INNOVATIONS

Using Castration Surgery in Male Rats to Demonstrate the Physiological Effects of Testosterone on Seminal Vesicle Anatomy in an Undergraduate Laboratory Setting

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Abstract: Rats can be used as a model organism to teach physiological concepts in a laboratory setting. This article describes a two-part laboratory that introduces students to hypothesis testing, experimental design, the appropriate use of controls and surgical techniques. Students perform both a castration and sham-control surgery on male rats and test the effects of reduced testosterone due to castration on the weight and histology of seminal vesicles. After performing the surgeries and collecting the data, students learn histological techniques, as well as empirical data collection, analysis and interpretation. Demonstrating the effects of testosterone through castration surgery bridges concepts learned in a traditional physiology class setting with data gleaned from research in a laboratory. Overall, the male castration surgery provides students with hands-on skills and an understanding of the work done by scientific researchers and health care professionals.

Key words: castration, physiology, seminal vesicles, testosterone, rat

INTRODUCTION

During their undergraduate studies in Biology, it is invaluable for students to gain hands-on experience in laboratory situations that they will carry with them into their postgraduate careers. Human physiology laboratory activities should give students hands-on demonstrations of different body systems at work and emphasize the interplay and interdependence of the body's organ systems. When combined with in-class discussions, engaging a student in research and laboratory activities aids in development of scientific processing skills, promotes conceptual understanding, and increases motivation and retention, especially in STEM-focused students (Lewis et al., 2003; Burrowes & Nazario, 2008). Primary reform efforts in undergraduate STEM education have focused on a conceptual shift to learner-centered and applied, hands-on learning (Fairweather, 2008; Mestre, 2008). In this vein it is important to present students with the opportunity not only to follow the "scientific method" but also to involve them in a research experience that will enhance understanding and long-term retention of scientific concepts. We therefore developed a two-part lab in an effort to expose students to the practical experiences of performing surgery using an animal model, to learn histological techniques and to collect empirical data. We use this lab exercise in Biology 4640: Physiology Laboratory, an upper-level lab course intended for junior and senior Biology, Biochemistry and Chemistry majors at the University of Detroit Mercy.

In this course, students participate in a laboratory research project designed to involve students in a hypothesis-driven activity that will generate data regarding the effects of testosterone exposure on male accessory sex organ anatomy. In addition to hearing a pre-laboratory lecture on reproductive anatomy and physiology, students generate hypotheses, learn surgical techniques, are introduced to the use of appropriate scientific controls, practice data interpretation, and evaluate data statistically. Students use male rats (*Rattus norvegigicus*) as a model organism and perform both a castration and sham-control surgery. This lab teaches that the physiologic workings and products of the endocrine system influence almost every organ system of the body. Specifically, students learn that the hormone testosterone has a substantial impact on the anatomy and physiology of the seminal vesicles.

It has long been understood that testosterone production by Leydig (interstitial) cells in the testes helps to regulate growth and maintenance of secondary sex organs. Leydig cells produce approximately 95% of a male's testosterone. Testosterone and testosterone derivatives regulate growth and development of all male reproductive organs (e.g., penis, seminal vesicles and prostate glands). During development, increases in the production of testosterone plays a major role in the maturation, physical growth and function of the male reproductive organs (Kandeel et al., 2001). The seminal vesicles contain pseudostratified columnar epithelium and consist of secretory cells that produce an alkaline seminal fluid rich in fructose, fibrinogen,

vitamin C and prostaglandins (Burkitt et al., 1993). Fluid produced in the seminal vesicles typically forms more than half of the seminal volume and is necessary for the formation of the sperm coagulum, the regulation of sperm motility and the suppression of immune function in the female genital tract (Freud, 1933, Higgins et al., 1976; Kandeel et al., 2001; Rudolph & Starnes, 1954). It has been reported in primary literature that when the effects of testosterone are blocked or when the testes are removed and testosterone is not produced in male animals, a significant decrease in seminal vesicle weight follows and the seminal vesicles display abnormal histology (Rudolph & Starnes, 1954; Karri et al., 2004).

