS, Inc., Cary, NC) with treatment as a fixed effect. Pen number and body weight at death were tested as covariates and removed from the model in a stepdown progression at $P < 0.05$.

Results

Average seminiferous tubule diameter was not different (P = 0.88) between the control group (201.78 ± 7.32 μm), 0.02% gossypol treatment group (206.55 ± 6.34 μm), and 0.04% gossypol treatment group (205.37 ± 6.78 μm). The results of the study are summarized in Figure 2.

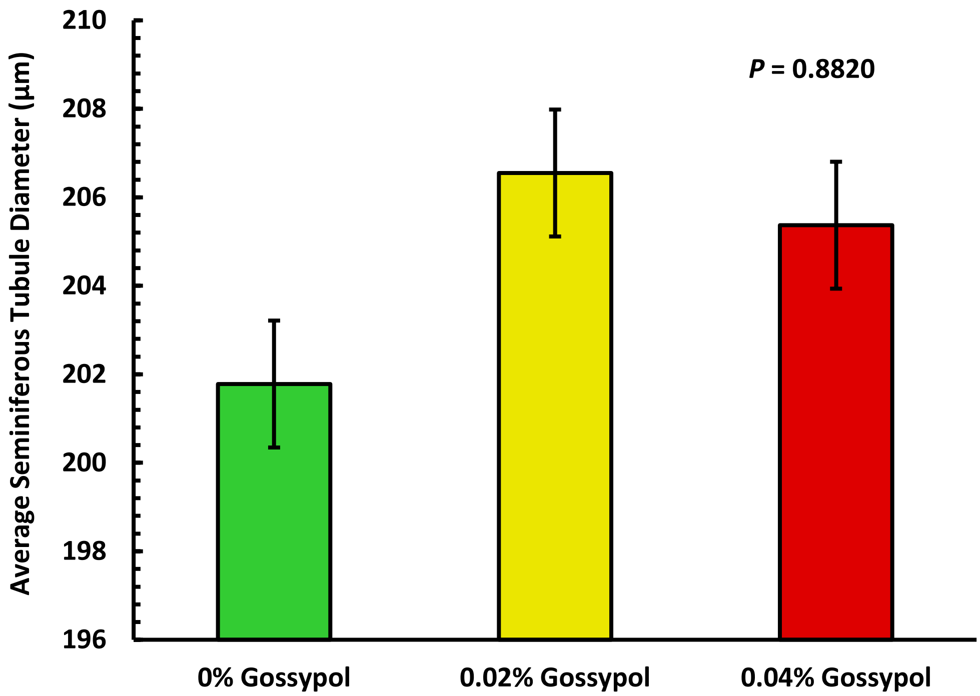

Figure 2. Average seminiferous tubule diameters in boars fed diets containing 0% gossypol, 0.02% gossypol, or 0.04% gossypol from cottonseed meal.

Discussion

It is worth noting that there were numerical differences in the seminiferous tubule diameters between the two treatment groups fed CSM and the control group fed no CSM. Specifically, the average seminiferous tubule diameters of the 0.02% and 0.04% treatment groups were both numerically larger than that of the control group. One possible explanation for this numerical difference is the use of gossypol from CSM instead of alternative methods. The hogs in the treatment groups that received CSM in addition to their nutrient adequate control diet had a higher protein content than the hogs in the control group. The numerical differences in seminiferous tubule diameter may be attributed to the higher protein content in the feed of the treatment groups that received CSM.

A potential limitation of the study is the timeline between the conclusion of receiving gossypol from CSM in the feeding trial and the date of harvest. The boars were exposed to gossypol during the development of their germinal epithelium before they were sexually mature. They last received gossypol on 105±1 d and were harvested on 266±3 d. This approximate 161-day gap in the timeline could allow for the regeneration of the germinal epithelium within the seminiferous tubules if it had been damaged during the feeding trial. The regeneration of the germinal epithelium is due to the process of spermatogenesis which takes 39 days to complete. In spermatogenesis, generations of germ cells develop within the germinal epithelium with the most immature diploid germ cells closest to the basement membrane and the most mature haploid gametes closest to the lumen. Within 39 days, there would be enough time for the most immature germ cell to develop into a mature spermatozoon which would regenerate the damage to the germinal epithelium, if any. By harvesting the boars in less than 39 days after the conclusion of the feeding trial, there would not be enough time for any damaged germinal epithelial cells to heal.

It is known that testes containing a larger number of Sertoli cells generally produce a larger number of spermatozoa (Senger, 2012). Any damage to the Sertoli cells could result in a decreased number of spermatozoa produced in the testes. The Sertoli cells are a part of the blood-testis barrier which prevents materials such as immunoglobulins and immune cells from accessing the developing gametes (Senger, 2012). Damages to the Sertoli cells could also allow for these cells to mount an immune response on the developing gametes.

The timing of gossypol exposure might have had an impact on the effects of this study. The gossypol from CSM was fed to prepubertal growing boars before their reproductive system was finished developing. Feeding gossypol from CSM to mature boars might have led to different results because mature boars would have a fully developed germinal epithelium.

Additionally, increasing the length of the feeding trial could result in an increased effect of gossypol on the reproductive system of boars. The toxic effects of gossypol are cumulative and can therefore increase in severity the longer it remains in the diet (Morgan, 2017). By exposing the boars to gossypol for a longer period of time, there is potential for the negative effects of gossypol on the reproductive system to increase in severity.

Conclusion

The objective of this research study was to determine the effects of gossypol from CSM on the diameter of the seminiferous tubules in boars. In this study under the set conditions, a diet containing 0.02% or 0.04% gossypol from CSM fed to growing boars did not influence seminiferous tubule diameters. Future directions may include increasing the amount of gossypol content used in the feed, lengthening the feeding trial, or decreasing the time between the end of the feeding study and the date of harvest.

Acknowledgments

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