The rat serves a useful animal model of the male reproductive system that can be manipulated to induce the loss of, or mimic a failure to produce, the male hormone testosterone. Additionally castration will demonstrate the effects of testosterone loss on the growth and maintenance of accessory sex organs. Freud (1933) stated that the seminal vesicles make a good model for studying the effects of testosterone because they are easy to prepare histologically and they respond rapidly to castration. Castration surgeries on female rats have been performed in other undergraduate labs (Kirkpatrick, 2009), supporting the idea that learning and performing small animal surgical techniques is ideal for undergraduate physiological lab courses that seek to give a hypothesis-driven research experience. The male castration surgery is relatively simple and the completion of a recovery surgery provides students not only with hands-on skills but also with an understanding of the work done by scientific researchers or health care professionals.

One week following the castration surgeries, students sacrifice the rats and weigh them, remove and weigh the seminal vesicles and prepare the seminal vesicles for histological evaluation in order to determine any testosterone-deletion effects. Post-fixation, the students embed, section and stain the seminal vesicle tissue from both castrated and sham-treated control rats. The second week of lab provides students with the opportunity to practice data collection, data interpretation and hypothesis testing using a real animal model of hormone action. As a final exercise in scientific data interpretation, students generate research posters in order to provide the opportunity to solidify background knowledge, use primary research references, and place collected data in the arena of reproductive scientific inquiry. Overall, this laboratory research activity provides exposure to biological practices such as animal surgery and care, tissue fixation, histology, tissue staining, hands-on hypothesis testing, statistical analysis and the interpretation and scientific presentation of data.

MATERIALS AND METHODS

Presurgical Preparation:

Male Sprague Dawley CD rats (*Rattus norvegigicus*) weighing between 125 and 136 grams were obtained from Charles Rivers Laboratories International, Inc. (Wilmington, Massachusetts). The rats were housed in groups of four in the animal care facility at the University of Detroit Mercy, and were fed rodent chow and given water *ad lib* (IACUC approved by UDM Animal Care and Use Committee; July 2011). Before the lab began, all instruments were sterilized in an autoclave and wrapped in paper. Prior to surgery, laboratory benches were sanitized and prepared with an absorbent liner at the surgery site. Each laboratory bench was stocked with 70% ethanol and sterile cotton pads, a 50-milliliter beaker with a cotton pad or gauze strips at the bottom soaked in ether (ether cone) and a covered bottle of ether as ether evaporates quickly. Each group of students also had access to a pair of electric clippers. Students were provided gloves when they entered the lab and were advised to wipe their surgical equipment with 70% ethanol. Surgical tools consisted of scissors, forceps, straight hemostats, silk suture and size 18 surgical needles. A recovery cage was also set up in a quiet area with a light mounted above it to provide warmth to the recovering rats.

Student Exercise:

Students are initially given a lecture on male reproductive anatomy and physiology that includes information on the effects of androgens. The use of appropriate experimental controls and the generation of hypotheses and expectations based on background information from both textbooks and primary literature is also explained. Given the appropriate background information, students are then required to generate a working hypothesis on what anatomical changes they predict they will occur in the seminal vesicles after castration. Students are also informed about the proper surgical techniques, anesthesia, post-surgical care and husbandry. The surgery, data collection and histological analysis take two to three laboratory sessions (or weeks) depending on the histology preparation. To conserve time, the teaching assistants and professor may complete the tissue embedding and sectioning.

Student Lab Week 1:

Anesthesiology:

Ether is used to anesthetize rats for castration surgery because it can be easily administered and regulated. The specific action of ether is unknown; however, there is experimental evidence suggesting that ether reduces the number of phospholipid interactions. Ether has been shown to decrease excitability of muscles and neurons by inhibiting the sodium conductance responsible for depolarization during an action potential and may therefore modulate consciousness (Inoue & Frank, 1965).

Anesthetics that could replace ether in this exercise include halothane, nitrous oxide, isoflurane, barbiturates, and opioid derivatives.

Prior to anesthetizing the rats, cotton soaked with ether was placed on the bottom of a glass desiccator (25 cm in diameter and 13 cm in depth) and the cover of the desiccator was sealed by placing a thin film of Vaseline around the lip. Rats are not introduced to the desiccator for ten minutes following the addition of ether to the desiccator so that the desiccator could become saturated with ether vapors. After this time period, a rat was quickly transferred to the ether chamber after removing it from its cage. The lid to the desiccator was reapplied quickly so that very little ether vapors were released. Students test the level of anesthesia by examining the rat's breathing and by using a toe pinch. A rat that has reached an appropriate plane of anesthesia should be breathing slowly. More importantly, it should show with no response to a toe pinch. Forceps are used in this exercise to test the rat's "readiness" by gently pinching its toes. If the rat does not respond by pulling (withdrawing) its leg in response to the pinch, the rat has reached a level of anesthesia appropriate for surgery.

The rat is then transferred to the prepared laboratory bench and its nose is placed in an ether cone. During the entire surgical procedure, anesthesia should be maintained by a student who has been appointed as the anesthesiologist. Breathing and the level of anesthesia should be closely monitored by the anesthesiologist. The student should ensure that the gauze in the ether cone is continually saturated with ether. Furthermore the student should adjust the cone, move the cone further away from the nose or remove the cone entirely, if the rat's breathing becomes very shallow until breathing returns to normal. If the rat stops breathing, it may be resuscitated using a six inch piece of aquarium tubing cut at a 45 degree angle at one end. The angled end of the tubing is placed over the rat's nose and the anesthesiologist breathes into the opposite end of the tubing in short, rapid puffs. The student's should apply gentle pressure on each side of the abdomen with their thumb and fingers to prevent air from entering the rat's stomach. The thumb and fingers should then be placed on either side of the chest and the chest gently massaged between intervals of puffing. Throughout the surgery, breathing should be shallow, the eyes should be clear pink (not cloudy) and the rat's feet should be flesh tone (not blue).

Male Castration:

Students are advised to put on gloves and to use 70% ethanol to wipe surgical equipment. The testes and surrounding area are shaved using electric clippers. The incision areas are prepared by wiping 70% ethanol over the shaved skin of the scrotum. The male gonads (testes) rest in the perineum. The perineum is the portion of the pelvic cavity that is isolated by a muscular diaphragm, which consists of the levator ani and coccygeus muscle. The testis, epididymis and lower spermatic cord (crematic muscle, vas deferens, testicular artery and pampiniform plexus) are contained in a pouch of skin called the scrotum. The testes are mobile organs; therefore, prior to shaving fur and making the initial incision on the rat's scrotum, the testes should be gently pushed into the scrotum. Students will make their initial incision with scissors; scalpels can cause unnecessary damage when pressing down on an organ. The incision should be straight (caudal to cranial) and about 3 to 4 cm in length. Each testis is surrounded by the tunica vaginalis and several layers of fascia. Using forceps, the testis is freed from the scrotum. A ligature is then tied tightly around the spermatic cord. The ligature should be at least 2 cm from the testes, which leaves enough room to cut through the spermatic cord with scissors. The loose ends of the ligature are then trimmed to 0.5 cm in length. Care should be taken not to trim too closely to the knot, so that it will not slip. The ligature should occlude the vasculature of the spermatic cord, thereby preventing blood loss. The incision is closed with three to four interrupted over-and-over sutures (Serag-Weissner, 2006). Next the procedure is repeated to remove the other testis in each experimental rat.

Following the first surgery, the students switch roles such that the anesthesiologist becomes the surgeon. A sham-control surgery is performed in which the control rat undergoes the initial cutaneous incisions described above, but does not have its testes removed. The incision is closed with sutures as described earlier. As a control animal, its gonads will remain undamaged and intact. This control condition will eliminate any question of variability in data collected that may be attributed to the surgical procedure, and not by the removal of sex hormones produced by the gonads.

Postsurgical Procedure:

After completion of the surgery, students punch ear marks to identify the castrated and control rats for follow-up experiments. The unconscious rats are placed in the recovery cage, making sure that it is under the warming lamp. The lamp provides low-level heat that facilitates the expulsion of ether from the rat, hastening its revival. Recovery from surgery typically takes approximately 20 to 30 minutes. Once conscious, the rats are relocated into a cage with clean litter and an ample supply of water (needed for replacement of lost blood volume) and food. Students are required to check on their rats throughout the next week.

Student Lab Week 2:

Removal of Seminal Vesicles:

One week after the castration and control surgeries, both the control and castrated rats are euthanized by overdose of ether and subsequent

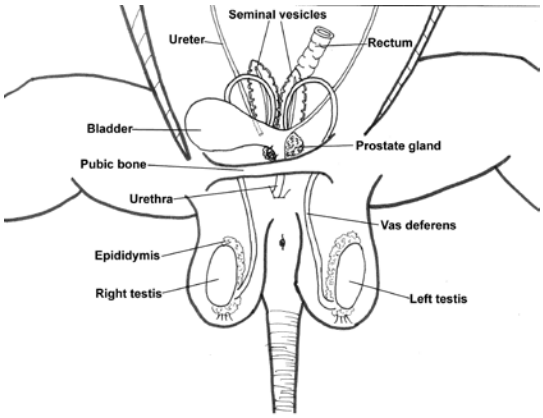

Fig. 1. Anatomy of the urinogenital system in male rats. Photo credit: Elizabeth Grabowski.

cervical dislocation (AVMA, 2013) and the body weights of both rats are obtained. An abdominal incision is made and the seminal vesicles, located on each side of the bladder, are identified and exposed (Figure 1). To determine if sex hormones affect the seminal vesicle anatomy, the students remove the seminal vesicles and weigh them. Seminal vesicle weight is expressed as a percent of total body weight to emphasize data standardization. We typically have students remove the seminal vesicles from control rats first as they are much larger than in the castrated rats. This makes locating the seminal vesicles in the castrated rats less challenging.

Histological Preparation:

The seminal vesicles from the control and castrated rats are then placed into Bouin's fixative (Sigma-Aldrich) in separate containers overnight and prepared for paraffin embedding. Histological preparation and staining is then completed using Bancroft and Stevens (1990) as a guide. The following morning, the tissues are placed in an ethanol series: 75%, 80%, 95% and 100% for 45 minutes each. The tissues are then placed in two changes of 100% ethanol for 60 minutes each. Following this, the seminal vesicles are placed into two changes of xylene for 60 minutes. Seminal vesicles then undergo three changes in paraffin for 60 minutes each (Bancroft & Stevens, 1990).

Embedding the tissue begins with selecting a mold that corresponds to the size of seminal vesicle tissue, leaving a margin of 2 mm around the tissue. A small amount of hot paraffin is poured into the mold and, using forceps, the seminal vesicle tissue is transferred into the mold (cut side down). The mold is then transferred to a cold plate and the tissue is centered within the paraffin wax. A tissue cassette is then placed on top of the mold as a support and hot paraffin is added to the mold, covering the cassette. The mold and cassette are kept cold, and the paraffin will solidify within about 30 minutes.

A microtome with a fresh blade is then used to cut 10 µm sections. These sections are transferred to a warm water bath (~37^{o}C) and the sections are then floated onto the surface of a clean glass slide marked as either control or castrated seminal vesicle. Slides, with paraffin sections, are then placed on a hot plate or warming block (~ 65°C) for 20 minutes in order to melt the wax to the glass. Slides can then be stored at room temperature until stained.

Hemotoxylin and Eosin Staining:

The processed and sectioned seminal vesicle tissue is then stained to give contrast to the tissue so that the seminal vesicle tissue from control and castrated rats may be compared. We use a series of Coplan jars for paraffin removal and Hemotoxylin and Eosin staining. First the slides are deparaffinized by putting them through two changes of xylene for 10 minutes each. Following this, the sections are rehydrated in 2 changes of 100% ethanol for 5 minutes each, then 95% and 70 % ethanol 2 minutes each. The slides with the tissue sections are then placed briefly in distilled water. Harris' Hemotoxylin is the first stain used and the slides are placed in the solution for 8 minutes, followed by washing for 5 minutes in distilled water. Tissues are then placed in acid alcohol for 30 seconds for differentiation and washed with distilled water for 1 minute. The water is blotted with blotting paper and the slides are placed in the counterstain Eosin for 30 seconds to 1 minute. Following the counterstain, tissue is then dehydrated using two changes of 95% and 100% ethanol for 5 minutes each. Finally slides are cleared using two changes of xylene, 5 minutes each and mounted using a xylene-based mounting medium such as Permount (Fisher Scientific) and a coverslip. The coverslip should be angled and allowed to fall gently, so that the Permount may spread evenly beneath the coverslip and bubbles may be avoided (Bancroft & Stevens, 1990; Kiernan, 2008).

RESULTS

While removing and weighing the seminal vesicles, students notice that the castrated rats have much smaller seminal vesicles. Students then compare the weights of control versus castrated seminal vesicles as a function of body size (weight of seminal vesicles/weight of rat x 100), which allows for comparison of rats of different weights. Students in each laboratory section are instructed to share data with their classmates. Figure 2 is similar to a figure that students create for their posters. It demonstrates that there is a significant difference ($p < 0.0001$; $t = 6.720$) in the percent weights (mean ± standard error) of the seminal vesicles between control (0.17 ± 0.02%) and castrated rats (0.05 ± 0.01%). Further, students perform a histological evaluation of the seminal vesicles. This evaluation typically demonstrates that control seminal vesicles are composed of columnar or pseudostratified columnar epithelium that consists of secretory cells arranged in folds surrounded by smooth muscle. A normal (control) seminal vesicle typically has an irregular

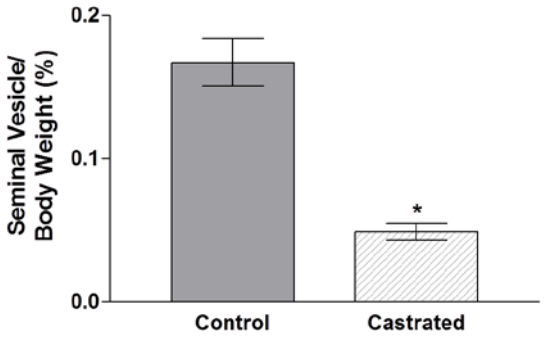

Fig. 2. One week after castration, seminal vesicles were removed from both castrated and control rats and weighed. Percent seminal vesicle weight (mean ± S.E.) as a function of overall body weight was calculated for both control and castrated male rats (N=15). One week post-surgery, the seminal vesicles of castrated rats were significantly smaller than control rats. * indicates $p < 0.0001$.

lumen, giving it a honeycombed appearance (Fig. 3A). In contrast, students notice that in castrated rats, the seminal vesicles are regressed and show abnormal histology. The seminal vesicle epithelium in castrated rats exhibits atrophy and is not organized into glandular folds, which inhibits secretion of seminal fluid (Fig. 3B). Because students prepare all of their own histological samples, after staining, students can trade coded (or unlabeled) slides with another group of students in order to reduce bias during analysis.

Laboratory Evaluation:

To evaluate learning in the BIO 4640 Physiology Laboratory, we compared overall student grades for the Physiology lecture course (BIO 4630) of Biology majors who took the course concurrently with the laboratory with Biology majors who did not enroll in the lab portion of this course (IRB protocol #1314-08). We found that Biology majors who take and complete the laboratory portion of this course have a higher overall grade in the Physiology lecture when the laboratory and lecture are taken concurrently ($p > 0.05$; t-test; N=35). The grade for students who are enrolled in the lab averaged 82.5% (B) while those who did not take the lab course averaged 79.5% (B-).

DISCUSSION

This laboratory teaches students that testosterone, produced by the Leydig cells of the testes, is physiologically important for the development and maintenance of the seminal vesicles. Using seminal vesicles to demonstrate the importance of testosterone is advantageous for this lab exercise because seminal vesicles are easy to prepare histologically and the secretory cells of the seminal vesicle respond quickly to castration (Frey, 1933; Burkitt et al., 1993). Within a week, students notice that there is a significant decrease in seminal vesicle weight in the castrated rats and that the castrated rats display abnormal seminal vesicle histology (Figures 2 and 3). Mean seminal vesicle weight expressed as a percentage of total body mass (seminal vesicle weight/body weight x 100) decreases by 0.12% one week post-castration. Further, when the Hemotoxylin- and Eosin-stained seminal vesicle tissue is prepared and examined by the students, it is evident that the control tissue has a large number of folds of pseudostratified epithelium or secretory tissue. The castrated rats' epithelium was regressed with distinctly fewer, less prominent folds.

The students enrolled in this laboratory gain a first-hand understanding of male reproductively physiology and how the hormone testosterone affects the structure and functioning of the seminal vesicles. Biology majors enrolled in the lab concurrently with the lecture had higher overall grades in the lecture course than students who did not take the laboratory. While we did not see statistically significant results from this preliminary analysis, we believe that this laboratory exercise greatly increases knowledge of reproductive physiology for those students participating in both the lecture and lab. Moreover, student data will continue to be collected and a larger sample size could ultimately yield a significant difference between students enrolled in both lecture and lab and those that are enrolled only in the lecture.

This lab exercise examining the effects of castration on the seminal vesicles has been taught for several years at the University of Detroit Mercy. Although we have not in the past collected quantitative data on each laboratory exercise offered throughout the semester, during end of the term course evaluations students often rate this course very highly (4.85 out of 5). When providing written feedback about the castration lab module, students generally have positive comments about what that lab taught them. Some comments that we have received from students following the completion of this lab were: "A tough class, but it was fun, interesting and an excellent complement to Physiology lecture. This class introduced me to my first live surgery, which is such an awesome experience." "I particularly enjoyed the rat surgery lab. I felt it helped me understand how

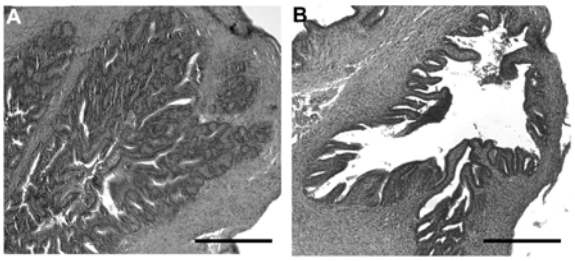

Fig. 3. Hemotoxylin and Eosin stained seminal vesicle tissue from control (A) and castrated (B) male rats, obtained one week after the castration and sham-control surgeries. Seminal vesicles are composed of pseuduostratified columnar glandular epithelium that is organized into folded mucosa (A). Castration surgery and the subsequent loss of testosterone caused a regression of seminal vesicle epithelium and abnormal histology (B). Scale bar in A = 300 µm and scale bar in B = is 200 µm.

testosterone functioned within the body; it also helped expand and clarify the knowledge I had about the reproductive system in general." "The Rat Reproduction lab was intriguing and rewarding for aspiring medical professionals. It made personal the tie between solid research techniques, the comprehension of reproductive physiology, and clinical proficiency." "I want to be a Physician Assistant, and the rat surgery lab made me feel like a doctor. We got to anesthetize and do surgery on our rats. Dissecting something that has already died is interesting, but doing surgery on live rats, successful ones at that is unlike an experiment I have ever done in my life actually. Loved this lab!" The feedback from this lab has been very positive and we have students in the hallways telling us they are looking forward to enrolling in this lab so that they can perform the rat surgeries. Overall, students gain an enhanced understanding and appreciation for animal research.

This laboratory module is an important component of Physiology Lab because it requires students to generate scientific hypotheses regarding the physiological effects of testosterone. Along with the generation of hypotheses, students are introduced to the appropriate use of scientific controls, and to the collection and evaluation of data. We believe that students gain hands-on experience in this laboratory that they will carry with them into their postgraduate careers. Many of our students are focused on gaining acceptance into Dental or Medical School or a Physician Assistant Program. Few of our students actually think about postgraduate programs such as an M.S. or Ph.D.; however, the number of student pursing these degrees has recently increased and some students who have completed this laboratory course have chosen to pursue research as a career. This lab module provides active participation in research that may help with retention of STEM-focused students in research fields. Students who complete the male castration surgery gain hands-on skills and an understanding and appreciation of the work done by scientific researchers and health care professionals.

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